Dissertation

Interacting microbes

– the microcosmos in our body and our homes –

Submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications, I followed the guidelines of “Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

Manuela - Raluca Pausan
Graz, January 2020

Disclosures

Parts of the introduction, materials and methods, results and discussion are already published in and contain the verbatim text of:

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Mahnert, A., Blohs, M., Pausan, M.-R., and Moissl-Eichinger, C. (2018). The human archaeome: methodological pitfalls and knowledge gaps. *Emerg. Top. Life Sci.* 2, 469–482. doi:10.1042/ETLS20180037.

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All co-authors presented here gave their consent to re-use the data from the publications within this thesis. The following co-authors contributed to the data shown in the thesis:

- **Corinna Bang**, **Anke Schilhabel**, **Ruth Schmitz** performed approach1 experiment in Koskinen *et al.*, 2017, wrote the approach1 results part in Koskinen *et al.*, 2017 and provided critical discussions for the article.
- **Michael Beck** performed parts of the experiments in Koskinen *et al.*, 2017.

- **Marcus Blohs** provided critical discussions throughout the House Microbiome and Human Archaeome projects, wrote parts of the review article Mahnert *et al.*, 2018, isolated the new strain of *Methanobrevibacter* used for the oxygen tolerance test and performed the oxygen tolerance test for the *Methanobrevibacter smithii.*
- **Cintia Csorba** performed some of the PCR for the Human Archaeome I project.
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- **Kaisa Koskinen** helped with analysing the data in Koskinen *et al.*, 2017 and wrote parts of the article, and provided critical discussions throughout the thesis.
- **Tobias Madl** and **Gesa L. Richter** performed the metabolomics analysis and wrote the metabolomics part in Pausan *et al.*, 2019b.
- **Alexander Mahnert** provided critical discussions throughout the thesis, helped with the ethics for the male cohort within the Human Archaeome II project, performed the analysis for the Fig. 41 and wrote parts of the review article Mahnert *et al.*, 2018.
- **Christine Moissl-Eichinger** provided critical discussions, and valuable help regarding all methods used throughout the whole thesis, revised the manuscripts of all publications and the thesis and helped in the development of the experimental design for each project presented in this thesis.
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Abstract

The human microbiome has become an essential topic for research in the last decade. Understanding the composition of the microbiome, how it interacts with its environment and especially with the host has been the main concern for many publications in the field of microbiome research.

The main goal of this thesis was to address some of the questions regarding the interactions of the microbiome with the host and the environment; therefore we tried answering the following questions: Could the environment be a source of human-associated anaerobic microorganisms? Are anaerobic microorganisms able to survive in an aerobic environment? Is preterm labour linked with any changes in the vaginal and urinary microbiome or changes in the human milk oligosaccharides' (HMOs) composition? Are there any associations between human milk oligosaccharides and the microbiome in urine and vagina? Are the microbiome universal approach methods sufficient to determine the archaeal communities within the human body? What is the composition of the archaeal communities in different body sites? Samples from all projects were analysed by different molecular methods, namely microbial amplicon sequencing and quantitative PCR, and some project-specific methods such as fluorescence *in situ* hybridization, oxygen tolerance test, and human milk oligosaccharides analysis.

In the house microbiome project, we explored the composition of the microbial communities found on the bathroom floor and distinguished between free DNA and DNA obtained from cells with intact cell wall or membrane. By analysing both the bacterial and archaeal communities, we identified that most of the microorganisms present on the bathroom floor are often associated with the human skin or the gastrointestinal and genitourinary tract. Anaerobic microorganisms were also detected, even though the relative abundance of these microorganisms was decreased in the samples which contained DNA only from intact cells. Additionally, we showed that the methanogens present on the bathroom floor are of human origin and that methanogens could survive in an aerobic environment up to 24h. Therefore, the indoor environment could act as a source of anaerobic microorganisms.

The results of the UMIC project showed that specific microorganisms found in the vaginal and urinary microbiome were associated with preterm labour, short cervix and preterm birth. Moreover, we observed associations between the sialylated HMOs, in particular, 3'sialyllactose, with preterm birth, high inflammation and short cervix, and confirmed that HMOs influence the microbiome profile.

Abstract

In the last project, the Human Archaeome, we demonstrated that the often-used universal approaches are unable to detect the archaeal communities in human samples, indicating the need for an archaeal-targeting approach for future microbiome studies. Also, we reconfirmed the body site-specificity of the archaeal communities. Most body sites share specific taxa such as *Methanobrevibacter*, *Methanobacterium* and *Methanosphaera*, although specific body sites have high microbial diversity, especially the nasal cavity.

This thesis expended the knowledge of microbiome regarding indoor environments, the microbiome composition in pregnancy and especially in women at high risk of preterm birth and the human archaeome. These results will contribute to the development of future studies addressing the human microbiome and how it interacts with its host and the environment.

Zusammenfassung

Das menschliche Mikrobiom hat sich im letzten Jahrzehnt zu einem essentiellen Thema in der Forschung entwickelt. Viele Publikationen im Feld der Mikrobiomforschung beschäftigten sich hauptsächlich mit der Zusammensetzung des Mikrobioms und seinen Interaktionen mit Umwelt und insbesondere dem Wirt.

Das Hauptziel meiner Doktorarbeit war, die folgenden Fragen bezüglich Interaktionen des Mikrobioms mit Wirt und Umwelt zu beantworten: Könnte die direkte Umwelt eine Quelle für Menschen-assoziierte anaerobe Mikroorganismen sein? Sind anaerobe Mikroorganismen in der Lage in einer aeroben Umgebung zu überleben? Stehen frühgeburtliche Wehen im Zusammenhang mit Veränderungen des vaginalen und Urin-Mikrobioms, oder mit Veränderungen der Zusammensetzung der sogenannten humanen Milch-Oligosaccharide (HMOs)? Gibt es Zusammenhänge zwischen HMOs und dem Mikrobiom in Vagina und Harn? Sind sogenannte universale Analysemethoden ausreichend um archaeelle Gemeinschaften im menschlichen Körper zu bestimmen? Welche archaeellen Profile von archaeellen Gemeinschaften sind in verschiedenen Körperbereichen zu finden?

Proben von allen Projekten wurden mit verschiedenen molekularen Methoden analysiert, nämlich mikrobielle Amplikonsequenzierung and quantitative PCR, projektspezifisch wurden weiterhin Fluorescence *in situ* hybridization, Sauerstoff-Toleranztest und HMO Analyse hinzugezogen.

Im Häuslichen Mikrobiom Projekt haben wir das Mikrobiomprofil von Proben des Badezimmerbodens untersucht und zwischen freier DNA und DNA aus Zellen mit intakter Zellwand oder Membran unterschieden. Die Analyse ergab, dass Proben vom Badezimmerboden hauptsächlich Signaturen von Mikroben aufweisen, die grundsätzlich mit der menschlichen Haut, dem Darm und dem Urogenitaltrakt assoziiert werden. Anaerobe Mikroorganismen konnte ebenfalls detektiert werden, obwohl die relative Abundanz dieser Mikroorganismen in Proben mit DNA aus Zellen mit intakter Zellwand deutlich verringert war. Darüberhinaus konnten wir zeigen, dass die auf dem Badezimmerboden nachweisbaren archaeellen Methanogene vom Menschen stammen und dass Methanogene grundsätzlich in aeroben Umgebungen bis zu 24 Stunden überleben können. Daher könnte das häusliche Mikrobiom durchaus eine Quelle für anaerobe Mikroorganismen darstellen.

Die Resultate des UMIC Projekt haben gezeigt, dass bestimmte Mikroorganismensignaturen in Vagina und Harn mit vorzeitigen Wehen, kurzem Gebärmutterhals und Frühgeburten assoziiert werden können. Darüber hinaus beobachteten wir Assoziationen zwischen sialylierten HMOs, insbesondere 3'Sialyllactose, mit Frühgeburten, hohen Entzündungswerten

und kurzem Gebärmutterhals, und konnten bestätigen dass HMOs das Mikrobiomprofil beeinflussen.

Im letzten Projekt – das Menschliche Archaeom – demonstrierten wir die Unzulänglichkeiten von oft genutzten, universellen Methoden Archaeen in menschlichen Proben im vollen Umfang nachzuweisen, und betonen mit unseren Analysen die Notwendigkeit der Verwendung von speziellen, archaeen-fokussierten Methoden in zukünftigen Mikrobiomstudien. Darüber hinaus bestätigten wir erneut die Körperstellen-spezifische Distribution von verschiedenen Archaeen. Die meisten Körperstellen tragen gleicherweise spezifische Taxa, z.B. Methanobrevibacter, Methanobacterium und Methanosphaera, obwohl manche Körperstellen eine hohe archaeelle Diversität aufweisen, wie z.B. die Nasenhöhle.

Diese Arbeit erweitert das Wissen um häusliche Mikrobiome, die Mikrobiom-Zusammensetzung während der Schwangerschaft – besonders bei Frauen mit hohem Frühgeburtenrisiko – und das menschliche Archaeom. Diese Resultate werden zur Entwicklung zukünftiger Studien zum menschlichen Mikrobiom und seinen Interaktionen mit Wirt und Umgebung beitragen.

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List of Abbreviations

‘	minutes
“	seconds
°C	degree Celsius
µg	microgram
µL	microLiter
µM	microMolar
2’FL	2’-FucosylLactose
2AB	2-AminoBenzamide
3’SL	3’-SialylLactose
3’SLN	3’-SialylLactosamiNe
6’SLN	6’-SialylLactosamiNe
aF	archaeal Forward
aR	archaeal Reverse
AUC	Area Under the Curve
BAL	BronchoAlveolar Lavage
bF	bacterial Forward
bp	base pairs
BSA	Bovine Serum Albumin
Ca	Candidatus
CH_4	methane
CI	Confidence Interval
cm^2	centimetre squared
Cp	Crossing point
CRP	C-Reactive Protein
CST	Community State Types
CY3	Cyanine 3
CY5	Cyanine 5

List of Abbreviations

DADA2	Divisive Amplicon Denoising Algorithm
DAPI	4′,6-DiAmidin-2-PhenylIndol
DNA	DeoxyriboNucleic Acid
dNTP	Nucleoside TriPhosphate
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	DiThioThreitol
F	Forward
FISH	Fluorescence *In Situ* Hybridization
g	gram (weight) or g-force (centrifugal unit)
gDNA	genomic DeoxyriboNucleic Acid
h	hour
HMOs	Human Milk Oligosaccharides
HPLC	High Performance Liquid Chromatography
HS	High Sensitivity
IQR	InterQuartile Range
iTOL	interactive Tree Of Life
KU	KiloUnits
LDFT	LactoDiFucoTetraose
LEfSe	Linear discriminant analysis Effect Size
LSM	Laser Scanning Microscope
M	Molar
MEGA7	Molecular Evolutionary Genetics Analysis version 7
mg	milligram
MgCl2	Magnesium Chloride
min	minutes
mL	milliLiter
mm	millimeter
mM	milliMolar
mmol/L	milimolar per Liter

List of Abbreviations

NaCl	Sodium Chloride
Na-EDTA	Sodium Ethylenediaminetetraacetic Acid
ng	nanogram
NGS	Next-Generation Sequencing
nm	nanometer
nM	nanoMolar
No.	numbers
OD	Optical Density
PBS	Phosphate-Buffered Saline
PCoA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PMA	Propidium Monozide
PTB	PreTerm Birth
qPCR	quantitative Polymerase Chain Reaction
R	Reverse
RDA	Redundancy Analysis
RNA	RiboNucleic Acid
rpm	rotation per minutes
rRNA	ribosomal RiboNucleic Acid
RSV	Ribosomal Sequence Variant
s	seconds
SDS	Sodium Dodecyl Sulphate
SPE	Solid-Phase Extraction
TMA	TriMethylAmine
TMAU	TriMethylAminUria
Tris-HCl	Tris-HydroChloride
TSS	Total Sum Normalization
U	Units
UMIC	**U**rinary and vaginal **MIC**robiome in preterm labour

uR	universal Reverse
V4	Variable region 4
w/v	weight per volume

I. Introduction

I.1. Human Microbiome

The human body is inhabited by trillions of microorganisms which co-evolved together. These microorganisms are estimated to be at least as abundant as the human cells, harbouring a far larger gene pool than the human genome (1,2). Recent studies estimate the number of bacteria inhabiting the human body to be around 1.3 times higher than the number of human cells (1). Approximately 1,000 species of bacteria are present in/on the human body at any time point (3). These complex microbial communities consist of taxa from all three domains of life (Bacteria, Eukaryota and Archaea), as well as viruses. All these microbial communities, together with their genomes, represent the microbiome, often depicted as “human microbiome” (4).

Interestingly, different people harbour different microbial communities, defined as inter-individual variations (5). Still, it is not well known the factors leading to this variability and how it is regulated. The human microbiome is body site-specific and is influenced by different factors like human genetics, immune system interactions, environment, diet, medication, and lifestyles (2,4). The gut is the most studied body biotope due to the high variability between individuals and the highest diversity (compared to other body habitats) (6). The gut microbial community of a healthy individual is usually dominated by bacteria of two phyla: Bacteroidetes and Firmicutes (7). On the other hand, the skin microbiome composition and profile vary across the different sites, the moist areas of the skin being dominated by *Staphylococcus* and *Corynebacterium* species, while the sebaceous sites are less diverse and are colonised mainly by *Propionibacterium* (8). The oral microbiome varies from person to person, but remains relatively stable in the same individual over time (9–11), and different oral sites (saliva, teeth, sub-gingival pockets) harbour relatively different microbial communities (12). The vaginal microbiome in healthy females is dominated by various species of *Lactobacillus* and diverse anaerobic taxa, based on its composition, the vaginal microbiome can be classified into community state types (13). The microbial composition of other body habitats has also been studied concerning different diseases, for example, the microbial community of airway/lung has been explored regarding various conditions such as lung cancer and cystic fibrosis (14).

The functions of the microbiome

The human microbiome confers several essential functions in relation to the human body. Overall the microorganisms offer colonisation resistance, acting as the first line of defence against

pathogens and modulate the development of the immune system. Some other roles of the microorganisms are body site-specific, for example, the microorganisms inhabiting the gut support the transformation of indigestible food components through fermentation into absorbable metabolites, participate in synthesis of essential vitamins, removal of toxic substances, and in modulating and stimulating the immune system (15). The microorganisms present in the oral cavity and the vagina, regulate the pH of these body habitats (6). Furthermore, recent studies have shown possible interactions between the gut microbiome and the human brain, suggesting that microorganisms could even influence the host behaviour (16).

Factors influencing the microbiome

The future of microbiome studies is to alter the microbiome deliberately for preventive and therapeutic treatment. To do this, first, we need to understand the factors influencing the composition of the microbiome.

It is well known that the human microbiome shows high plasticity. Still, at the same time, it remains stable, indicating that the human microbiome composition can remain unaltered over more extended periods and in response to different factors. Changes can occur over time in the microbiome, but most of the variation within the human microbiome is still not entirely explained by the majority of the analysed phenotypic metadata (2,7).

A substantial proportion of the composition of the human microbiome is individualised, and the differences in the microbial communities among individuals are higher than the microbial discrepancies in an individual over time (5). Interestingly, individuals living in the same geographical region share some characteristics in their gut microbial communities with individuals of the same ethnic background, for example, Turks and Moroccans living in Amsterdam have a similar gut microbiota, while the Dutch or other ethnical groups living in the same city show a different microbial composition (17,18). Furthermore, when the microbiome of individuals from different geographic areas are compared, differences in the microbial composition have been observed as well (19,20). These results indicate that the environment has a strong influence on the microbiome.

Studies on twins have shown that the microbiome is also influenced by host genetics; one of the most heritable taxa is the family Christensenellaceae (21). Recent studies have shown that human genetics have a relatively small influence on shaping human microbiome, compared to other factors such as diet. The assembly of the microbial communities is mostly determined by environmental factors, rather than host genetics (22).

The influence of the host immune system on shaping the human microbiome is not entirely understood, although the microorganisms play an essential role in inducing, training and influencing the function of the host immune system (23). Comprehensive studies have focused on the development of the gut microbiome in the first year of life as it influences the development of the immune system and has a long-term impact on the host metabolism and immune health (24).

The effect of the diet has been extensively studied in relation to the gut microbiome. A dietary change strongly influences the gut microbiome composition, leading to changes in the microbial composition within a few days. Different diets have different effects on the gut microbiome. For example, a Western Diet, which is characterised by high-fat and high-carbohydrates, leads to a decrease in the microbial diversity and richness, and a reduction in *Bifidobacterium*, *Lactobacillus* and other beneficial bacteria, this diet often being associated with some diseased states such as obesity, colon cancer and type 2 diabetes. On the other hand, a Mediterranean diet or a diet based on plants is associated with an increase in the diversity and richness of the gut microbiome, characterised by an increase in *Bifidobacterium*, *Lactobacillus* and a decrease in *Clostridium* (25,26). It is well established that the diet can change the microbiome composition, and it is discussed whether the microbiome can influence dietary preferences (27).

Antibiotics have a substantial effect on the microbiome and are the most studied drugs regarding their influence on the human microbiome (28). Although antibiotics are beneficial in fighting infections, they strongly alter the microbial composition, often seen as a reduction in microbial diversity (29). Interestingly, early administration of antibiotics has a long-term effect on the development of a healthy microbiome and has been linked with the development of obesity, asthma, inflammatory bowel disease and other disorders (30,31).

Another important factor which strongly influences the microbiome composition is the lifestyle. Living together with pets and exposure to livestock or living in a rural area has a positive influence on the microbiome, especially in the first years of life (32–34). Furthermore, smoking and stress not only influences the health of an individual but also leads to changes in the microbial composition, which could favour the emergence of pathogenic microbes (25).

Dynamics of the human microbiome

The microbiome establishment and early colonisation are essential in developing a healthy microbiome. Many factors are known to influence the early colonisation such as mode of birth, type of feeding and the introduction of solid foods. C-section born infants are often colonised by skin-like microbes (*Staphylococcus*, *Propionibacterium*, and *Corynebacterium*), while the natural-

born infants acquire beneficial microorganisms (species of *Lactobacillus*, *Prevotella*, *Bifidobacterium*, and *Bacteroides)* from their mother's gut and vagina as they pass through the birth canal (35). The other two major factors influencing the development of a healthy microbiome in the first year of life are breastfeeding and the time when solid foods are introduced. Breast milk contains not only beneficial microbes but also prebiotic components, human milk oligosaccharides (HMOs), which shape the microbiome. The microbial gut community of breastfed infants is often dominated by species of *Bifidobacterium* and *Bacteroides* (36), while the gut microbial community of formula-fed infants is characterised by a higher proportion of Clostridiales and Proteobacteria (37). The introduction of foods leads to a new shift in the microbiome towards an adult-like one (38).

The environment also plays an essential role in the development and maintenance of a healthy microbiome, especially as a large proportion of the human population spends most of their time indoors (39). Previous studies have demonstrated that humans and their environment interact and that most of the microbes found on surfaces in the built environment are of human origin (40). The inhalation of dust from the built environment was showed to lead to asthma development in children living in cities (41), but not in children from rural regions. This observation suggests that the microorganisms present in the built environment in rural areas have a protective role against asthma in comparison to those present in cities (42). It is not entirely understood if microorganisms found in built environments can colonise humans; this aspect has been mainly studied for pathogens and how pathogens are carried across built environments, but there is still lack of information regarding other microorganisms (43).

Moreover, several studies have shown that the environment has an influence on the development of the gut microbiome in the first year of life (44). While many studies on the gut development are focused on the transfer of microorganisms between mother and infant (45–47), it is still widely unknown if the environment could harbour anaerobic microorganisms, and if the environment could act as a source of anaerobes. In our study, we were interested in determining whether human-associated anaerobic microorganisms can survive in an indoor environment and act as 'seeds' for the human microbiome.

The human microbiome can demonstrate high plasticity especially as we know that human interaction with the environment, including pets and other people, creates possibilities for other microorganisms to colonise the body, sometimes leading to observable changes in the microbiome (48). At the same time, the microbiome shows robustness; many studies have shown

that a disruption in the microbiome (which sometimes results due to medication or entry of a pathogen, or a dietary change) is often followed by a return to a healthy state (49,50).

An example of microbial change over an extended period is pregnancy. Studies have shown that during pregnancy, microbial communities from different body niches are changed during the first and third trimester of pregnancy (51). The vaginal microbiome undergoes significant changes during pregnancy, characterised by a decrease in the overall diversity and richness, and an increase in the *Lactobacillus* species (52). These changes are correlated with a decrease in vaginal pH, which has a positive effect, protecting pregnant women from possible vaginal infections. Studies have suggested that the mother's microbiome influences the pregnancy outcome (53). It is hypothesised, that vaginal dysbiosis, infections or changes in specific taxa could lead to preterm birth (13). Therefore previous comparative studies have shown that the vaginal microbiome of women who delivered preterm was characterised by a significantly decreased vaginal microbial richness, diversity and evenness; changes that occurred between the first and third trimester, but no distinct taxa could be associated with preterm birth (54). Other researchers, such as Kindinger et al. (55) have shown that *Lactobacillus iners* was associated with preterm birth, while *Lactobacillus crispatus* has been linked to term birth, suggesting that a vaginal microbiome dominated by *Lactobacillus crispatus* could support in preventing preterm labour. Other studies have indicated that a community state type characterised by low *Lactobacillus* and an increase in the bacterial vaginosis-like microbiome (CSTIV) may be associated with increased risk of preterm delivery (56,57). Even though studies have shown a possible correlation between vaginal microbiome and preterm delivery, there is no causal link between vaginal microbiome composition and preterm delivery. As to further address this topic and explore associations between the microbiome and preterm birth, we investigated the connection between preterm labour and microbiome and hypothesised that changes in the vaginal and urinary microbiome, together with changes in the human milk oligosaccharides could play a role in triggering preterm labour/preterm birth.

I.2. The Human Archaeome

Archaea, together with Bacteria, viruses and small eukaryotes (fungi, protozoa), inhabit the human body. Archaea are classified within their domain of life. Although archaea are prokaryotes like bacteria and share similarities to eukaryotes, they possess unique features, namely a unique cell wall, distinctive metabolic pathways and enzymes, and especially differences in the ribosomal RNAs and ribosomal proteins compared to Eukarya and Bacteria (58,59).

In the human body, archaea colonise different niches, such as the gut, the oral cavity, the skin, the nose, the lungs and the vagina. Previous studies have shown that archaeal communities are body site-specific, similar to bacteria (60,61). The skin is dominated by Thaumarchaeota but also colonised by species belonging to the Euryarchaeota phylum (61–63). The nasal cavity is inhabited by archaeal species belonging to both Thaumarchaeota and Euryarchaeota phyla and shares similarities to archaeal communities present in the gut and on the skin. The lungs are colonised mainly by archaeal species belonging to Woesearchaeota. The oral cavity is inhabited by *Methanobrevibacter oralis*, but other archaeal species have also been identified such as *Methanosphaera* (64), *Methanosarcina* (65), *Methanobacterium* species (66,67) and signatures belonging to Thermoplasmata (68). The gut is mainly colonised by methanogenic species belonging to Methanobacteriaceae and Methanomassiliicoccaceae families, especially: *Methanobrevibacter smithii*, *Methanosphaera stadtmanae*, and *Methanomassiliicoccus luminyensis*. Other methanogenic archaea have been found in the human gut such as Candidatus *Methanomassiliicoccus intestinalis* and Candidatus *Methanomethylophilus alvus*, along with several unknown members of Methanosarcinales, Methanobacteriales, Methanococcales, Methanomicrobiales and Methanopyrales (69). However, not all human gut-associated archaea are methanogens; unknown members of Desulfurococcales, Sulfolobales, Thermoproteales, Nitrososphaerales, and Halobacteriales have also been detected in the human gastrointestinal tract (69). Furthermore, a new species of halophilic archaeon has been isolated from the human gut, *Haloferax massiliensis* (70), indicating that halophilic archaea could be permanent residents and not only transitory microbes as it has been suggested previously (71). In our previous study, we have shown that the gastrointestinal tract has different archaeal communities dependent on the location, for example, the ileum is dominated by archaeal species belonging to *Methanobacterium* and not *Methanobrevibacter* (61). This suggests that the archaeal community composition in the gastrointestinal tract is different compared to the archaeal communities identified in stool samples. These results indicate that some of the archaeal taxa could be attached to other microorganisms or the mucus. Previous studies have shown that the genomes of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* present adhesin-like proteins (72), furthermore other studies have shown that these methanogens can form biofilms (73).

In the vagina, only one study has identified archaeal signatures similar to *Methanobrevibacter smithii*, and one isolate of *Methanobrevibacter smithii* was obtained from a patient with bacterial vaginosis (74).

The most studied archaea associated with the human body are the methanogens due to their correlations with some diseases such as periodontal disease or gastrointestinal disorders. To date, four species of methanogenic archaea have been isolated and cultivated from the human body: the first isolated and described human-associated archaeon *Methanobrevibacter smithii* (75), *Methanosphaera stadtmanae* (76), and most recently *Methanomassiliicoccus luminyensis* (77), were all isolated from human faeces. The fourth isolate, *Methanobrevibacter oralis* (78), was cultivated and described from oral mucosa. It is estimated that up to 96% of all people carry *M. smithii* in their gut (69,79). Several other species have been reported as being isolated from the human body, such as *Methanobrevibacter arbophilus*, *Methanobrevibacter massiliensis*, and two candidate species Ca. *Methanomethylophilus alvus* and Ca. *Methanomassiliicoccus intestinalis* (80), but to date, no other research groups reported the presence of these microorganisms in the human body. Therefore, it is inconclusive if these species are human-associated. The abundance of archaea in the human gut is highly individual, but *Methanobrevibacter smithii* can account up to 10% of gut microbiota and is considered to be present in at least 90% of the population (72,79). Besides *M. smithii, Methanosphaera stadtmanae* appears to be the next abundant archaeon, found in about 30% of all test persons (79). The abundance of *Methanomassiliicoccus luminyensis* is lower compared to the other two, being around 4% in all studied samples (81).

The functions of methane-producing archaea are manifold. Their metabolism allows the use of (small) carbon sources and hydrogen for CH_4 production, and thus their activity is placed instead at the end of the metabolic chain. As a carbon source, *Methanomassiliicoccus luminyensis* can use methanol, and trimethylamine (77,82); whereas *Methanosphaera stadtmanae* uses acetate and methanol (83), and *Methanobrevibacter smithii*, carbon dioxide and formate (72). All methanogenic archaea need hydrogen, which is produced as an end product of bacterial fermentation processes (84). When the hydrogen partial pressure is kept low, then the metabolism of anaerobic bacteria is enhanced. Thus archaea, by using the hydrogen in their metabolism maintain the hydrogen partial pressure low, therefore influencing the metabolic function and growth of the bacterial populations (85). Methanogenic archaea are believed to be “keystone” species that could have a more significant influence on the whole gastrointestinal microbial community composition and function than we currently know (86).

Moreover, archaea can closely associate with specific syntrophic bacteria as it has been previously shown by Samuel and Gordon, the association between *Methanobrevibacter smithii* and *Bacteroides thetaiotaomicron* (87). Although methanogenic archaea have been correlated

with many diseases as being involved in the development of inflammatory bowel disease and dental disease, especially periodontitis (88), not all discovered interactions with archaea are harmful to humans. Recently, *Methanomassiliicoccus luminyensis* (89) was discovered to be able to reduce methanol, as well as mono-, di-, and trimethylamine (TMA) in the presence of hydrogen. TMA is produced in the gut, and in its oxidized form is a risk factor for cardiovascular diseases like atherosclerosis. It is believed to contribute to the symptoms of trimethylaminuria (TMAU), fish odour syndrome. Consequently, *Methanomassiliicoccus luminyensis* and possibly other archaea in the same lineage carrying the same metabolic pathways in their genome could be used as probiotics, or “archaeabiotics”, to prevent these diseases (82,90).

Despite the many roles that archaea have in the human body and their influences over the whole microbial communities, the number of studies focusing on human archaea are scarce. Most studies are focused instead on methanogens, especially on the presence/absence or abundance of particular archaeal species such as *M. smithii* or *M. oralis* rather than on the whole archaeal diversity (see TableS1 from (61)). Additionally, there are several reasons why only some microbiome studies also include archaea (91). Firstly, the identification of archaea in medical samples is often hindered due to differences in abundance between bacteria and archaea and the 16S rRNA gene copy numbers (92); insufficient primer coverage, especially the use of universal primers in many microbiome studies that are not efficient in detecting archaea (60,93); insufficient sample processing and inadequate DNA extraction protocols, in particular, the use of DNA extraction kits based on lysozyme lysis of the cells which would not affect the archaeal walls since they lack bacterial peptidoglycans (94). Another problem is that the archaeal primers often cross-react with human DNA (95). This problem can be overcome by using a nested PCR approach. Secondly, there is no archaeal pathogen known which led to a habit of just overlooking these species within the field of clinical microbiology (96,97).

In our study, we first addressed the problem of inappropriate primer pairs for the detection of archaea, by determining the coverage of 27 different primer pair combinations both *in silico* and experimentally through amplicon sequencing, to identify an optimal approach in detecting archaea in human samples. Secondly, we further explored the archaeal diversity in human samples from different body sites.

I.3. Hypothesis and objectives

This thesis is based on three projects covering different topics related to the human-associated microorganisms and their interaction with the host and the environment.

The **first project** is about the indoor microbiome, with particular focus on the human-associated microorganisms found on surfaces near toilets. Our objective was to explore the microbial composition of these areas and assess the survivability of anaerobic microorganisms associated with the human body. The hypothesis is that certain groups of anaerobic microorganisms can survive under aerobic conditions for a short period; therefore, the environment could act as a source of anaerobic microorganisms.

The **second project** is about the urinary and vaginal microbiome in women at high risk of preterm birth. The overall objective was to assess whether urinary and vaginal microbiome and/or human milk oligosaccharides (HMOs) are altered in women at high risk of preterm birth and to investigate the interaction of HMOs with the microbiome. This project hypothesises that specific changes in the vaginal and urinary microbiome and the profile of HMOs in urine and serum occur in women at high risk of preterm birth. Therefore, understanding these changes could lead to the development of new methods and strategies to identify women at high risk of preterm birth.

The **third project** is focused on investigating the human archaeome present in different body sites. The objectives of this project were: 1. To optimise the detection of archaeal communities in different samples from different body sites using an NGS approach; 2. To use the new method to explore the archaeal communities in various body sites. The hypothesis for this project is that the archaea are present in all body sites where bacterial communities have been previously identified and that the archaeal communities are also body site-specific like bacteria.

II. Material and methods

II.1. Cohorts and sample collection

II.1.1. Ethics statement

Research involving human material was performed in accordance with the Declaration of Helsinki and was approved by the local ethics committees (the Ethics Committee at the Medical University of Graz, Graz, Austria). The ethics votes are mentioned for each project after the description of the samples collected.

II.1.2. House Microbiome

Samples have been collected from 10 different houses to determine if human-associated anaerobic microorganisms can survive in the indoor environment. The sampled surface was selected based on the main source of these anaerobic microorganisms. Two areas of 30 cm^2 have been selected in the proximity of the toilet, cleaned with a bleach solution and sterile water, and left uncovered and untouched for 7 days. After 7 days, the areas were sampled using a sterile nylon swab (FLOQSwabsTM, Copan, Brescia, Italy) that has been dipped in 0.9% saline (NaCl) solution. Each area was sampled with the swab three times, by rotating the swab every time before sampling the area again.

After being transported to the lab on ice packs, one of the samples was treated with propidium monoazide (PMA) to mask the free DNA as described previously (98). From each PMA and nonPMA treated tube, 100 µl was fixed using paraformaldehyde (3% w/v) for fluorescence *in situ* hybridization (FISH) (99).

An overview of the House Microbiome project and the methods used can be found in Appendix 4. Appendix 4 was created in BioRender.com and further processed in Inkscape 0.92.4.

II.1.3. UMIC (Urinary and vaginal microbiome in preterm labour)

The UMIC cohort consisting of 60 pregnant women was recruited during a hospital visit due to possible preterm contractions. All recruited patients had a viable pregnancy >23 weeks gestation, but not more than 34 weeks gestation. All women were healthy, were 18 years of age or older, willing to consent to all aspects of the protocol. Subjects were excluded if they had any recent genitourinary infections, multiple pregnancies, or more than 3 consecutive miscarriages. Women were also excluded if they had any known fetal anomalies associated with possible growth, genetic anomalies, pre-pregnancy diabetes type 1 or 2 or gestational diabetes mellitus, and pre-

pregnancy hypertension. Additionally, we excluded women who took any antibiotic/probiotic treatment in the last 6 months. Clinical metadata from all subjects can be found in Supplementary Table 1 (available only on the compact disk attached to the thesis). Gestational age is provided as weeks and days. Blood, urine and vaginal samples were collected from each patient by trained personnel. All urine samples were collected using urinary catheters in sterile urine tubes. Vaginal swabs were collected using FLOQSwabs (FLOQSwabsTM). All samples were stored at 4°C and aliquots were prepared within 4h after collection and stored at -80°C until further processing. The UMIC study aimed to investigate possible changes in the urinary and vaginal microbiome in preterm labour. An overview of the UMIC study can be seen in Appendix 5. Appendix 5 was generated in BioRender.com and further processed in Inkscape 0.92.4.

The samples included in this study have been obtained covered by the ethics vote with the code 525 ex15/16.

II.1.4. Human Archaeome I

Two different sample sets have been used for improving the detection of archaeal communities in the human samples. The **first sample set** was used to test different amplification protocols for characterizing the archaeal communities in human samples. This sample set was comprised of one sample from different body sites: stool and appendix samples (gastrointestinal tract), oral and nasal samples (head cavities) and a skin sample. The **second sample set** was used to confirm the results from the sample set 1 and was composed of samples from the same body areas: stool (n=5) and appendix (n=5) samples, oral (n=6) and nasal (n=6) samples, and skin (n=5) samples. A schematic overview of the samples and methods used within this study can be found in Appendix 6. Appendix 6 was created in BioRender.com and further processed in Inkscape 0.92.4.

All samples have been obtained covered by ethics votes: stool samples (27-151 ex 14/15), appendix samples (25-469 ex12/13), oral samples (as described in (100)), nasal samples (39/80/63 ex 2014/15) and skin samples (27-263 ex 14/15).

The nasal samples were taken from healthy volunteers from the olfactory mucosa located at the ceiling of the nasal cavity using ultra mini tip nylon flocked swabs (Copan, Brescia, Italy; n=7) (101). A standardized protocol based on paper point sampling was used to obtain the oral samples from healthy volunteers who participated in a microbiome study investigating the subgingival biofilm formation (n=7) (100,102). Appendix samples have been obtained from collaboration partners at the Department of Pediatric and Adolescent Surgery and the Institute of Pathology, both part of the Medical University of Graz. Appendix samples were collected during pediatric

appendectomies from either acute or ulcerous appendicitis (n=6). Stool samples were obtained from healthy volunteers (n=5), and from one patient with above-average methane production after metronidazole treatment (n=1; this sample was used for comparing different amplification protocols). Skin samples were obtained from healthy volunteers from either the back (n=1), this sample was used for comparing different amplification protocols, or the left forearm (n=5) using BD Culture Swabs™ (Franklin Lakes, New Jersey, USA)).

II.1.5. Human Archaeome II

To further explore the archaeal diversity within the human body, samples from different body locations, such as oral cavity, skin, gastrointestinal tract (stool), vagina, lung (bronchoalveolar lavage - BAL), nasal cavity, and urinary tract (urine), have been obtained according to ethics votes: 28-524 ex 15/16 (for the samples obtained from the female participants in the study), 27-289 ex 14/15 (for the samples obtained from the male participants in the study), 39/80/63 ex 2014/15 (nasal samples), 25-221 ex12/13 (BAL).

The nasal swabs were taken from healthy volunteers from the olfactory mucosa as described in the Human Archaeome I section of the sample collection (n=36). Stool samples (n=46) were collected using a sterile stool collection tube from healthy volunteers. Skin samples (n=26) were collected from healthy volunteers from the left arm using BD Culture Swabs™ (Franklin Lakes, New Jersey, USA) that have been dipped in 0.9% NaCl solution. Oral samples (n=46) have been collected from the healthy volunteers from the vestibule of the mouth using a sterile nylon swab (FLOQSwabsTM, Copan, Brescia, Italy). Vaginal samples (n=27) were taken using a sterile nylon swab (FLOQSwabsTM, Copan, Brescia, Italy) by the healthy volunteers. Midstream urine (n=46) was collected from healthy participants using a sterile collection Falcon tube. BAL samples (n=59) were obtained from intubated patients, as described previously (103).

Metadata obtained from all participants can be found in Supplementary Table 2 (available only on the compact disk attached to the thesis).

In total, 286 samples have been tested for the presence of archaea through archaeal specific NGS methods. A schematic overview of the study can be found in Appendix 7. Appendix 7 was generated in BioRender.com and further processed in Inkscape 0.92.4.

II.2. Molecular Approach

II.2.1. DNA extraction

Genomic DNA was extracted from all collected samples using a combined mechanical and enzymatic lysis approach. The samples from the UMIC cohort and all samples from Human Archaeome II, except for the skin and nose samples, were processed using the QIAamp DNA Mini Kit (QIAGEN) with some modification. Before the extraction with the kit, the urine samples were centrifuged at 4400xg for 15 min; the supernatant was removed with the exception of 500 µL that was used to resuspend the pellet. 500 µL of Lysis Buffer (sterile filtered, 20 mM Tris-HCl pH 8, 2 mM Na-EDTA, 1,2% Triton X-100) was added to the vaginal, oral and the stool samples. The BAL samples were mixed with 0.15% of DTT and incubated at 37°C for 20-30 min, followed by centrifugation at 10000 rpm for 10 min. Most of the supernatant was removed except for around 500 µl which was used to resuspend the pellet. To all samples, 50 µL of Lysozyme (10 mg/mL) and 6 µL of Mutanolysin (25 KU/mL) was added, followed by incubation at 37°C for 1 h. The obtained mix was transferred to Lysing Matrix E tubes (MP Biomedicals) followed by a step of mechanical lysis at 5500 rpm for 30 s two times using the MagNA Lyser Instrument (Roche, Mannheim, Germany). After the mechanical lysis, the samples were centrifuged to separate the beads from the supernatant at 10000xg for 2 min. Afterwards, the DNA was extracted according to the provided instructions. The DNA was eluted in 100 µL of Elution Buffer for the vaginal samples, in 60 µL for the urine, BAL and oral samples, and in 200 µL for the stool samples. The genomic DNA concentration was measured using Qubit HS. Most urine samples had a DNA concentration under the detection limit.

The indoor samples, the skin and the nose samples were processed using the FastDNA Spin Kit (MP Biomedicals, Germany) according to the provided instructions. The gDNA was eluted in 100 µL of elution buffer.

The stool samples (around 200 mg) used for improving the detection methods for archaea (Human Archaeome I) were processed using the E.Z.N.A. stool DNA kit according to the manufacturer's instruction. The DNA from the appendix samples was obtained using the AllPrep DNA/RNA/Protein Mini Kit (QIAGEN), before the DNA extraction, small pieces of cryo-tissue were homogenised 3 times for 30 s at 6500rpm using the MagNA Lyser Instrument (Roche Molecular Systems) with buffer RTL and β-mercaptoethanol (according to the manufacturer's instructions). The DNA from the oral samples and the skin samples from the back were isolated using the

MagnaPure LC DNA Isolation Kit III (Bacteria, Fungi; Roche, Mannheim, Germany) as described by Santigli et al. (100) and Klymiuk et al. (104)

II.2.2. 16S rRNA gene amplification through PCR

The gDNA obtained from all samples was used to prepare amplicons by amplifying through PCR the V4 region of the 16S rRNA gene. Table 1 contains the primer pairs used for each cohort. The *in silico* evaluation of the primer pairs and the primers' sequence are given in Table 10 in the Results section.

The PCR reaction mixture for amplifying the V4 region of the bacterial 16S rRNA gene contained: TAKARA Ex Taq® buffer with $MgCl_2$ (10 X; Takara Bio Inc., Tokyo, Japan), primers 300 nM, BSA (Roche Lifescience, Basel, Switzerland) 1 mg/ml, dNTP mix 200 µM, TAKARA Ex Taq® Polymerase 0.5 U, 1 - 50 ng/µl of gDNA and water (Lichrosolv®; Merck, Darmstadt, Germany) up to total volume of 25 µl. The BSA was removed for the UMIC samples due to contamination issues in the new BSA batches.

The V4 region of the archaeal 16S rRNA was amplified using a nested approach to avoid the formation of strong primer dimers by the Illumina tag primers and to increase the specificity for archaea.

In the first PCR, each reaction was performed in a final volume of 20 µl containing: TAKARA Ex Taq® buffer with $MgCl_2$ (10 X; Takara Bio Inc., Tokyo, Japan), primers 500 nM, BSA (Roche Lifescience, Basel, Switzerland) 1 mg/ml, dNTP mix 200 µM, TAKARA Ex Taq® Polymerase 0.5 U, water (Lichrosolv®; Merck, Darmstadt, Germany), and DNA template (1-50 ng/µl). The BSA was no longer used for the Human Archaeome II due to contamination issues in the new BSA batches.

For the Human Archaeome I, the resulted amplicons were purified either with MinElute DNA purification kit (QIAGEN), Monarch PCR&DNA Cleanup Kit (BioLabs), or innPREP DOUBLEpure Kit (Analytik Jena) as indicated in Table 2 and eluted in 10 µl. For the house microbiome study, the amplicons were purified using Monarch PCR&DNA Cleanup Kit (BioLabs), and the obtained amplicons were eluted in 10 µl. For the Human Archaeome II project, this step was omitted, and no purification was performed after the first PCR.

The resulting PCR products were used in a subsequent PCR containing the following mixture: TAKARA Ex Taq® buffer with $MgCl_2$ (10 X; Takara Bio Inc., Tokyo, Japan), primers 500 nM, BSA (Roche Lifescience, Basel, Switzerland) 1 mg/ml, dNTP mix 200 µM, TAKARA Ex Taq® Polymerase 0.5 U, 2 – 8 µL of PCR product, and water (Lichrosolv®; Merck, Darmstadt, Germany)

up to a volume of 25 µL. The BSA was removed for the Human Archaeome II due to contamination issues in the new BSA batches. The PCR cycling conditions are listed in Table 3, according to the primer pairs used.

Table 1. The primer pairs used to amplify the microbial 16S rRNA gene according to each project.

Project	16S rRNA gene	Primer pairs
UMIC	Bacterial V4 region	515FB-806RB
House Microbiome	Bacterial V4 region	515F-806uR
	Archaeal V4 region	344F-1041R/519F-806R
Human Archaeome II	Archaeal V4 region	344F-1041R/519F-806R
Human Archaeome I – sample set 2	Archaeal V4 region	344F-1041R/519F-806R
Human Archaeome I – sample set 1	See Table 2	

Table 2. The PCR primer pairs tested with the sample set 1 of the Human Archaeome I project. The table is taken from (60).

PCR #	Primer combination 1st PCR	Primer combination 2nd PCR
PCR21	349F-915R	Illu 349F-Illu519R
PCR22		Illu 519F-Illu785R
PCR23		Illu 519F-Illu806R
PCR31	344F-1041R	Illu 349F-Illu519R
PCR33		Illu 519F-Illu785R
PCR34		Illu 519F-Illu806R
PCR41	349F-1041R	Illu 349F-Illu519R
PCR42		Illu 519F-Illu785R
PCR43		Illu 519F-Illu806R
PCR61	349F-806R	Illu 349F-Illu519R
PCR62		Illu 519F-Illu785R
PCR63		Illu 519F-Illu806R
PCR71	519F-1041R	Illu 519F-Illu785R
PCR72		Illu 519F-Illu806R
PCR81	519F-806R	Illu 519F-Illu785R
PCR82		Illu 519F-Illu806R
PCR91	344F-519R	Illu 349F-Illu519R
PCRQ1	344F-915R (QIAGEN)	Illu 349F-Illu519R
PCRQ3		Illu 519F-Illu785R
PCRQ4		Illu 519F-Illu806R
PCRM1	344F-915R (NEB Monarch)	Illu 349F-Illu519R
PCRM3		Illu 519F-Illu785R
PCRM4		Illu 519F-Illu806R
PCRA1	344F-915R (Analytik Jena)	Illu 349F-Illu519R
PCRA3		Illu 519F-Illu785R
PCRA4		Illu 519F-Illu806R
PCRQ5	344F-806R (QIAGEN)	Illu 349F-Illu519R
PCRQ6		Illu 519F-Illu785R
PCRQ7		Illu 519F-Illu806R
PCRM5	344F-806R (NEB Monarch)	Illu 349F-Illu519R
PCRM6		Illu 519F-Illu785R
PCRM7		Illu 519F-Illu806R
PCR8-Uni	n.a.	Illu 515F-Illu806uR
PCR9-Uni		Illu 515FB-Illu806RB
PCR10		Illu 519F-Illu806R
PCR11-Uni		Illu 519F-Illu785R

Table 3. The PCR conditions for all the primer pairs used in this thesis. The table is taken from (60).

Target	*Archaea* (16S rRNA gene)			"Universal" (16S rRNA gene)	
(Nested) PCR, round	1°	1°	2°	1°	1°
Primer pair	344F / 915R 349F / 915R 344F / 806R 349F / 806R 519F / 806R	344F / 1041R 349F / 1041R 519F / 1041R	All Illumina tagged primer pairs	Illu519F /Illu806R Illu519F/Illu785R	Illu515F/Illu806uR Illu515FB/Illu806RB
Initial denaturation	2', 95°C	5', 95°C	5', 95°C	5', 95°C	3', 94°C
Denaturation	30", 96°C (first 10 cycl.), 25" 94°C	30", 94°C	40", 95°C	40", 95°C	45", 94°C
Annealing	30", 60°C	45", 56°C	2', 63°C	2', 63°C	1', 50°C
Elongation	1', 72°C	1', 72°C	1', 72°C	1',72°C	1' 30", 72°C
Final elongation	10', 72°C	10', 72°C	10', 72°C	10', 72°C	10', 72°C
No. of cycles	25	25	30	40	40

II.2.3. Quantitative PCR of the bacterial and archaeal 16S rRNA genes

A quantitative approach based on qPCR was used to determine the ratio between the number of archaeal and bacterial copies of 16S rRNA genes in the samples from the **house microbiome** project. Additionally, a qPCR approach was used for the **UMIC study** to compare the bacterial copies of 16S rRNA genes of the urinary and vaginal samples between the cases and controls. The qPCR was performed based on SYBR chemistry, and the used primer pairs can be found in Table 4.

The reaction mix contained: 1x SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, USA), 300 nM of forward and reverse primer, 1-2 µL of gDNA template (1-50 ng/µl), and water (Lichrosolv®; Merck, Darmstadt, Germany). The qPCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The qPCR conditions used are given in Table 5.

Table 4. Primer pairs used for archaea and bacteria qPCR.

Approach and target	Name	Primer name*	Sequence (5'-3')	Reference
SYBR (Archaea) House Microbiome	806 aF	S-D-Arch-0787-a-S-20	ATTAGATACCCSBGTAGTCC	(105)
	958 aR	S-D-Arch-0958-a-A-19	YCCGGCGTTGAMTCCAATT	(106)
SYBR (Bacteria) House Microbiome	338 bF	S-D-Bact-0337-a-S-20	ACTCCTACGGGAGGCAGCAG	(107)
	517 uR	S-*-Univ-0517-a-A-15	GWATTACCGCGGCKGCTG	(108)
SYBR (Bacteria) UMIC project	331 bF	S-D-Bact-0340-a-S-19	TCCTACGGGAGGCAGCAGT	(109)
	797 bR	S-D-Bact-0781-a-A-26	GGACTACCAGGGTATCTAATCCTGTT	(109)

*according to (110)

Table 5. Quantitative PCR conditions. For denaturation, annealing and elongation, the corresponding time and temperature are given. The last two columns contain the efficiency and R^2 values.

		QPCR conditions						Standard curves	
Target gene	**Primer pair**	**Initial denaturation**	**Denaturation**	**Annealing**	**Elongation**	**No. of cycles**	**Melting curve**	**Efficiency**	**R^2**
Bacteria (16S rRNA gene)	338 bF-517 uR	15', 95°C	15", 94°C	30", 60°C	40", 72°C	40	60-95°C	85.26	0.956
Bacteria (16S rRNA gene)	331 bF – 797 bR	15', 95°C	15", 94°C	30", 54°C	40", 72°C	40	60-95°C	90-105	>0.9
Archaea (16S rRNA gene)	806 aF-957 aR	15', 95°C	15", 94°C	30", 53°C	40", 72°C	40	60-95°C	88.8	0.974

Crossing point (Cp) values were determined using the regression method within the Bio-Rad CFX Manager software version 3.1. Absolute copy numbers of bacterial and archaeal 16S rRNA genes were calculated using the Cp values and the reaction efficiencies based on standard curves obtained from defined DNA samples from *Nitrososphaera viennensis* and *Escherichia coli* (63). The qPCR efficiency and R^2 values of the standard curves can be found in Table 5 (last two columns).

Detection limits were defined based on the average Cp values of non-template controls (triplicates) and the corresponding standard curves of the positive controls. The detection limits were 1224 copies for archaea and 523 copies for bacteria in the House Microbiome project and varied between 1300 to 320 copies for urine and vaginal samples in the UMIC project.

All qPCR reactions were performed in triplicates. Only samples with positive results in 2 out of 3 or 3 out of 3 replicates were considered for further analysis. The number of copies identified in the negative controls was subtracted from all samples; therefore, the results are only the number of copies of 16S rRNA from the samples.

II.2.4. Amplicon sequencing and metagenomics

Library preparation and sequencing of the amplicons were carried out at the Core Facility Molecular Biology at the Center for Medical Research at the Medical University Graz, Austria. In brief, DNA concentrations were normalized using a SequalPrep™ normalization plate (Invitrogen), and each sample was indexed with a unique barcode sequence (8 cycles index PCR). After pooling of the indexed samples, a gel cut was carried out to purify the products of the index PCR. Sequencing was performed using the Illumina MiSeq device and MS-102-3003 MiSeq® Reagent Kit v3-600cycles (2x251 cycles).

The data processing of the obtained MiSeq sequence data was performed using either the open-source package DADA2 in R (Divisive Amplicon Denoising Algorithm; (111)) as described previously (98) or by using DADA2 method directly in QIIME2 (112) as described previously (113). Shortly, the DADA2 turns paired-end fastq files into merged, denoised, chimaera-free, and inferred sample sequences called ribosomal sequence variants (RSVs). The taxonomic affiliations were determined using SILVA v128 (for Human Archaeome I) or SILVA v132 (for all the other projects) database as the reference database (114). In the resulting RSV table, each row corresponds to a non-chimeric inferred sample sequence with a separate taxonomic classification. Negative controls (extraction controls and no-template controls) were included during PCR amplification. The RSVs overlapping the negative controls and samples were either subtracted or completely removed from the data sets. The bacterial sequences were removed from analysis for the projects Human Archaeome I and II. The obtained RSVs tables without the negative controls were used for further analysis.

II.3. Data analysis and statistics

II.3.1. House microbiome

For the house microbiome study, all analyses were done by comparing the nonPMA with the PMA treated samples. Bar charts were constructed for both the bacterial and archaeal communities at phylum and genus level using the *ggplot2* package in R (115). Before analysis in Calypso, the data was normalized using total sum normalization (TSS) combined with square root transformation. Alpha diversity based on Shannon and richness indices, the bar plots and PCoA plots based on Bray-Curtis index, together with the statistical analyses for testing the differences between PMA and nonPMA treated samples were performed and generated in Calypso. To test for differences in the beta diversity between PMA and nonPMA treated samples, Adonis test was performed for the Bray-Curtis index in Calypso (116). Bar plots showing differences between PMA and nonPMA treated samples of the relative abundance of specific taxa were also plotted in Calypso. Bug Base was used to determine the phylotypes such as aerobic, anaerobic, facultative anaerobic and stress-tolerant communities which were present in the PMA and nonPMA treated samples.

A phylogenetic tree was constructed to determine if the sequences belonging to methanogens identified in the analysed samples are of human origin. All sequences classified within the genera *Methanobacterium*, *Methanobrevibacter* and *Methanomassiliicoccus* from our analysed samples were used for creating the phylogenetic tree. Additionally, 16S rRNA sequences of species of

Methanobacterium, *Methanobrevibacter* and *Methanomassiliicoccus* previously identified in the human body or the environment were included. The alignment was performed using the SILVA SINA alignment tool (117). The sequences were cropped to the same length using BioEdit and used afterwards to construct a tree based on the maximum-likelihood algorithm with a bootstrap value of 1000 using MEGA7 (118). The phylogenetic tree was further processed using the online tool iTOL (119).

II.3.2. UMIC

After classification with the SILVA database v132, the sequences classified as *Lactobacillus* genus were further used to classify to species level allowing the clustering of the vaginal microbiome into community state types based on the hierarchical clustering. The classification was performed through EzBioCloud (120). To further test that the classification is correct, a Maximum Likelihood tree was constructed in MEGA7 (118) using the sequences classified into the *Lactobacillus* genus. The alignment was performed in SINA (117), and the closest neighbours were picked only from the All-Species Living Tree project (121). The tree can be found in the Supplementary Figure S1 (Appendix 1).

The hierarchical clustering was performed in R on the relative abundance at species level based on Bray-Curtis dissimilarity and the method *ward* using the packages *vegan* (122) and *gplots* (123). Alpha diversity indices were calculated using the *vegan* package in R. For alpha diversity analysis; the data was rarefied to the lowest number of reads, which was over 2000 reads. Plots of the alpha diversity indices were generated in R using *ggplot2* package (115). Differences in the alpha diversity indices between the groups were tested in R using Wilcoxon Rank test. PCoA plots were constructed based on Bray-Curtis dissimilarities using *phyloseq* (124) and *ggplot2* packages in R. The differences in the microbial composition between the analysed groups was tested in R using Adonis test. Bar plots based on relative abundance at genera level were plotted for both the urinary and vaginal microbiome using *ggplot2*. LEfSe analysis plots and Spearman correlation heatmaps were generated using Calypso (116). Microbial functional profiles from 16S rRNA gene data were predicted by Tax4Fun (125). The results were visualised *via* Calypso. Before data analysis in Calypso, the data was normalized using total sum normalization (TSS) combined with square root transformation, and the rare taxa below 1% were removed. All statistical analyses were performed in SPSS (IBM Corp.) if not stated otherwise.

II.3.3. Human Archaeome I

Processing of sequencing data was performed using DADA2 (111), and subsequent statistical analyses were performed in R version 3.4.3 (126). Alpha diversity was calculated using the Shannon diversity. To identify differences between the archaeal diversity, Wilcoxon Rank Test was performed. The diversity of the archaeal communities within the **sample set 2** was determined using two diversity matrices (Shannon and Richness). Analysis of variance (ANOVA) was performed to test for differences in the archaeal diversity based on the body location. Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances was used to visualise differences between the samples from different body site. Redundancy discrimination analysis (RDA) was used to analyse the association between archaeal community composition and the body site location. RDA, alpha diversity and PCoA analysis were performed using Calypso Version 8.62 (116). Before data analysis in Calypso, the data was normalized using total sum normalization (TSS) combined with square root transformation. The RSV tables obtained were used to summarize taxon abundance at different taxonomic levels. The taxonomic profiles obtained at the genus level were used to generate bar plots for all samples.

A phylogenetic tree was constructed with the obtained archaeal RSVs from the **sample set 1**, from the universal approach with the primers 515F-806uR and 519F-785R, the archaeal primer pair 519F-806R, and the archaeal specific primer pair combination 344F-1041R/519F-806R. The alignment of the sequences was performed using the SILVA SINA (117) and the 5 most closely related available sequences (neighbours) were downloaded together with the aligned sequences. All sequences were cropped to the same length and used to construct a tree based on the maximum-likelihood algorithm using MEGA7 (118), with a bootstrap value of 500. The Newick output was further processed with iTOL interactive online platform (119).

II.3.4. Human Archaeome II

II.3.4.1. Amplicon Sequencing

The obtained RSV tables after QIIME2 analysis were used to summarize taxon abundance at different taxonomic levels. The taxonomic profiles obtained at phylum and genus level were used to generate bar plots for all the samples classified within the body-location from where the samples were collected.

The data was further analysed in Calypso. All the data analysed in Calypso was normalized using total sum normalization (TSS) combined with square root transformation. Alpha diversity based

on Chao1, Shannon and Inverse Simpson indices was performed only on samples with more than 500 reads. PCoA based on Bray-Curtis index and RDA plots were performed to explore the differences in the archaeal communities present in the various body sites. The differences in the relative abundance of specific taxa were assessed for the various body sites analysed. To observe the influence of sex and age on the archaeal communities, we performed LEfSe analysis and ANOVA tests based on the relative abundance.

A microbial community network was constructed using Cytoscape 3.7.2 (127). Using the *make_otu_network.py* script in QIIME 1.9.1 (128) the G-test for independence and edge weights were calculated on the archaeal RSV table. The obtained network table was further processed in Cytoscape 3.7.2 and visualised as a bipartite network of body sites (hexagons) and RSV nodes (circles) connected by edges. The size of the hexagons and circles was correlated with the RSV abundances, and the intensity of the connecting line refers to the RSV presence in the analysed samples from the different body sites.

II.3.4.2. Metagenomics analysis

Based on the results from the amplicon sequencing approach, 20 samples were selected (4 BAL, 5 nasal samples, 4 oral, 4 urinary and 3 vaginal samples) and prepared for metagenomics sequencing. The libraries were prepared using the TruSeq DNA Nano DNA Library Prep kit, and the sequencing was performed on Illumina HiSeq X with 2 x 150 bp (600 Mio. reads per lane). Macrogen performed the library construction and sequencing.

The raw data was first quality checked using FastQC. Based on the FastQC report, the sequencing adaptors were removed from sequences and quality filtered according to the Phred score (>q35) as well as length filtered (min. 50bp) by trimming at the 3' prime end using the trimmomatic tool (129). The filtered-samples were used for a genomic-centric approach. The assembly was performed using MEGAHIT with default settings (130). The obtained contigs were further binned using MaxBin 2.0 (131), and the binning quality of the contigs was validated by CheckM (132).

II.4. Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization was performed on the samples collected from the House Microbiome project as described previously (63). The FISH was performed both in solution and on slides. Shortly, the samples were fixed in paraformaldehyde (3% (w/v) after collection. For detection of archaea, a mix of three CY3 labelled probes (ARC344, ARC915, ARC1060) was used, and for bacteria, the probe CY5 labelled EUB338 was used. The hybridization was performed at

46°C for 2 h. In the initial step, hybridization buffer containing 20% (v/v) formamide, 1% (w/v) SDS, 20 mmol/L Tris-HCl, pH 7 (AMRESCO, OH, USA), and 0.9 M NaCl (VWR Chemicals, Pennsylvania, USA) was added to the samples, followed by an incubation for 15 min at 46°C. After the incubation step, the mixture of probes (50 ng each) was added to the samples. A NONEUB-probe (Eurofins) was used as a nonsense negative control. The sequences of the used probes are given in Table 6. After hybridization, the samples were washed using a buffer containing 10 mM Tris-HCl (pH 7), 0.225 M NaCl and 1% sodium dodecyl sulfate and incubated at 46°C for 15 min. Afterwards, samples were washed with cold water and allowed to dry at room temperature before counterstained with DAPI (Sigma Aldrich). The samples were analysed using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) using the following filter settings (excitation/emission): DAPI (358 nm/463 nm), CY3 (549 nm/562 nm) and CY5 (646 nm/664 nm). The figures were visualized and prepared in Carl Zeiss ZEN 3.0 Black Edition software.

Table 6. Probes used for FISH analysis and their sequences.

Name of probes	Sequence (5'-3')	References
EUB338	GCTGCCTCCCGTAGGAGT	(133)
ARC344	TCGCGCCTGCTGCICCCCGT	(105)
ARC915	GTGCTCCCCCGCCAATTCCT	(134)
ARC1060	GGCCATGCACCWCCTCTC	(135)
NONEUB	ACTCCTACGGGAGGCAGC	(136)

II.5. Cultivation and oxygen tolerance test

An oxygen sensitivity test (Fig. 1) was used to determine if methanogenic archaeal strains can survive under aerobic conditions for a specified period. Two human-associated methanogens strains (*Methanosphaera stadtmanae* DSM no. 3091 and *Methanobrevibacter smithii* DSM no. 2375) and a newly isolated strain of methanogen (*Methanobrevibacter sp*.) have been tested for their ability to survive under aerobic conditions. The human-associated methanogens cultures were obtained from DSMZ and grown on special medium at 37 °C (see Appendix 1). The new *Methanobrevibacter* strain was isolated in our lab by our PhD colleague, Marcus Blohs, from a stool sample obtained from a healthy donor using the MS medium (see Appendix 1).

Cultures have been used after 3-5 days of growth as it follows: 1 mL culture has been transferred to sterile 1,5 mL Eppendorf tubes and centrifuged at 10000xg for 10 min, the cell pellet was washed twice either anaerobically for the controls or aerobically for the oxygen exposed

microorganisms with 1 mL sterile 1% PBS and centrifuged again at 10000xg for 10 min. After removing most of the PBS, the cell pellet was resuspended in the left liquid in the tube and exposed to aerobic or anaerobic conditions for different time points: 0h (30 minutes after transfer), 6h, 24h, 48h, and 168h (7 days). After exposure, the liquid from the tubes was transferred to a sterile medium in Hungate tubes and the cultures were left to grow for 2 weeks. After 2 weeks, the first OD measurements (600 nm) and methane measurements were done using a spectrometer and methane sensor (BCP-CH4 sensor, BlueSens). For the methane measurement, 10 mL of gas was taken from the Hungate tubes using an air-tight glass syringe. For each experiment, two sterile unopened Hungate tubes with media served as control and two inoculated Hungate tubes with 0,5 mL of the culture served as a positive control. In addition, a tube in which 1% PBS was added served as a control for 168h. The experiment was performed in triplicates. After OD measurements, microscopic observations were done to determine the purity of the culture and the shape of the microorganisms growing in the medium.

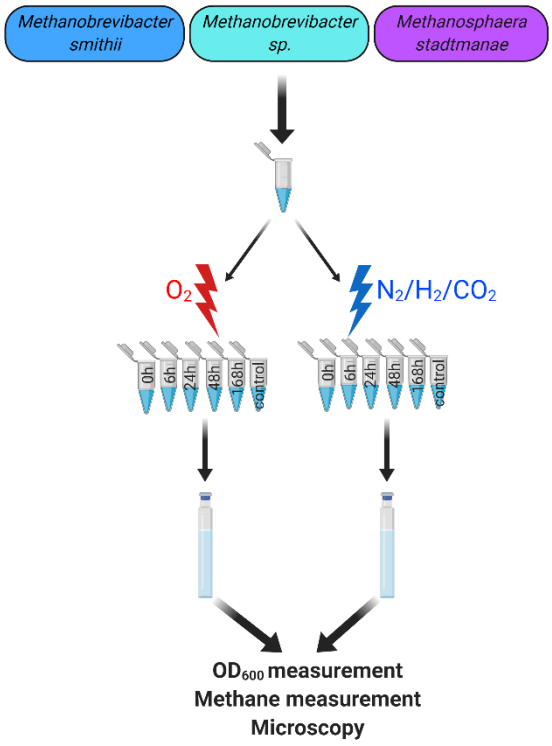

Figure 1. Oxygen tolerance test developed to test the tolerance of methanogens to aerobic environment.

II.6. Human milk oligosaccharides (HMOs) analysis

Oligosaccharides from maternal serum were isolated, as previously described (137). In brief, 50 µL serum was diluted with H_2O containing the internal standard Linear B6-Trisaccharide (Dextra Laboratories), and samples were subjected to 2 cycles of chloroform/methanol (2:1) extraction, followed by solid-phase extraction (SPE) using C18 columns (Thermo Fisher Scientific) and graphitized carbon columns (Thermo Fisher Scientific). Urinary HMOs were isolated by exposing 10 µL urine samples with internal standard directly to C18 columns, then following the same protocol as for serum samples.

Isolated HMOs from serum and urine samples were fluorescently labelled with 2-aminobenzamide (2AB), as previously described (138). The 2AB-glycans were separated by HPLC with

fluorescence detection (360 nm/425 nm) on a TSKgel Amide-80 column (Tosoh Bioscience, Japan), using a linear gradient of a 50mmol/L-ammonium formate/acetonitrile solvent system. Commercially available standards for 2'-Fucosyllactose (2'FL), Lactodifucotetraose (LDFT), 3'-Sialyllactose (3'SL), 3'-Sialyllactosamine (3'SLN), and 6'-Sialyllactosamine (6'SLN) (Prozyme, Hayward CA) were used to annotate HPLC peaks. The amount of each HMO was calculated based on pre-determined response factors.

For normalizing the HMO concentration in urine samples, total osmolality indicative of solute concentration was measured in urine samples using a cryoscopic osmometer (Osmomat 030, Gonotec, Germany) according to the manufacturer's protocol. HPLC Chromatogram area under the curve (AUC) for individual HMO peaks were divided by osmolality and multiplied by the mean of the osmolalities of all urine samples. Secretor status was determined based on the relative abundance of 2'FL and LDFT.

HMO data are presented as median and IQR for skewed data. Changes in HMOs between groups were tested using Mann-Whitney-U-test, and graphs were plotted in GraphPad Prism (version 8.00; GraphPad Software, La Jolla California, USA).

III. Results

III.1. House Microbiome

Samples were obtained from two surface areas of around 30 cm^2 from the floor near the toilet from ten different houses to determine whether toilets are a source of anaerobic microorganisms and if these microorganisms can survive in an aerobic environment. In order to make a distinction between cells that have an intact membrane or not, one of the collected samples from each house has been treated with PMA (propidium monoazide). PMA is a photoreactive DNA binding dye. When a cell is damaged, the PMA can enter the cell and intercalate the DNA, and upon light activation, an irreversible DNA modification happens, which leads to an inhibition of the DNA amplification. On the other hand, PMA cannot enter cells which have an intact membrane or cell wall. Therefore, in the downstream analysis, only the DNA from these cells can be further amplified, thus preventing the detection of dead microorganisms (139).

III.1.1. Microbial composition

Two amplicon sequencing approaches were used in our study, one universal approach based on primers 515F-806uR (140) to explore the overall microbial communities and one archaeal-targeting approach, as previously described (60), to determine mainly the archaeal communities present in the analysed samples.

The universal approach allowed the detection and classification of most sequences within the Bacteria domain (99.86%), and only a small percentage of the sequences were classified as part of the Archaea domain (0.14%) for both the nonPMA and PMA treated samples. A total of 32 phyla were observed and most sequences classified within four phyla: Firmicutes (52.2% nonPMA, 41.2% PMA), Actinobacteria (20.3% nonPMA, 26% PMA), Proteobacteria (18.8% nonPMA, 23.3% PMA), and Bacteroidetes (6% nonPMA, 4.3% PMA) (Fig. 2A). Within these dominant phyla, taxa associated with human skin (e.g. *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Neisseria*, *Micrococcus*, *Cutibacterium*), gastrointestinal and genitourinary tract (e.g. *Finegoldia*, *Lactobacillus*, *Bacteroides*, *Anaerococcus*, *Peptoniphilus*, *Lachnospiraceae*) have been identified in both nonPMA and PMA treated samples (Fig. 2B).

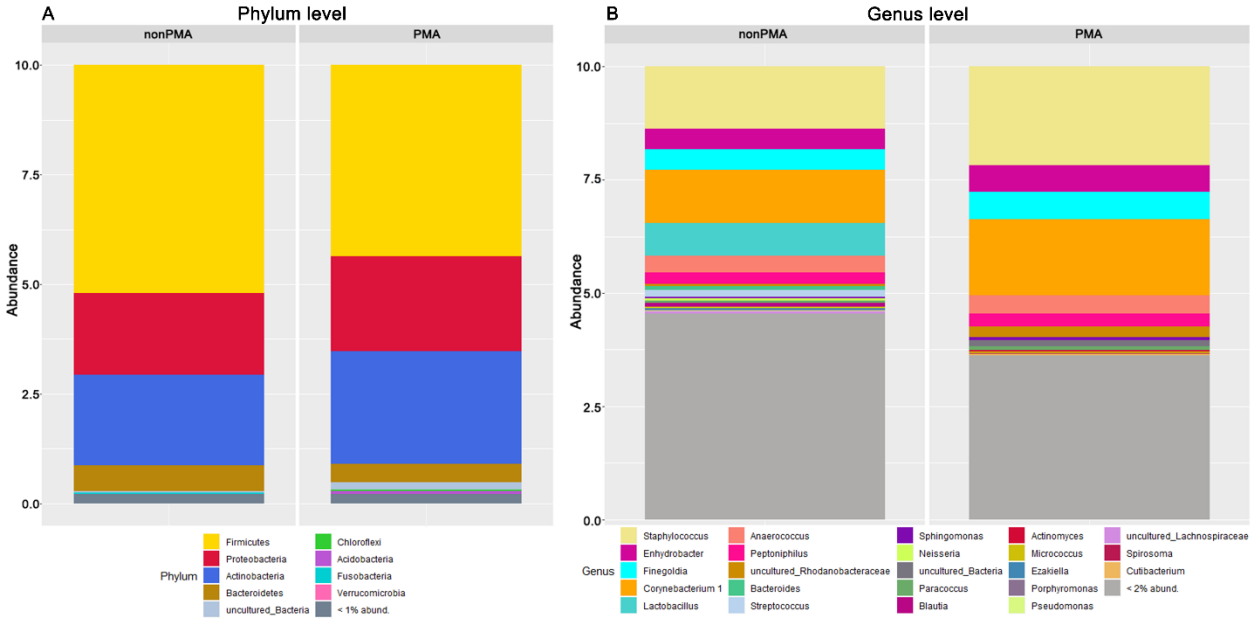

Figure 2. The bar charts show the microbial composition of the nonPMA and PMA treated bathroom floor samples at (A) phylum and (B) genus level.

With the archaeal-targeting approach, most sequences were classified within the Archaea domain (98,4% nonPMA and 98.3% PMA) and only a small proportion within Bacteria (1.6% nonPMA and 1.7% PMA). The bacterial reads were removed bioinformatically, and only the archaeal reads were further processed. The universal approach allowed only the detection of signatures from Euryarchaeota and Thaumarchaeota phyla, while the archaea-targeting approach led to the identification of signatures belonging to four different archaeal phyla (i.e. Euryarchaeota, Thaumarchaeota, Crenarcheota, and Nanoarchaeota). Most of the archaeal sequences were classified within the phylum Thaumarchaeota (62.2% nonPMA, 64.1% PMA), followed by Euryarchaeota (36.6% nonPMA, 35.0% PMA), and only less than 1% were classified within Crenarchaeota (0.8% nonPMA, 0.5% PMA) and Nanoarchaeota (0.4% nonPMA, 0.5% PMA) phyla (Fig. 3A).

Most reads were classified within the Nitrososphaeraceae family (62.10% nonPMA, 64.06% PMA), followed by Halococcaceae family (19.74% nonPMA, 28.71% PMA). Within the Nitrososphaeraceae family, the dominant genus identified was Candidatus *Nitrosocosmicus* (22.5% nonPMA, 15.9% PMA), but sequences classified within Candidatus *Nitrososphaera* (0.9% nonPMA, 0.2% PMA) were also identified (Fig. 2B). From the Halococcaceae family, the dominant genus determined in our samples was *Halococcus* (13.7% nonPMA, 28.6% PMA) (Fig. 3B).

Besides these two families, signatures belonging to methanogens have also been identified, the most dominant genera being: *Methanosarcina* (3.2% nonPMA, 1.8% PMA), *Methanobrevibacter* (2.4% nonPMA, 0.2% PMA), *Methanobacterium* (1.3% nonPMA, 3.3% PMA), and *Methanomassiliicoccus* (0.4% nonPMA, 0.2% PMA) (Fig. 3B). Similar to the bacterial communities, the archaeal communities identified on the surface near the toilet were dominated by skin-associated taxa belonging to Thaumarchaeota phylum, and some methanogens, which are associated with the human gastrointestinal tract.

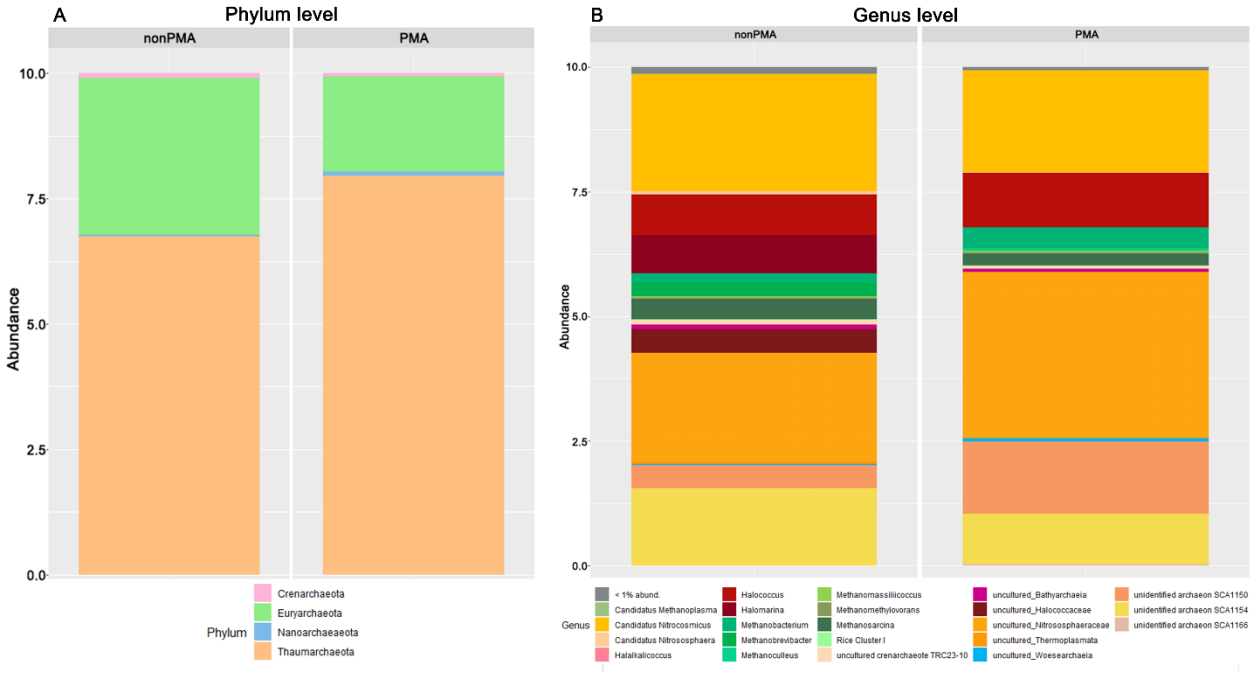

Figure 3. The bar charts show the archaeal communities present in the analysed nonPMA and PMA bathroom floor samples at (A) phylum and (B) genus level.

III.1.2. The influence of the PMA treatment on the microbial communities

To determine the influence of PMA treatment on the microbial communities, we explored the alpha and beta diversity between the samples treated with PMA and those untreated for both the bacterial and archaeal communities. The alpha diversity showed a slight decrease in the PMA treated samples, but the difference was not statistically significant (Fig. 4A, B).

PCoA plots based on Bray-Curtis showed no clear separation based on the PMA treatment, most PMA and nonPMA samples from the same house clustered nearby, despite the PMA treatment, although the bacterial composition was affected by the PMA treatment as indicated by the Adonis test (Fig. 5A, B).

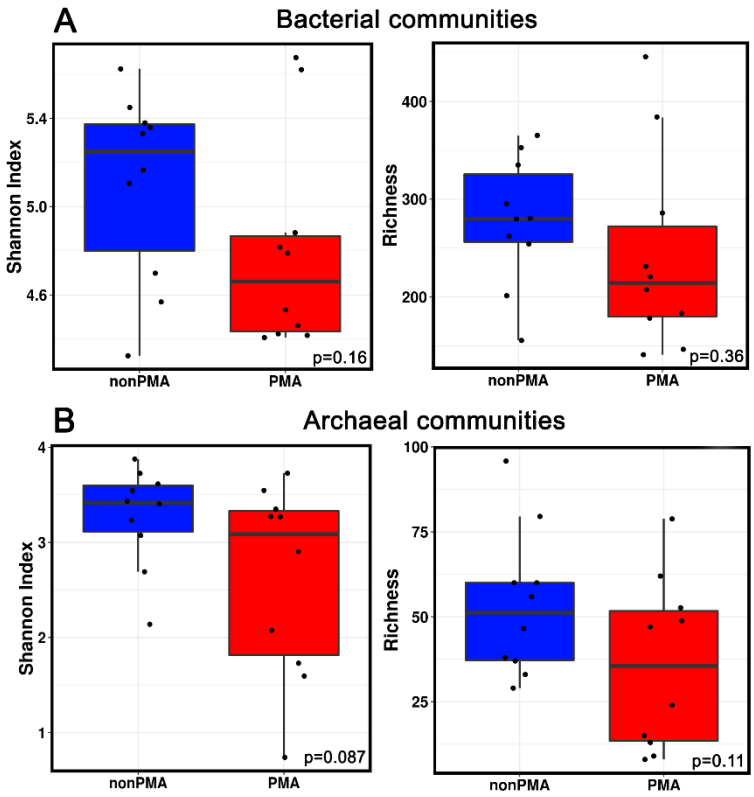

Figure 4. Alpha diversity based on Shannon index and Richness of the (A) bacterial and (B) archaeal PMA/nonPMA treated communities.

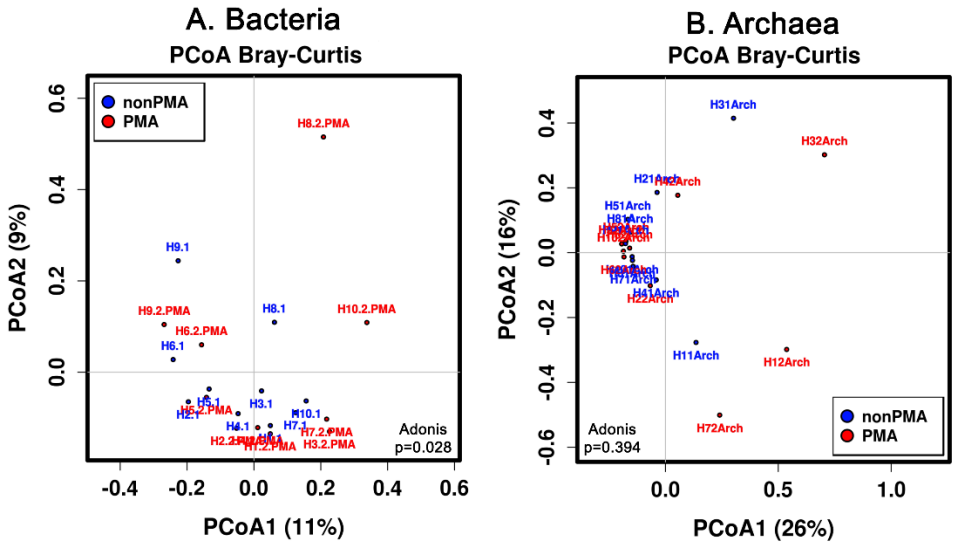

Figure 5. Principal coordinates analysis (PCoA) plots based on Bray-Curtis dissimilarity of the (A) bacterial and (B) archaeal PMA/nonPMA treated community composition.

The results of the quantitative PCR based on 16S rRNA gene revealed a significant decrease in the number of copies for both bacteria and archaea between the nonPMA and PMA treated samples, which is to be expected, as the PMA treated samples should contain DNA only from cells with intact cell wall or membrane (Fig. 6A, B). The ratio between bacteria and archaea was 100:1 in the nonPMA samples, and 70:1 in the PMA treated samples.

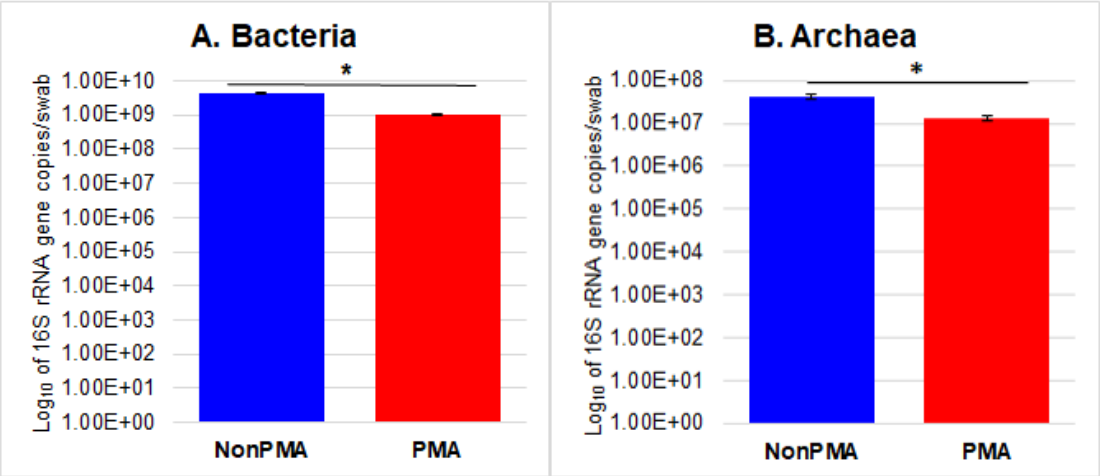

Figure 6. The number of 16S rRNA gene copies of (A) bacteria and (B) archaea of the nonPMA and PMA treated samples.

To explore the survivability of anaerobic microorganisms in the indoor environment, we performed RankTest analysis on the relative abundance for specific anaerobic taxa for the PMA treated and untreated samples. The analysis revealed a decrease in the abundance of most taxa, although the decrease was not always significant. Nevertheless, anaerobic microorganisms were detected in the PMA treated samples, indicating that these cells had still an intact membrane at the time of sampling (Fig. 7A, B).

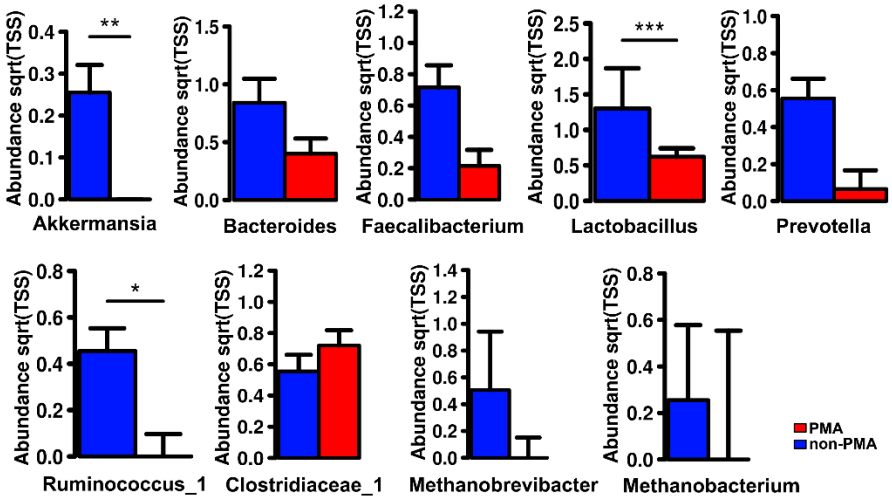

Figure 7. RankTest analysis of the relative abundance of specific bacterial and archaeal anaerobic taxa in the PMA and nonPMA treated bathroom floor samples.

III.1.3. Phenotype analysis

We used BugBase (141) to determine the phenotypes present in the analysed microbial communities for the nonPMA and PMA treated samples. BugBase is a microbiome analysis tool predicting the microbial phenotypes at the organism-level. The analysis showed that aerobic communities were predominant in the PMA treated samples, while the relative abundance of anaerobic communities was similar between the PMA and nonPMA treated samples. Additionally, the microbial communities present in the PMA treated samples showed a high relative abundance of facultative anaerobes and had a higher stress tolerance compared to the microbial communities present in the nonPMA samples (Fig. 8).

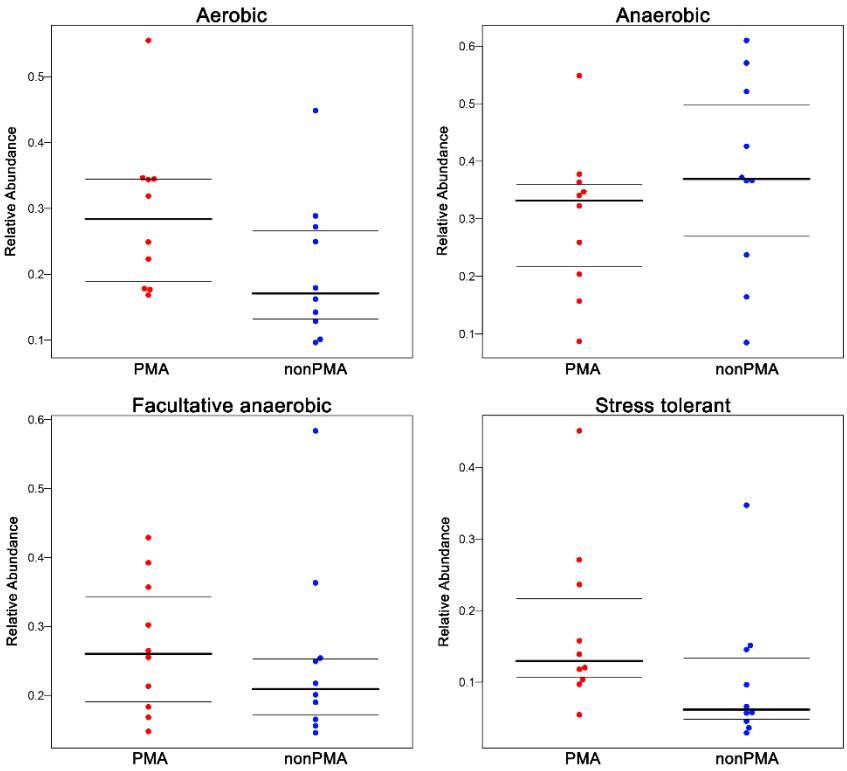

Figure 8. The relative abundance of different phenotypes as determined through BugBase between the microorganisms found in the nonPMA and PMA treated samples.

To further explore if anaerobic microorganisms identified in our experiment are of human-origin, we selected sequences belonging to methanogens known to be found both in humans and in the environment from the nonPMA, and PMA treated samples. The selected sequences were used to determine whether these methanogens identified in our samples are either human-associated or environment-associated. Additionally, we selected sequences from NCBI or other publications of similar methanogens isolated from the human body or different environments and constructed a phylogenetic tree. For *Methanobrevibacter*, the sequences obtained from our study classified within human-associated *Methanobrevibacter* taxa. *Methanomassiliicoccus* sequences clustered mainly into two different clades, one host-associated and one environment-associated following the observations made by Borrel et al. (142). Our samples clustered only in the environment-associated clade together with other strains isolated or identified in the human body, but also with the environment-associated *Methanomassiliicoccus* taxa. For *Methanobacterium*, the sequences identified in our study were mainly associated with environmental *Methanobacterium* taxa,

although some human-associated *Methanobacterium* were clustered in the same groups with the environmental taxa (Fig. 9).

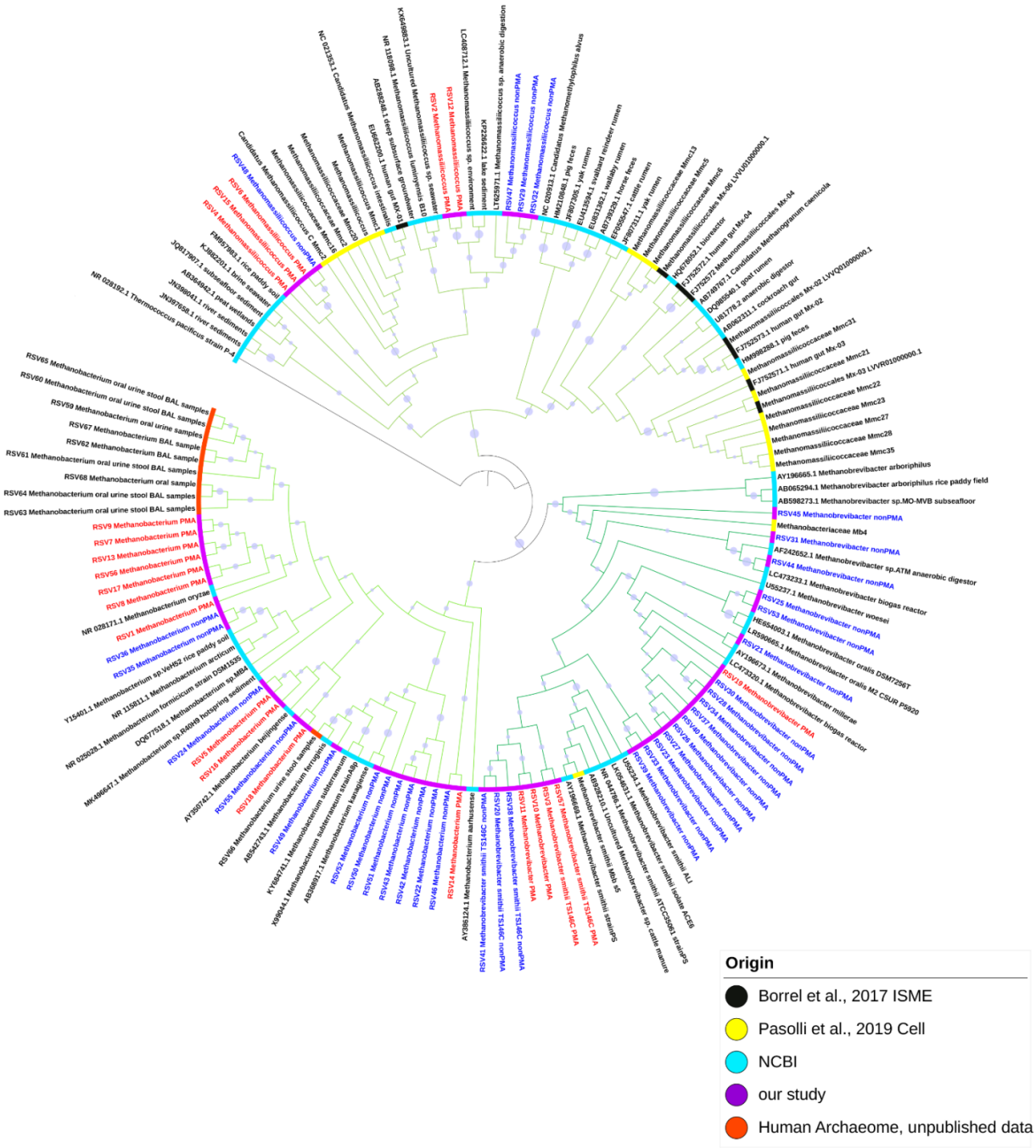

Figure 9. Phylogenetic tree based on the sequences obtained from our study, NCBI, and three other studies as indicated in the legend of the tree under the name “Origin”. The circle indicates the origin of the sequences used for creating the tree (see legend). The branches of the tree were coloured in different shades of green according to the genus it represents.

III.1.4. Fluorescence *in situ* hybridization results

We performed fluorescence *in situ* hybridization (FISH) to observe the microorganisms present in the areas sampled. In most of the samples, bacterial communities could be observed as indicated by the white colour in Fig. 10. An archaeal signal was detected in only two samples, as showed by the red colour in Fig. 10 G, H. The observed microbial communities were found in the nonPMA treated samples.

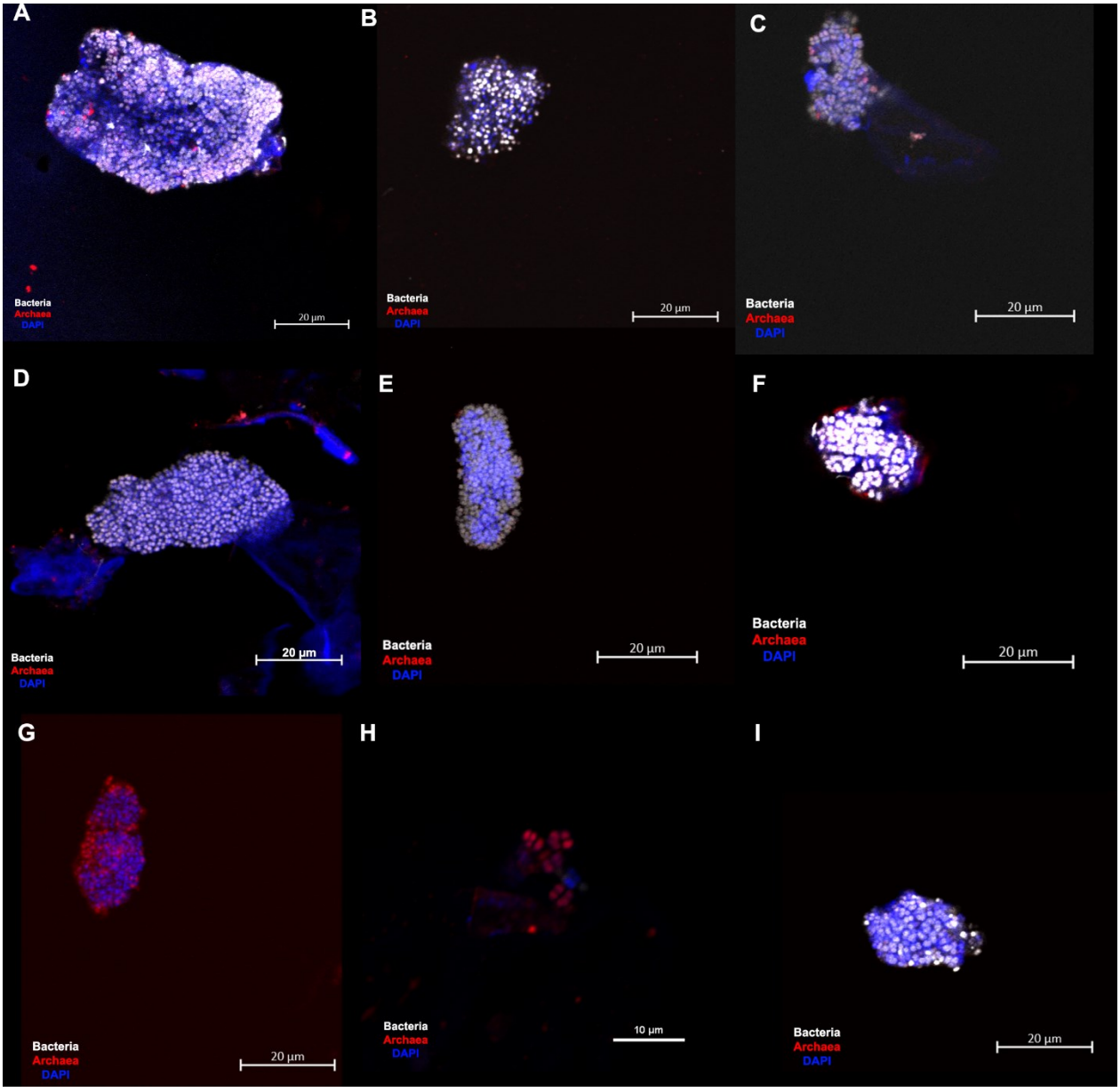

Figure 10. Confocal laser scanning microscopy images of FISH analysis of the collected samples. White cells represent bacteria, red cells represent archaea, and the blue colour represents the DAPI staining.

III.1.5. Survivability of methanogens in an aerobic environment

In order to evaluate if methanogens can survive for a specific time in an aerobic environment, we developed a test to determine the oxygen tolerance of three strains of methanogens. Two of the three strains tested were obtained from DSMZ, these strains have been isolated from faeces (*Methanosphaera stadtmanae* DSM no. 3091 and *Methanobrevibacter smithii* DSM no. 2375) and one strain (*Methanobrevibacter* sp.) has been isolated in our laboratory from a stool sample obtained from a healthy volunteer. All strains were exposed to an aerobic and anaerobic environment for 5 time points (0h, 6h, 24h, 48h and 168h). After exposure, the cells were transferred to fresh media and left to grow for 2 weeks before OD_{600} and methane measurements. All strains tested were able to survive in an aerobic environment for 6h as indicated by the growth measured through OD_{600} measurements and confirmed by the presence of methane in the cultures, which indicated that the cultures were active and alive (Fig. 11).

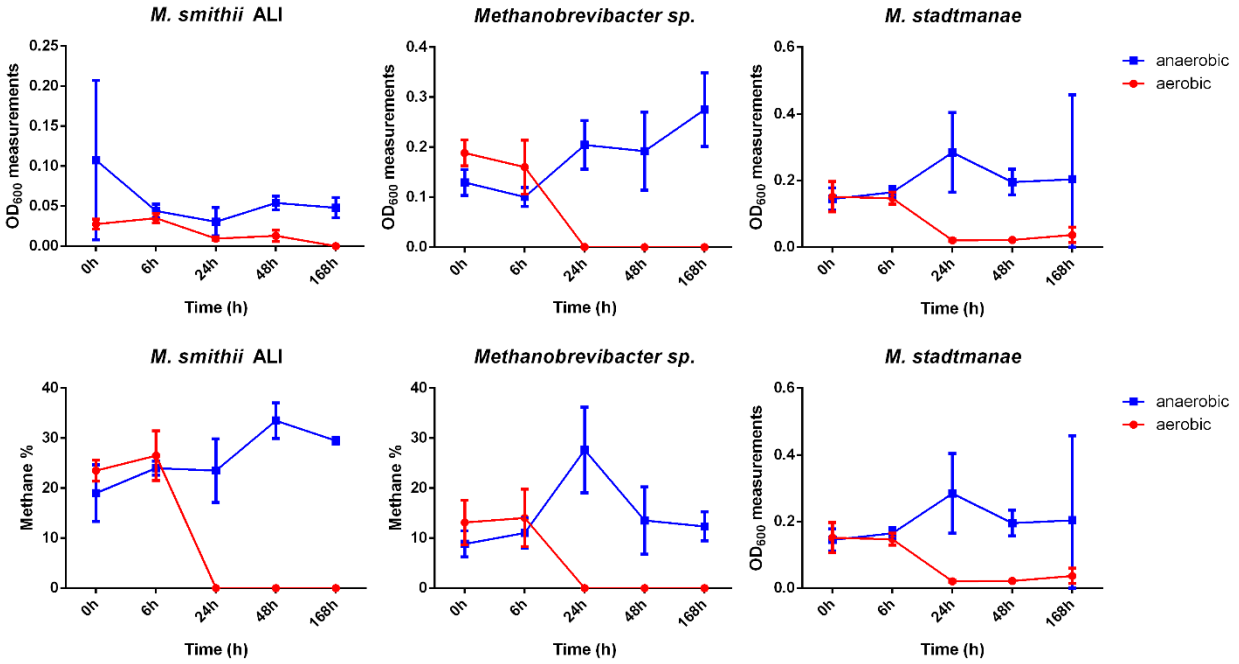

Figure 11. Plots are indicating the growth as determined by OD measurements and methane produced by the tested strains after exposure to an aerobic (red) or anaerobic (blue) environment for different time points.

With the oxygen tolerance test, we demonstrated that methanogens, which are known to be strictly anaerobic microorganisms, can survive exposure to oxygen for up to 24h. Our test demonstrated that all the cultures tested were still active, as indicated by methane production after 6h exposure to oxygen.

III.2. UMIC

III.2.1. Overview

In our study, a total of 60 pregnant women were recruited during a hospital visit due to possible preterm contractions. All women recruited were between weeks 23-34 of pregnancy (Fig. 12). From all patients, a vaginal swab, catheter urine and blood were collected during the recruitment before any treatment. After a medical examination and transvaginal scan for cervical length measurements, around 55% of the women received tocolytics to suppress the preterm labour (case group). Both groups (**case group**, those who received tocolytics, and **control group**, those that did not receive the tocolytic treatment) were analysed with respect to their: vaginal and urinary microbiome (qualitative), the bacterial load (quantitative), and HMOs (blood and urine). All the collected metadata regarding the recruited women were used for correlation analysis. Socio-demographic characteristics of the two groups of women are summarized in Table 7.

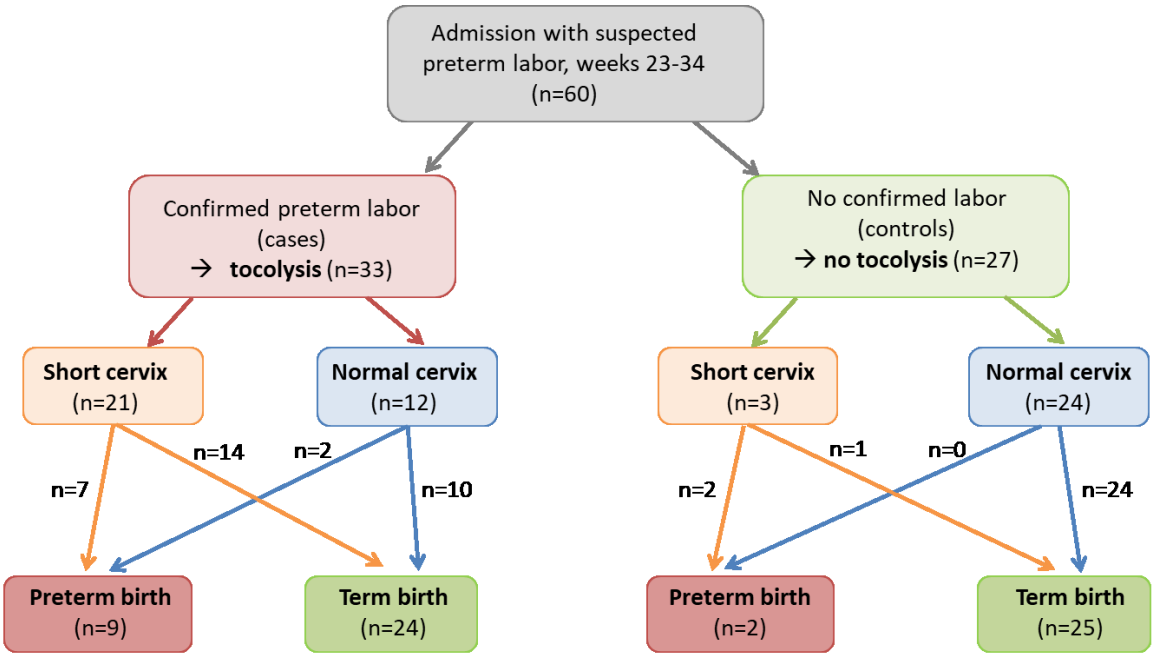

Figure 12. Study overview of the recruited women and distribution into study groups. The figure is taken from (143).

One characteristic, the cervical length, was highly correlated with the risk of preterm birth. At the moment of recruitment, 40% of the women had a short cervix (< 25 mm), and the majority of these women received tocolytic treatment. Women who received tocolytics did not differ significantly compared with the control group concerning race, body mass index, and age (Table 7). Only

18.3% of all recruited women delivered preterm, while the majority of the women delivered term, after 37 weeks of pregnancy. Most of the women (81.8%) who delivered preterm had a short cervix at the time of the recruitment, except two women. Not all women who delivered preterm received tocolytic treatment. The only significant difference between the two groups was the length of the cervix, as more women of the tocolytics group had a short cervix compared to the controls (Table 7). An overview of the study can be seen in Fig. 12.

Table 7. Group characteristics. The table is taken from (143).

	Case group	**Control group**	**p-value**
Received tocolytics	yes (n=33)	no (n=27)	
Age	29.45 ± 0.895 (20-39)	29 ± 1.089 (18-41)	0.746
18-25	7 (21.21%)	6 (22.22%)	
26-35	21 (63.64%)	16 (59.26%)	
36-41	5 (15.15%)	5 (18.52%)	
Cervical-length	22.45 ± 1.6504 (6-40)	34.92 ± 1.90 (6.8-50)	0.000*
BMI	22.27 ± 3.56 (15.43-30.86)	24.31 ± 4.5 (16.94-35.66)	0.055
Underweight (<18,5)	5 (15.15%)	4 (14.82%)	
Normal weight (18,51-24,9)	22 (66.67%)	11 (40.74%)	
Overweight (25,0-29,9)	4 (12.12%)	9 (33.33%)	
Obese (>30)	2 (6.06%)	3 (11.11%)	
Ethnicity			0.141
White	32 (97%)	23 (85.2%)	
Asian	0 (0.0%)	3 (11.1%)	
Black	1 (3%)	1 (3.7%)	
Gestation at sample (weeks)	29^{+2} ± 3,3 (23^{+3} – 33^{+4})	28^{+4} ± 3,2 (23^{+4} – 33^{+1})	0.659
Delivery			
Term	n=24 (72.7%)	n=25 (92.6%)	0.091
Preterm	n=9 (27.3%)	n=2 (7.4%)	
CRP	7.43 ± 1.3217 (0.9-30.1)	9.763 ± 3.2984 (0.8-86.2)	0.841
Secretor Status			
Secretor	n=29 (87.9%)	n=21 (77.8%)	0.322
Non-Secretor	n=4 (12.1%)	n=6 (22.2%)	
Vaginal pH	4.85 ± 0.1418 (4-7)	4.73 ± 0.1623 (3-7)	0.743
Urine pH	6.53 ± 0.1442 (5.5–8)	6.69 ± 0.311 (5-8)	0.213

*p-value < 0.05

III.2.2. Human milk oligosaccharides (HMOs) analysis

HMOs were detected in all serum and urine samples collected. The five most abundant HMOs were quantified using an HPLC-based method. In serum, median concentrations (95% confidence interval [CI] of median) for the five most abundant serum HMOs were as follows: 0.68 nmol/mL (95% CI of median 0.41-0.81) for **2'FL**, 0.23 nmol/mL for **LDFT** (95% CI of median 0.16–0.36), 0.37 nmol/mL (95% CI of median 0.32-0.41) for **3'SL**, 0.093 nmol/mL (95% CI of median 0.087 - 0.10) for **3'SLN** and 0.24 nmol/mL (95% CI of median 0.21- 0.27) for **6'SLN** (Fig. 13A). The same HMOs were identified in the urine samples, although total concentration was approximately 50-fold higher in urine (Fig. 13A). The urine profiles showed a significantly higher proportion of the fucosylated HMOs 2'FL, LDFT and smaller proportion of sialylated HMOs such as 3'SL and 6'SLN (Fig. 13B). The secretor active HMOs, 2'FL and LDFT, were strongly correlated between serum and urine samples. Only 10 out of 60 women were found to be secretor negative showing only a trace amount of 2'FL and LDFT in serum and urine samples (Fig. 13A). The sialylated HMOs, 3'SL and 3'SLN, were also correlated but to a lesser extent (Fig. 13C). The normalization of urinary HMOs with the osmolality resulted in a marked increase in R squared values of the correlations between serum and urine for 2'FL, LDFT, and 3'SL.

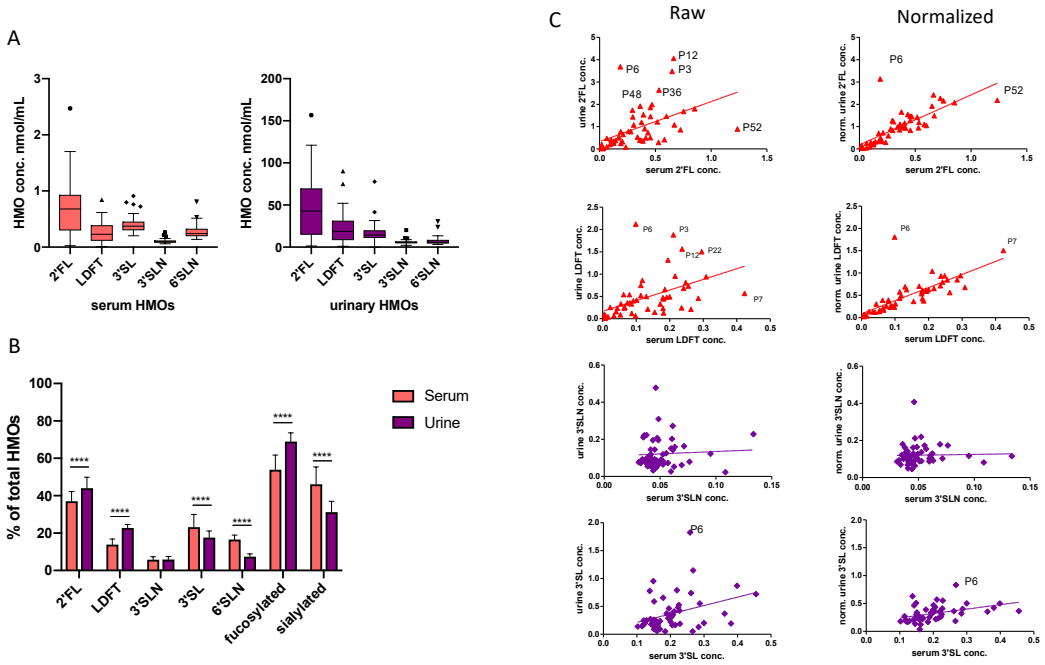

Figure 13. Serum and urinary measured HMOs. Box plots show (A) absolute HMOs concentration in serum (red boxes) and urine (purple boxes) and (B) relative HMO concentrations of serum and urine. (C) Linear regression of serum and urine 2'FL (first row), LDFT (second row), 3'SLN (third row) and 3'SL (fourth row) before and after normalisation with osmolality. The figure is taken from (143).

The case group had significantly higher LDFT concentrations in serum and urine compared to the control group (Mann Whitney t-test) (Fig. 14A). Two women in the control group who did not receive tocolytic treatment delivered their babies preterm 3 - 4 days after admission to the clinics. Excluding them from the analysis, did not change the results. The women with a short cervix had a significantly higher concentration of serum 3'SLN and higher, but not significant, serum concentrations of 2'FL and LDFT. In urine, we observed a trend towards a higher concentration of LDFT without reaching significance (Fig. 14B). By comparing the HMOs concentration in women who later delivered preterm to women who delivered at term, a significant increase of serum 3'SL was observed in women who delivered preterm. Urinary HMOs were not altered concerning preterm delivery (Fig. 14C).

Next, we performed Spearman correlation analyses to assess whether HMOs were associated with cervical length, gestational age at birth, CRP (a marker of inflammation) and maternal anthropometrics data. All HMOs, especially serum LDFT and 2'FL, were positively correlated with gestational age at the admission at the clinics (Spearman rho r= 0.36, p=0.004; r= 0.3, p= 0.015, respectively). Serum 3'SL was negatively correlated with gestational days of birth (r= -0.37, p= 0.004), and positively correlated with CRP levels (r=0.26; p=0.032). Serum 3'SLN was negatively correlated with cervical length (r= -0.42, p= 0.001). Serum 2'FL and LDFT were significantly negatively correlated with vaginal pH (r=-0.26, p=0.032; r=-0.22, p=0.009, respectively), whereas associations between urinary pH with urinary 2'FL and LDFT did not reach significance (Fig. 14D). When we controlled for the time of sampling by considering the gestational time, all the above associations remained significant.

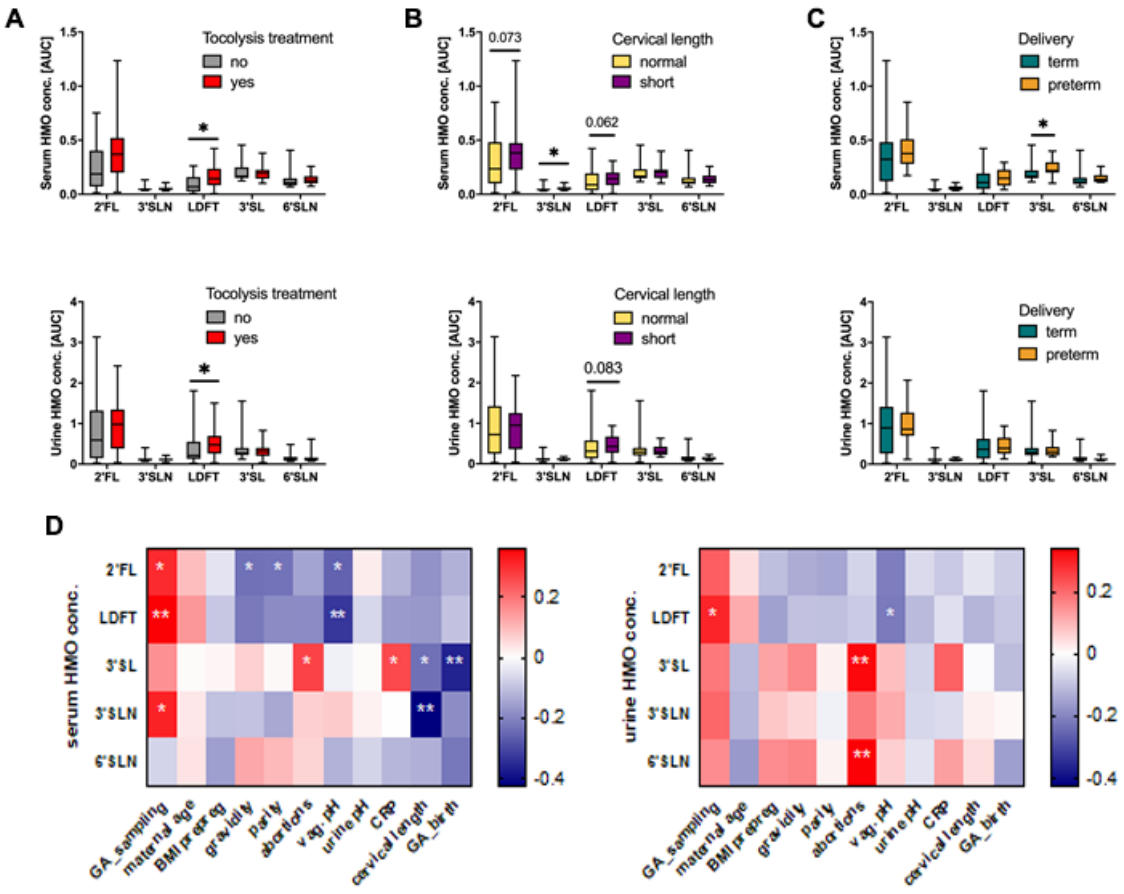

Figure 14. Serum and urinary HMO concentrations in different groupings. HMO concentrations in serum (upper row) and urine (lower row) (A) in pregnant women who received tocolytic treatment versus controls, (B) in women with short cervix versus normal cervix (B) and (C) in women who delivered preterm versus women with term deliveries. (D) Heap map of Spearman correlations. The figure is taken from (143).

III.2.3. Microbial density, richness and composition in vaginal and urine samples

The catheter urine samples and vaginal swabs collected from the recruited women were used for exploring the microbial density and composition in order to identify changes related to tocolytic treatment (yes/no), cervical length (normal/short), and delivery (term/preterm).

The qPCR results showed no significant differences in the number of copies of microbial 16S rRNA gene neither in urine nor vagina, within the analysed groups: the tocolytic group (no/yes), cervical length group (normal/short) or delivery group (term/preterm) (Fig. 15A, B).

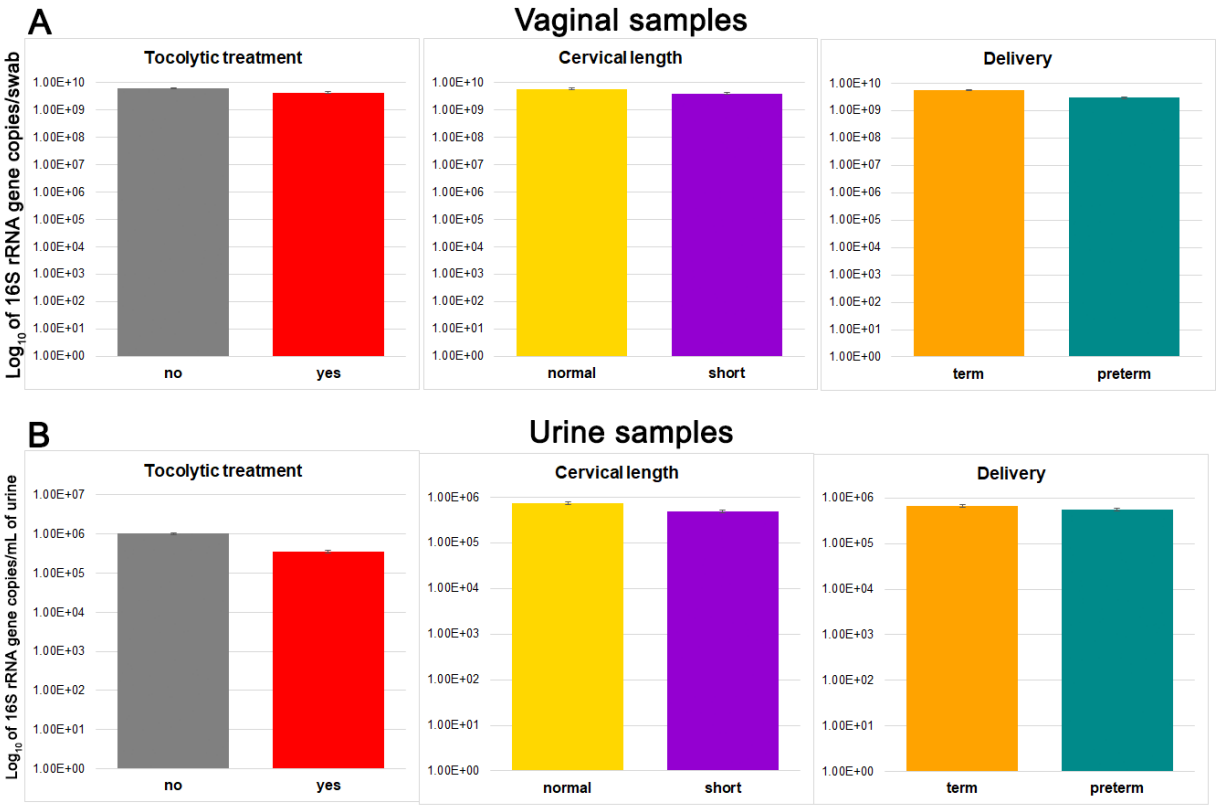

Figure 15. Quantitative PCR results of the bacterial 16S rRNA gene in (A) vaginal and (B) urine samples in the analysed groups: tocolytic treatment, cervical length and delivery. The figure is taken from (143).

When comparing the vaginal with the urinary microbial composition; however, a generally higher microbial load was observed in the vaginal samples (Fig. 16C). The number of bacterial 16S rRNA gene copies in urine varied between 10^2 and 10^7 16S rRNA gene copies/mL, mostly agreeing with previously published results (144). In general, the urinary microbiome was more diverse and more abundant compared to the vaginal microbiome, as shown by the alpha diversity measurements (Chao1, Shannon, and inverse Simpson) (Fig. 16A).

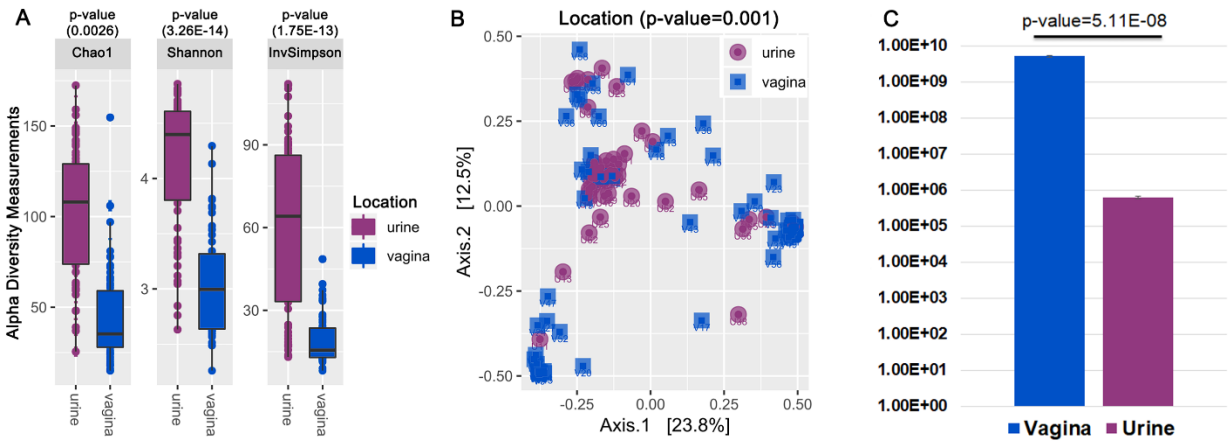

Figure 16. Alpha (A) and beta (B) diversity of urine and vaginal microbiome. 16S rRNA gene copies (qPCR results) in urine and vaginal sample are given in (C). The figure is taken from (143).

Furthermore, the beta diversity analysis (Fig. 16B) showed that the urinary and vaginal microbial communities were distinct (p=0.001), although both shared some microbial taxa such as *Lactobacillus*, *Prevotella*, *Gardnerella, Ureaplasma, Anaerococcus* and other microbial species (Fig. 17B).

Lactobacillus dominated the vaginal microbiome in our study, followed by *Gardnerella*, *Atopobium*, *Ureaplasma*, *Anerococcus*, *Finegoldia* and other genera (Fig. 17A), confirming the results of previous studies (13,145).

The urine samples were collected using a catheter; therefore, no correction was made in the analysis of the urinary microbiome as little influence of the vaginal microbiome was expected. The urinary microbiome was found to be very diverse and rich compared to the vaginal microbiome. The urinary microbial community in our study was composed of taxa previously associated with a healthy urinary microbial community (146). The microbial composition of the urine samples included signatures of species belonging to *Lactobacillus*, *Gardnerella*, *Streptococcus*, *Dialister*, *Finegoldia*, *Ureaplasma*, *Staphylococcus*, *Atopobium*, *Anaerococcus*, *Peptoniphilus* and many more (Fig. 17B).

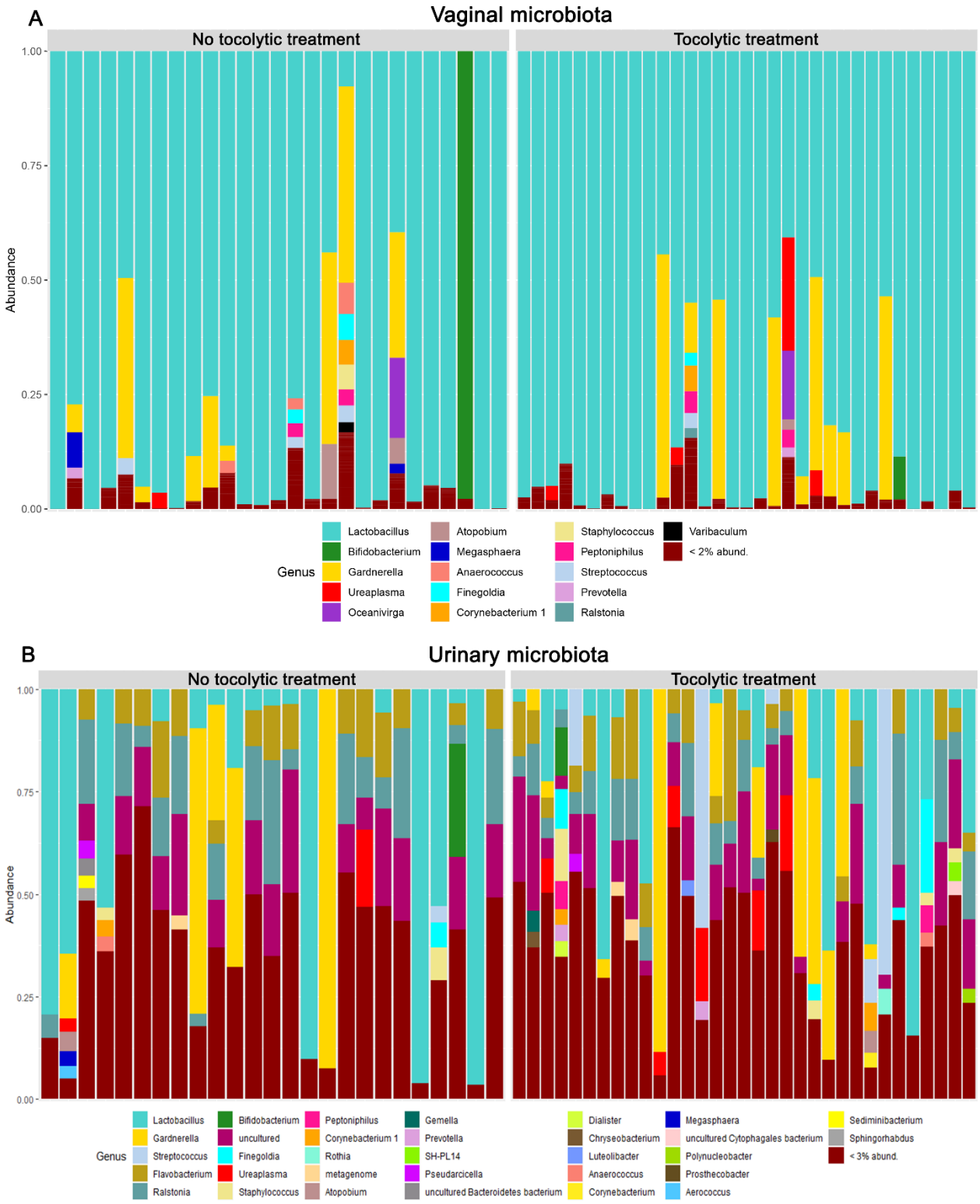

Figure 17. Composition of the vaginal (A) and urinary (B) microbiome at the genus level. The figure is taken from (143).

III.2.4. Vaginal microbiome related to tocolytic treatment, cervical length and delivery

After analysing the vaginal microbiome composition, the samples were classified into community state types (CSTs; (13)) by performing hierarchical clustering at the species level (Fig. 18). A vaginal community state type is characterised by the microbial composition and by the dominance of specific taxa. Therefore, based on microbial composition five community state types were characterised as follows: CSTI (*L. crispatus*), CSTII (*L. gasseri*), CSTIII (*L. iners*), CSTIV (diverse species), and CSTV (*L. jensenii*). The most prevalent CST observed in our cohort was CSTI (30 women out of 60, 50%), followed by CSTIII (14 women out of 60, 23.3%), CSTIV (9 women out of 60, 15%), CSTII (4 women out of 60, 6.7%) and CSTV (3 women out of 60, 5%).

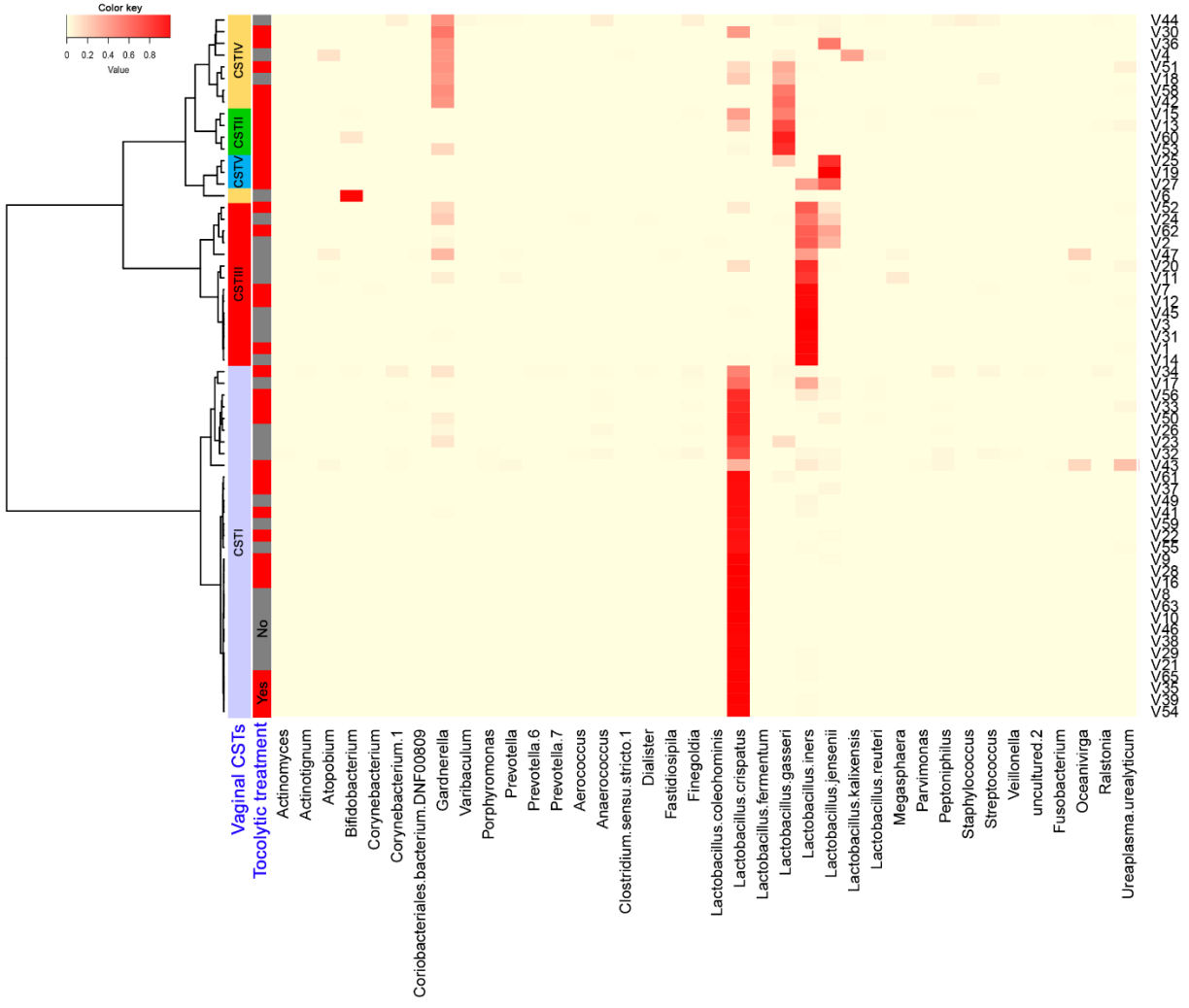

Figure 18. Vaginal community state types (CSTs) as found in our cohort (heat-map). The figure is taken from (143).

Two community state types, namely CSTII and CSTV, were found only in patients who received tocolytic treatment, and mainly in those with a short cervix (< 25 mm), but not in the subjects who delivered preterm (Fig. 19C), indicating that there could be an association between *L. jensenii/ L. gasseri* predominance, preterm contractions and short cervix. No significant differences were observed in the other CST between the groups formed by either the tocolytic treatment (no, yes) or delivery (term, preterm) (Fig. 19A, B). Significant differences in the CSTs were observed only between the group formed by cervical length (normal, short), as fewer women with short cervix had the CSTIII compared to those that had a normal cervix (Fig.19B).

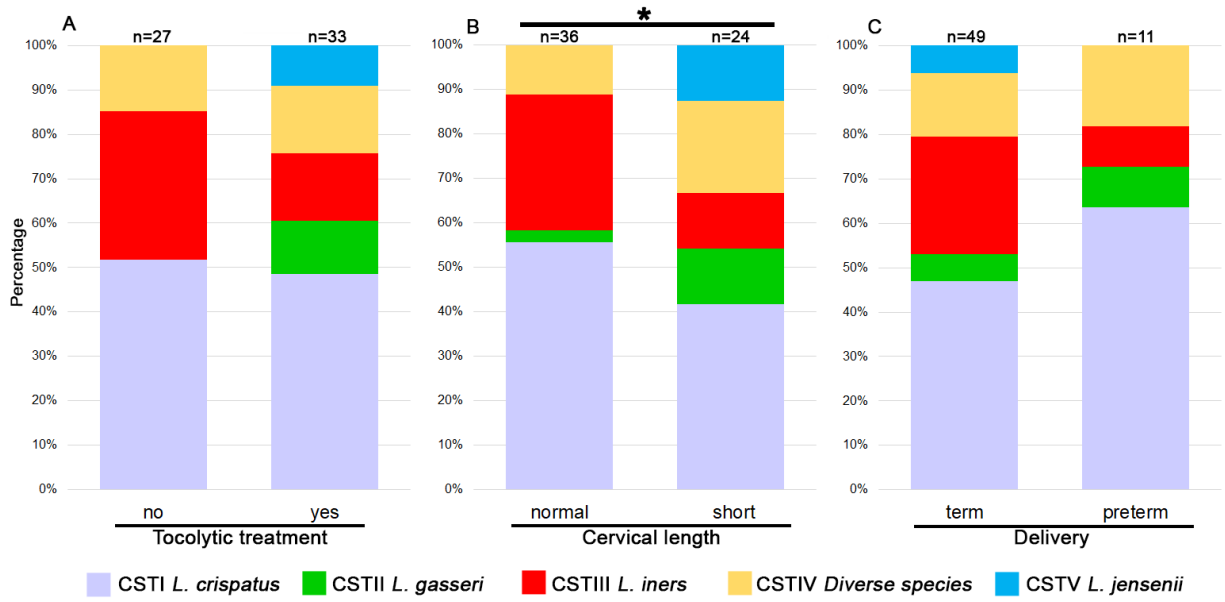

Figure 19. Vaginal community state types (CSTs) and their prevalence in patient groups. The figure is taken from (143).

Alpha (Chao 1, Shannon and inverse Simpson) and beta (principal coordinate analysis, PCoA) diversity were analysed for the vaginal microbiome for the groups formed by the tocolytic treatment (yes/no), cervical length (normal/short) and delivery outcome (term/preterm). No significant differences with respect to diversity, richness and clustering were observed, the microbial communities thus were similar in all analysed groupings (Fig. 20).

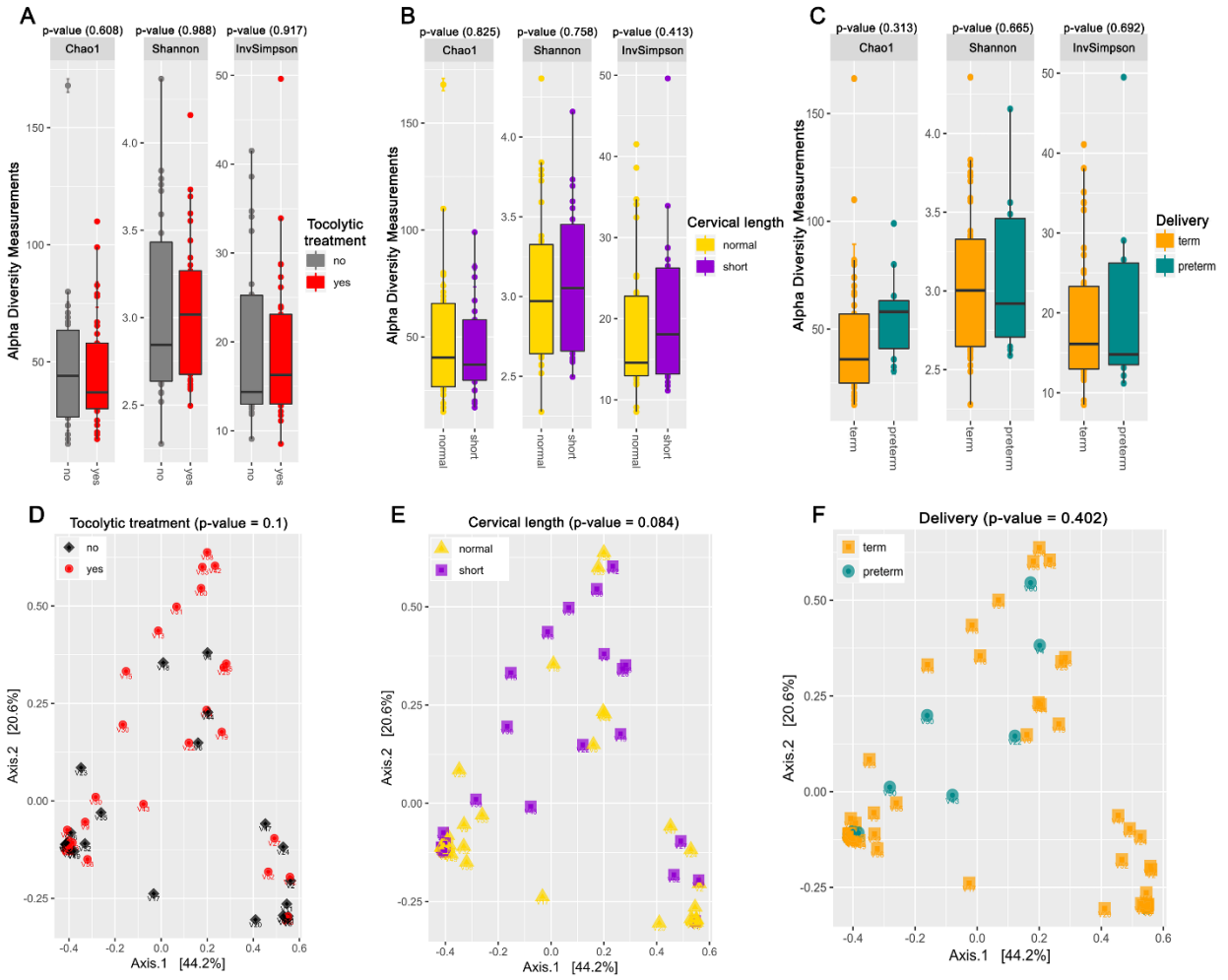

Fig. 20. Alpha (A, B, C) and beta (D, E, F) diversity of vaginal microbiomes in different groupings: tocolytic treatment (A, D), cervical length (B, E) and delivery (C, F). The figure is taken from (143).

To further investigate any association between specific taxa and preterm contractions, cervical length or preterm delivery, LEfSe (Linear discriminant analysis effect size) was performed for all three groups. The relative abundance of *Lactobacillus jensenii* was found to be increased in women who had preterm contractions and short cervix, but not in those who delivered preterm. Additionally, we observed an increase in *Lactobacillus iners* in women with a normal cervix and an increase in *Lactobacillus crispatus* in women who delivered preterm. A correlation between *Ureaplasma urealyticum*, preterm delivery and preterm contractions was also observed (Fig. 21A, B, C).

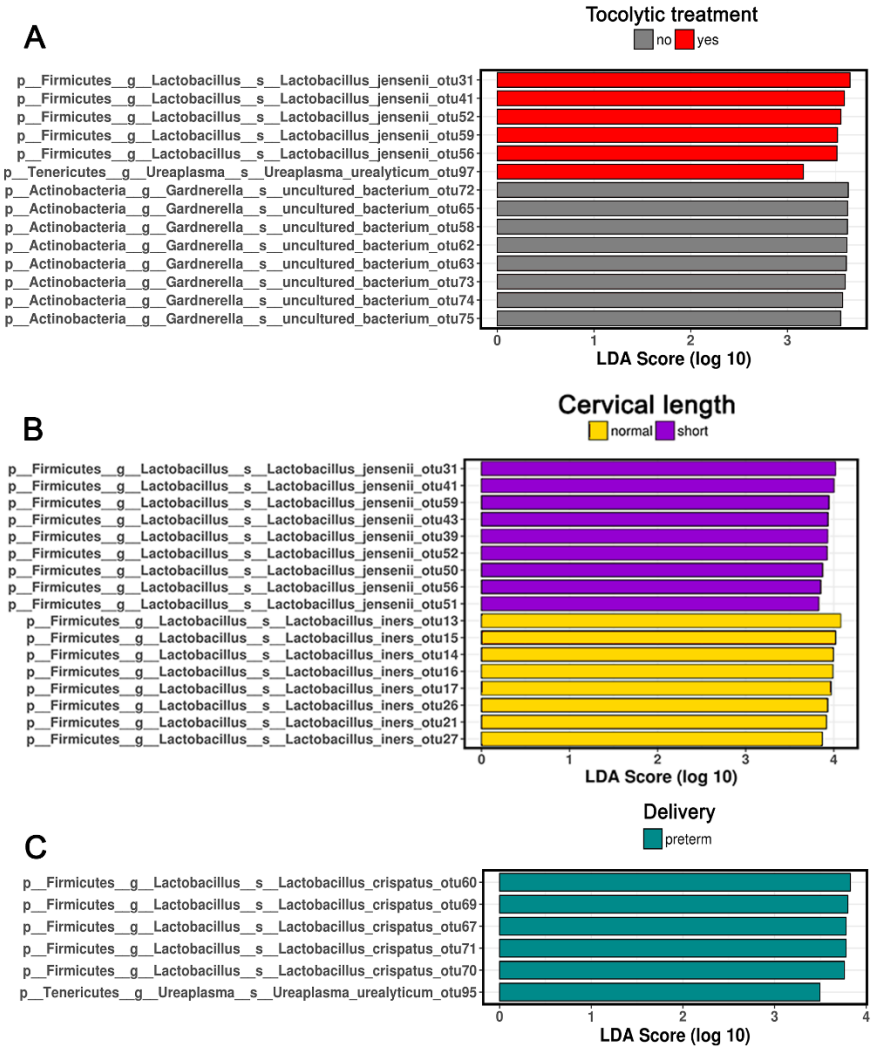

Figure 21. LEfSe (Linear discriminant analysis effect size) of vaginal microbiomes, indicating the association of specific lactobacilli with tocolytic treatment (A), cervical length (B) and delivery outcome (C). The figure is taken and modified from (143).

Furthermore, we calculated correlation heatmaps between taxa (Fig. 22A) and genera (Fig. 22B) with retrieved cohort characteristics based on the Spearman correlation coefficient, and we observed similar correlation patterns. *Lactobacillus jensenii* and *Lactobacillus gasseri* strongly correlated with preterm contractions and short cervix. Similar correlations have been observed between specific *Gardnerella* taxa and preterm contractions, short cervix, and preterm delivery, while other *Gardnerella* were correlated with term delivery. At the genus level, correlations between preterm delivery and specific genera such as *Staphylococcus*, *Ureaplasma*, *Streptococcus* and *Finegoldia* are noted. *Ureaplasma* was found to be correlated with tocolytic treatment, but not with a short cervix (Fig. 22B).

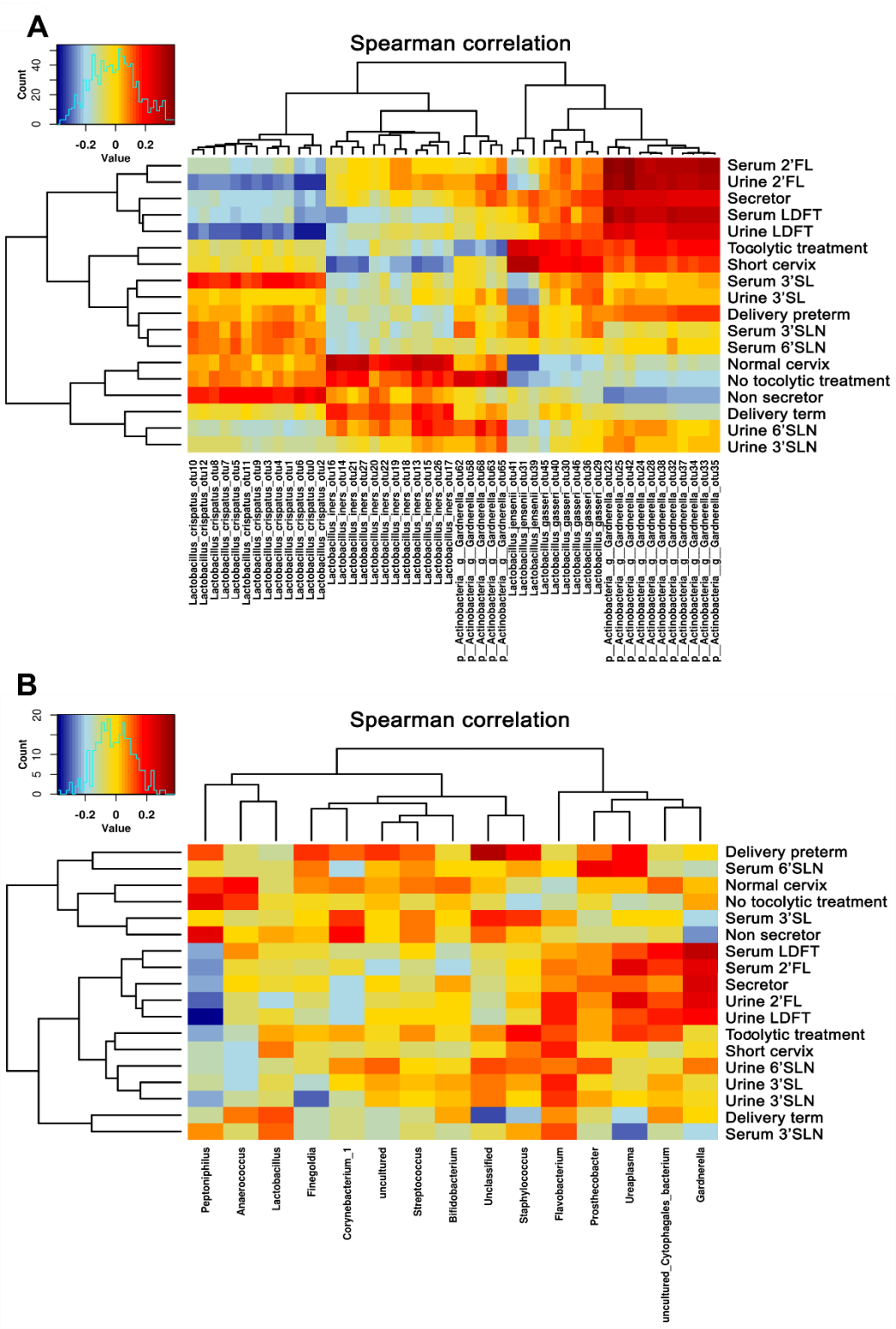

Figure 22. Spearman correlation heatmaps at RSV (A) and genus (B) level between the vaginal microbiome and the cohort characteristics. The figure is taken and modified from (143).

III.2.5. Urinary microbiome related to tocolytic treatment, cervical length and delivery

To explore differences in the urinary microbial communities between the groups formed by the tocolytic treatment, cervical length and delivery, alpha and beta diversity were determined for all the groups. Alpha diversity based on Chao1, Shannon, and inverse Simpson indices was calculated, differences between the groups were tested using the statistical test Wilcoxon Rank test. The results showed no significant differences in the alpha diversity in the three groups analysed (Fig. 23A, B, C). Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities were plotted, and Adonis tests were performed to test if the microbial communities in the three groups are different. The p-values were not significant, indicating that the microbial communities are similar between the analysed groups (Fig. 23D, E, F).

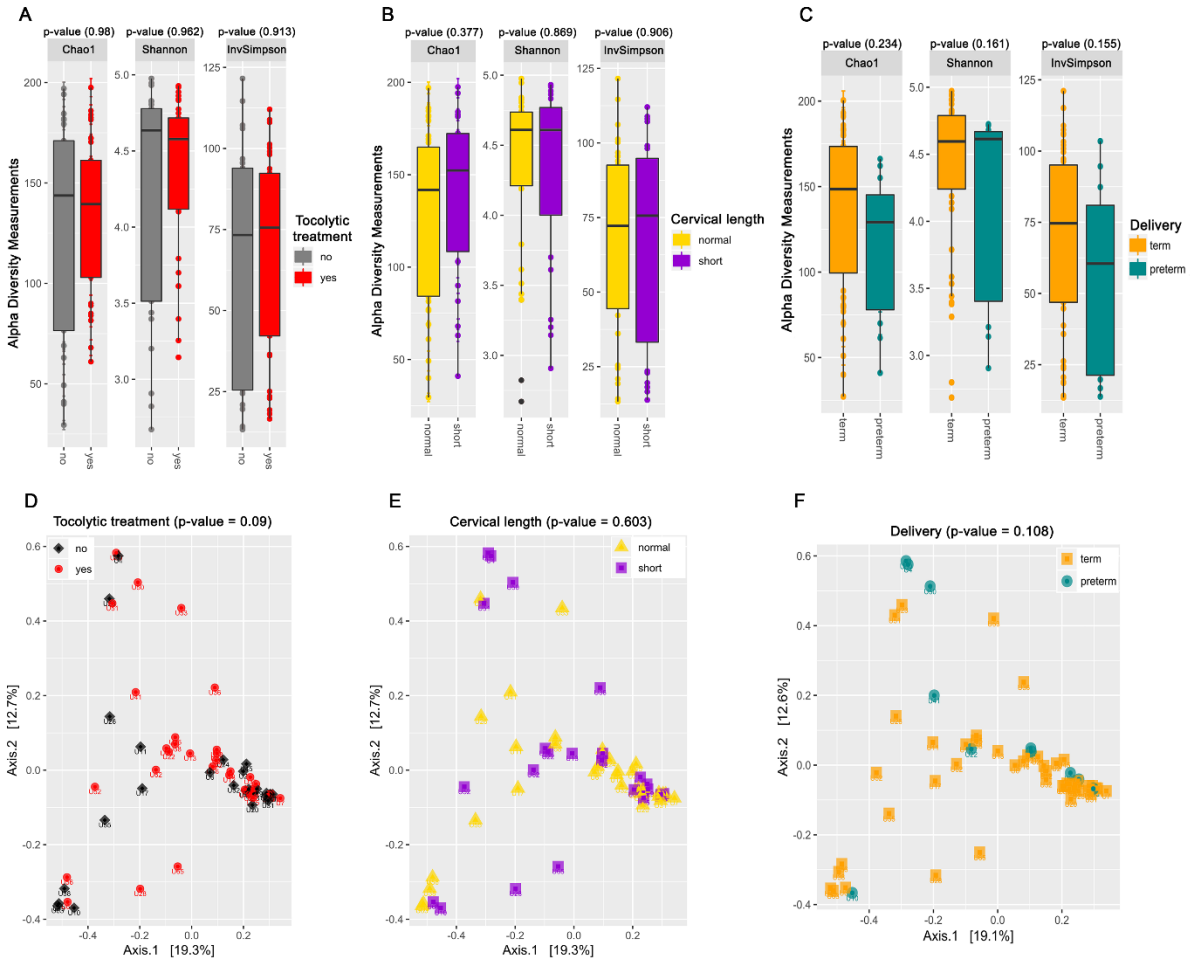

Figure 23. Alpha (A, B, C) and beta (D, E, F) diversity of urinary (catheterised) microbiomes in different grouping: tocolytic treatment (A, D), cervical length (B, E) and delivery (C, F). The Figure is taken from (143).

LEfSe analysis allowed the detection of several taxa being different between the analysed groups. The relative abundance of *Lactobacillus jensenii*, *Lactobacillus gasseri* and species of *Finegoldia* were increased in women with a short cervix, while species of *Pseudarcicella* were associated with the normal cervix. An increase in the relative abundance of *Lactobacillus crispatus* was observed in women who delivered term and women who did not receive tocolytics, while *Ureaplasma urealyticum* was associated with preterm delivery (Fig. 24A, B, C).

Similar observations were noted in the heatmap correlations performed between the urinary microbiome and some of the cohort characteristics based on the Spearman correlation coefficient. Also, specific signatures of *Gardnerella* were correlated with preterm delivery, short cervix, preterm contractions and all five HMOs investigated in serum and urine (Fig. 25A). At the genus level, we observed similar correlations between all measured HMOs, preterm delivery, short cervix, tocolytic treatment and *Gardnerella*. Signatures of *Ureaplasma* representatives were mainly correlated with preterm delivery and non-secretor status. Correlations between *Pseudarcicella* and normal cervix were also observed in the Spearman correlation heatmaps (Fig. 25B).

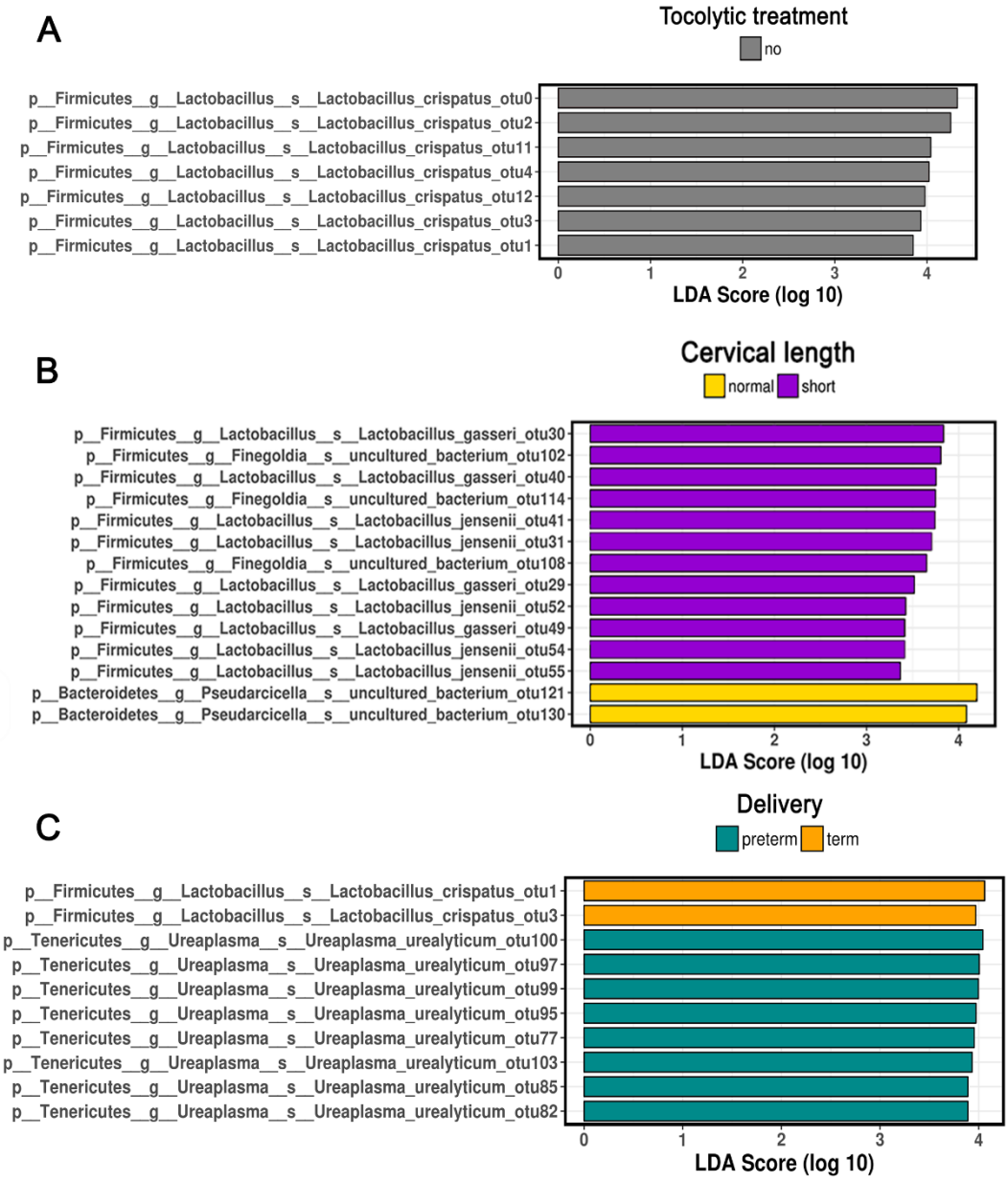

Figure 24. LEfSe (Linear discriminant analysis effect size) of urinary microbiomes, indicating the association of specific lactobacilli with preterm labour, cervix length and delivery outcome (A, B, C). The figure is taken and modified from (143).

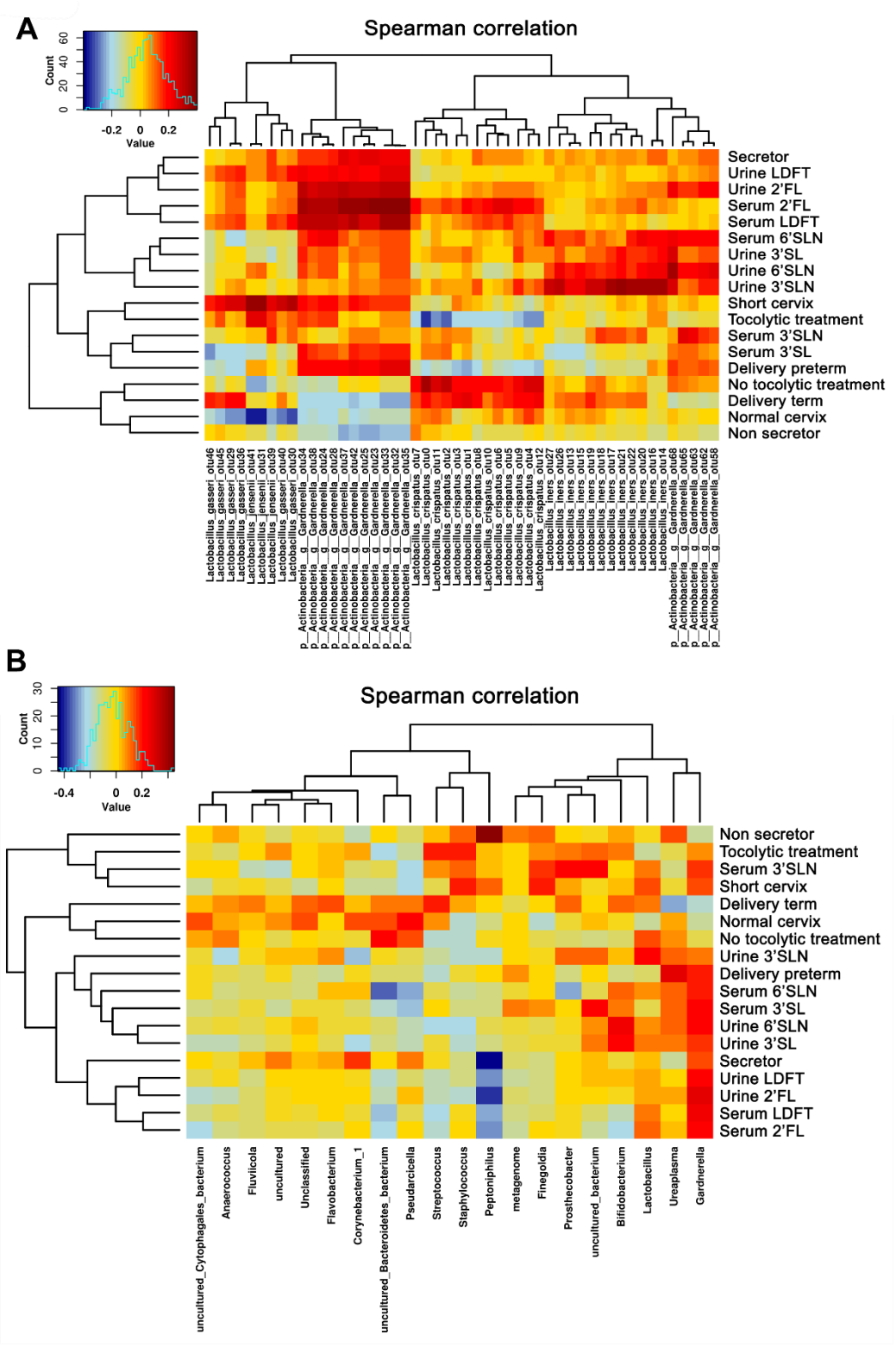

Figure 25. Spearman correlation heatmaps at RSV (A) and genus (B) level between the urinary microbiome and the cohort characteristics. The figure is taken and modified from (143).

III.2.6. Correlations between HMOs profile and microbiome in urine and vagina

We were further interested in identifying if there are any correlations between specific HMOs and the microbiome. We found strong correlations between serum and urine HMOs with the urinary and vaginal microbiome composition. Serum and urine HMO concentrations were strongly correlated with signatures of *Gardnerella* in the urinary and vaginal microbiome. Specific vaginal genera such as *Ureaplasma*, *Gardnerella*, and *Flavobacterium* were positively correlated with the concentrations of LDFT and 2'FL in both, urine and serum, and thus, with positive secretor status (Fig. 23B). Vaginal *Flavobacterium* genus was correlated with sialylated HMO concentrations in urine and with serum 3'SLN (Fig. 23B). Vaginal *Ureaplasma* was correlated with serum 6'SLN (Fig. 23B), while the *Ureaplasma* in urine was correlated with sialylated HMO concentrations except for serum 3'SLN. Serum 3'SLN was mainly correlated with *Finegoldia* (Fig. 25B).

We also observed correlations between *Bifidobacterium* in urine and 6'SLN and 3'SL in urine and serum (Fig. 25B). Furthermore, we found correlations between specific HMOs and *Lactobacillus iners* from urine samples (Fig. 25A). *Gardnerella* species were also highly correlated with LDFT and 2'FL concentrations in serum and urine, and thus, with positive secretor status (Fig. 25B). *Lactobacillus crispatus* species were highly positively correlated with serum 3'SL (Fig. 25A).

III.2.7. Processes which could lead to preterm birth

Next, we were interested in the microbial functions and their correlation with HMOs and other obtained metadata. For this, we predicted functional profiles from our 16S rRNA gene data using Tax4Fun (125). As shown in Fig. 26, the functional profiles of the urinary microbiome grouped into three major groups. Notably, one cluster of microbial functions was strongly influenced by the secretor status, reflected by an increase in sugar-degradation-related microbial functions (e.g. pentose and glucuronate interconversions ($p=0.031$, ANOVA), pentose-phosphate pathway, galactose metabolism, starch and sucrose metabolism). This observation explains the increase as mentioned above of particularly sugar-metabolizing microbial signatures, such as *Gardnerella,* along with secretor-associated HMOs LDFT and 2'FL.

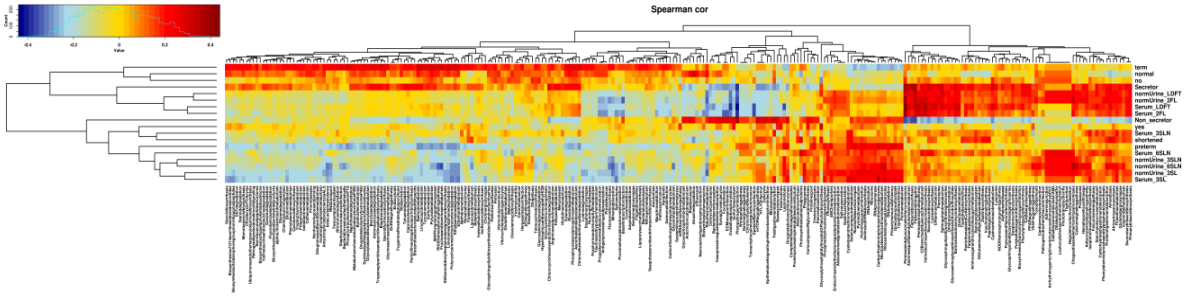

Figure 26: Estimated functional profiles of the urinary microbiome and correlations with HMOs and preterm delivery risk. The vaginal microbiome showed a similar but more blurry picture. The figure is taken from (143).

Notably, microbiome functional profiles in vagina and urine were not correlated with inflammation status (CRP), but microbial function profiles of urine, but not the vagina, were strongly correlated with the number of 16S rRNA gene copy numbers in the urine samples therein (two categories of copy numbers groups, high and low, cutoff- 10^5; p=0.001). These copy numbers were increased along with again sugar-degrading microbial functions (galactose metabolism, starch and sucrose metabolism; LEfSe analysis), and corresponding microbial taxa, such as *Gardnerella* (p=0.029), *Lactobacillus* (p=0.00064) and *Finegoldia* (p=0.0058) (Fig. 27), indicating an increased microbial load in the urine due to the secretor-associated HMOs. This observation was also reflected in the vaginal microbiome, as a high copy number in the urine correlated with an increase in *Gardnerella* and *Finegoldia* therein as well.

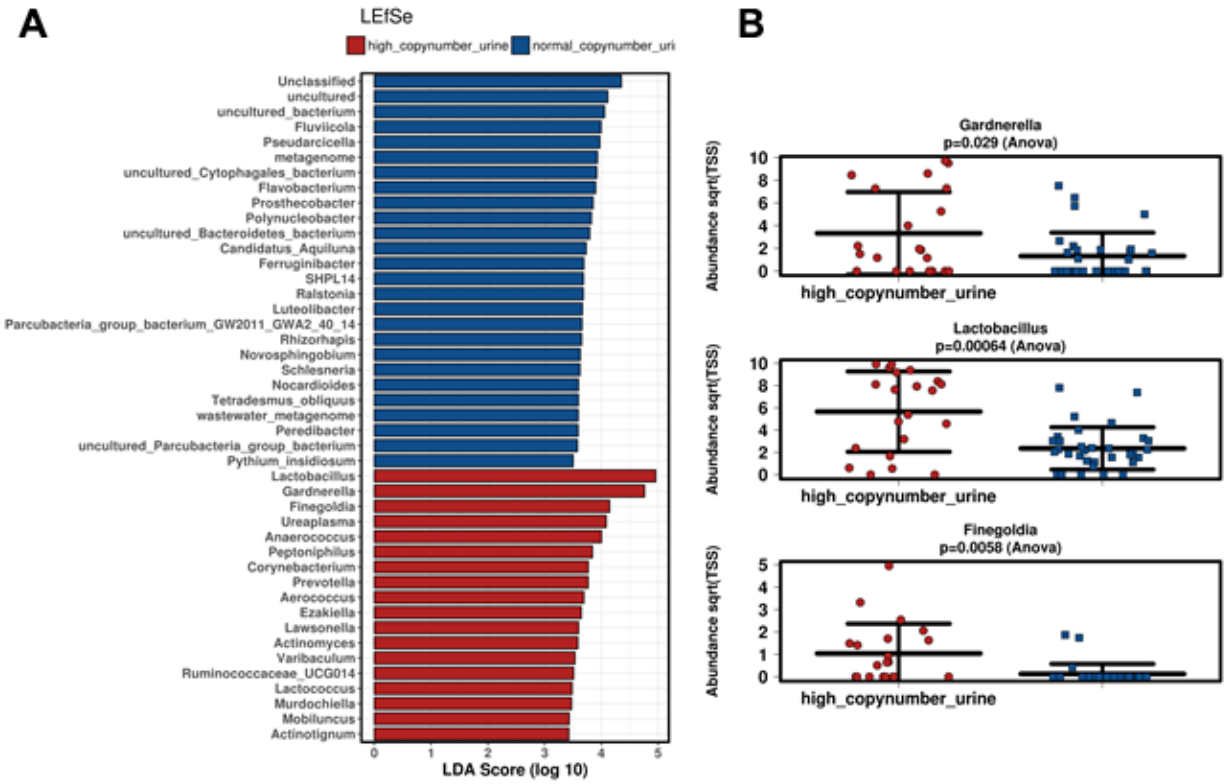

Figure 27. Distinct taxa associated with high/low 16S rRNA gene copy number in the urinary microbiome. The figure is taken from (143).

In Figure 28, we present an overview of the retrieved results from the study, in which we were able to identify potential key compounds and essential microorganisms in the process.

Sialylated HMOs, in particular, 3'SL, were found to strongly correlate with preterm delivery, short cervix, and increased inflammation. Notably, 3'SL did only partially correlate with key microbial signatures in urine and vagina, indicating an independent risk increased through a direct, but microbiome-independent mechanism. Key microbial signatures were *Lactobacillus jensenii*, *L. gasseri*, *Ureaplasma* sp., *Gardnerella* sp. and *Finegoldia* sp., which appeared to be correlated with a short cervix, preterm delivery and/or preterm contractions. Notably, most of the microorganisms mentioned above were associated with an increased bacterial load in the urine, indicating a potential low-inflammatory or the beginning of an infection therein. Mainly, *Gardnerella* signatures were found to be associated with the secretor status, in agreement with the observations on the functional level. The secretor status, however, was also found to be associated with a higher bacterial load in the urine (Fig. 28A).

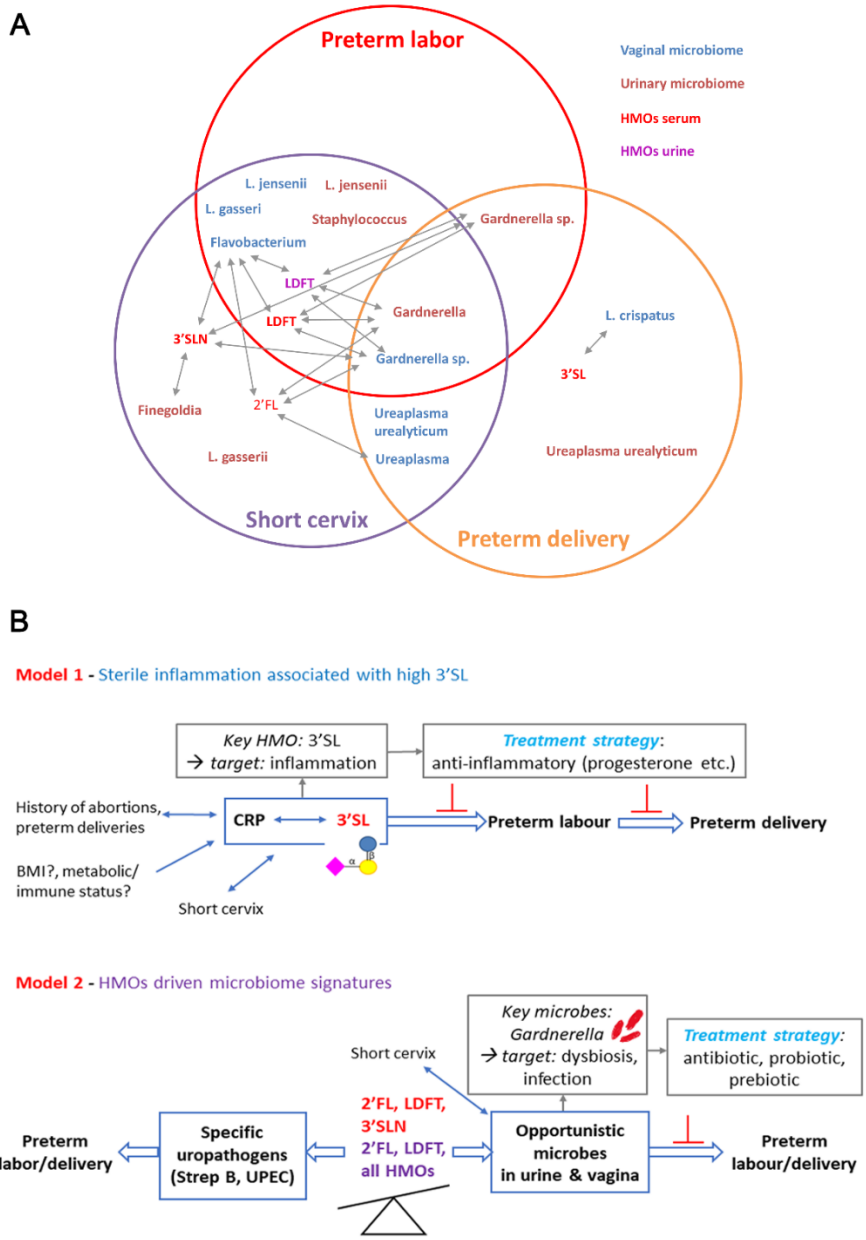

Figure 28. Overview of the results (A) and the proposed models (B). The figure is taken and modified from (143).

To summarise, our observations point at two different processes. One process appears to be driven by sterile inflammation, characterised by increased sialylated HMO concentrations in serum; another process appears to be indirect and microbiome-mediated, which could, however, be driven by secretor-associated HMOs. HMOs thus were identified as key molecules involved in the regulation of term birth (Fig. 28B). Fig. 28B indicates the two distinct models on how HMOs

might be involved in modulating the risk for preterm birth and potential treatment strategies are shown. Model 1 is characterised by an increased concentration of sialylated HMOs, i.e. 3'SL, associated with sterile inflammation, independent of the vaginal or urinary microbiome. Thus, anti-inflammatory therapies might be recommended. Model 2 is characterised by an imbalance in HMOs, leading to disturbances in the vaginal and urinary microbiome. This, combined with other risk factors (short cervix), might promote preterm labour and delivery. While specific HMOs might prevent colonisation with urogenital pathogens, the same HMOs might also foster overgrowth of certain opportunistic microbial species in the urinary tract and the vagina, leading to infections and promoting preterm labour and delivery.

III.3. Human Archaeome I

III.3.1. Overview

In our previous study, we have explored the archaeal communities in different body sites by using a specific amplicon sequencing approach based on the primer pair 349F-519R, which targets the V3 region of the 16S rRNA gene. In this study, we showed that archaea are present in other body sites as well and not only in the gastrointestinal tract, oral and skin as previous studies have shown (94). We found signatures of archaea also in the nasal cavity and lung (61). For this study, we analysed biopsies from different parts of the gastrointestinal tract, samples from the nasal cavity, bronchoalveolar lavage (BAL) and skin samples collected from the back of healthy participants. By analysing the archaeal communities in these samples, we observed that these communities are body site-specific like bacteria: the gastrointestinal tract is dominated by a Euryarchaeota community, while the archaeal community on the skin is mainly comprised of sequences assigned to the Thaumarchaeota phylum. Members of the Woesearchaeota group dominated the lung samples, and the nasal archaeal community shared similarities with the archaeal communities from the gastrointestinal tract and the skin and was composed of mostly members of the Euryarchaeota and Thaumarchaeota phyla (Fig. 29). Interestingly, the gastrointestinal tract was not entirely dominated by members belonging to the *Methanobrevibacter* genus, specific areas within the gastrointestinal tract showed a high abundance of *Methanobacterium* genus, especially in the ileum and left colon. Our results indicate that the gastrointestinal archaeal community is different than previously shown (86) and is dependent on the analysed samples, biopsies from some regions of the gastrointestinal tract could show a different archaeal community than stool samples (Fig. 30).

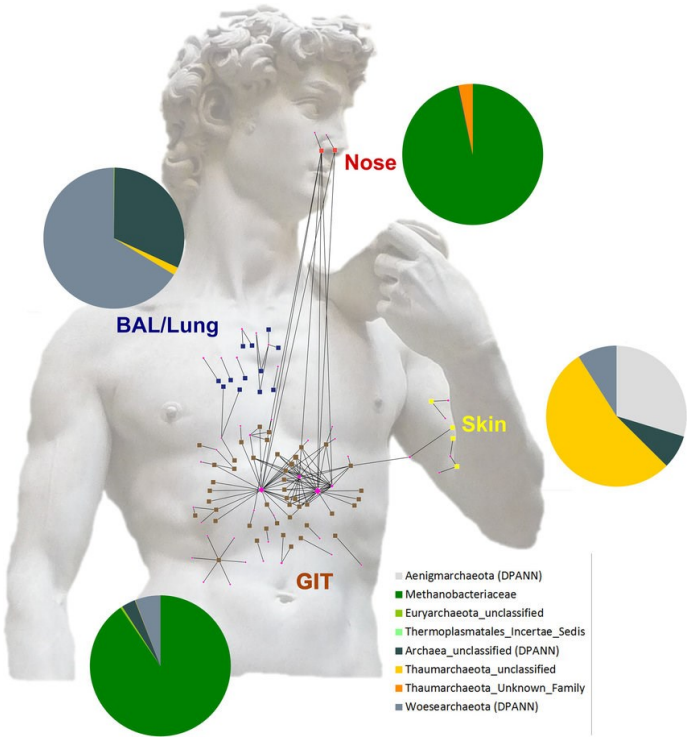

Figure 29. The archaeal communities present in the analysed different body-sites (nasal cavity, BAL/Lung, skin and gastrointestinal tract) arranged as a network. The pie charts indicate the archaeal composition of these body-sites. The figure is taken from (61).

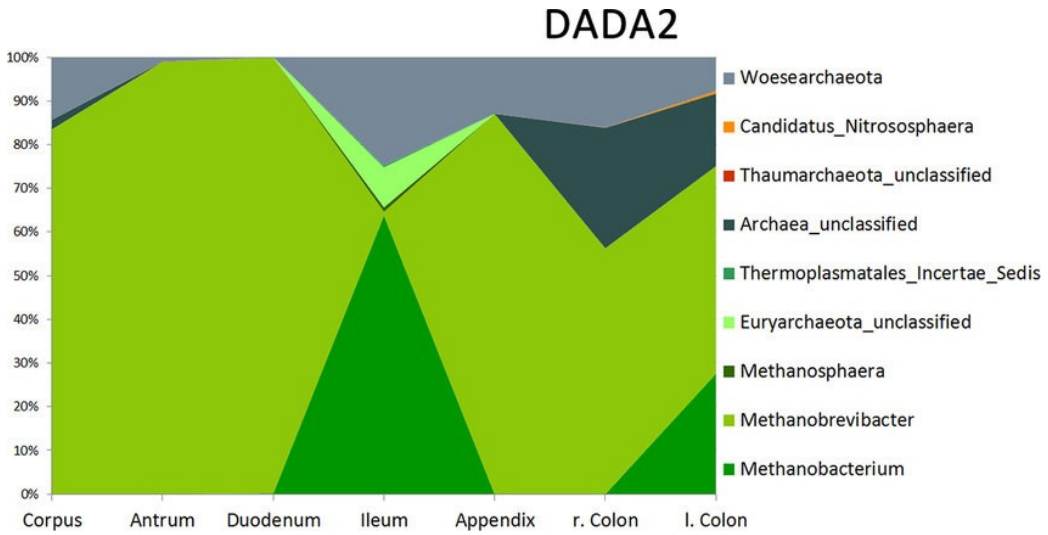

Figure 30. The area plot indicates the relative abundance of archaeal genera within the gastrointestinal tract, from corpus to left colon. The figure is taken from (61).

As a consequence of our previous study, we further tested both *in silico* and in wet-lab experiments different primer combinations, as presented in the following chapters, to identify a new primer pair combination which allows the amplification of the V4 region of the archaeal 16S rRNA gene,

resulting in a longer sequence (300 bp) for a better resolution to study archaeal communities present in human samples further.

III.3.2. Primer pair comparison

The evaluation of the primer pairs was done for the following characteristics. The primer pairs needed to have a high *in silico* specificity for archaeal 16S rRNA genes and allow the amplification of a sequence of 150 to 300 bp length, therefore being suitable for Next Generation Sequencing (NGS). Additionally, the primer pairs had to have the *in vitro* capability to amplify diverse archaeal 16S rRNA genes from a variety of human specimens.

Besides archaea-specific primer pairs, two widely used “universal” primers (515F-806uR original; 515FB-806RB modified; (140,147)) were evaluated along to assess the potential of “universal” primers to display archaeal diversity associated with the human body.

III.3.2.1. *In silico* analysis of different primer pairs

A total of 12 different primer pairs were evaluated *in silico* (Table 10). Most tested primer pairs indicated a high coverage for the archaeal domain ranging from 46% to 89% and revealed a high domain-specificity (8 of 12 primer pairs without matches outside of the archaeal domain). The coverage increased to values between 68% and 95% when one mismatch per primer was allowed. One designated archaeal primer pair, namely primer pair 519F-806R, was found to target not only the archaea domain but also the bacteria and eukarya domain when one mismatch per primer was allowed.

We further evaluated in detail the primer pairs *in silico* coverage for specific archaeal phyla and genera of particular interest for the human archaeome studies such as Euryarchaeota, Thaumarchaeota, and Woesearchaeota, as well as *Nitrososphaera, Methanobrevibacter*, *Methanosphaera* and *Methanomassiliicoccus*. For all subsequent *in silico* analyses, we allowed one mismatch per primer.

All primer pairs revealed a high coverage of over 90% for the Euryarchaeota phylum. All primer pairs analysed had coverage over 94% for *Methanobrevibacter* and over 92% for *Methanomassiliicoccus*. Most primer pairs showed coverage below 90% for *Methanosphaera* except for the primer pairs 519F-806R and 349F-519R, which had a coverage of 90% (Table 11). The coverage of the Thaumarchaeota phylum depended strongly on the primer pair used. Most analyses which included the forward primer 344F showed a low *in silico* coverage for Thaumarchaeota (below 30%), whereas all other primer pair combinations revealed a high

coverage of more than 90% of this phylum (Table 11). Although the coverage of the Thaumarchaeota phylum varied greatly, the coverage for *Nitrososphaera* genus was higher than 85%. The coverage for Woesearchaeota was entirely dependent on the primer pairs used and varied between 65.2% and 89.5%.

Table 10. Primer selection and sequences and the results of the pre-analysis *in silico* evaluation of all primer pairs tested. Coverage of Archaea, Bacteria and Eukarya are given in percentages, depending on whether no or one mismatch was allowed. Designated “universal” primers (primer pairs 10-12) are indicated in bold letters. The table is taken from (60).

				0 mismatch			1 mismatch		
Primer pair	**Name**	**Primer name***	**Sequence (5' -> 3')**	**Archaea**	**Bacteria**	**Eukarya**	**Archaea**	**Bacteria**	**Eukarya**
1	344F	S-D-Arch-0344-a-S-20	ACGGGGYGCAGCAGGCGCGA	46.1%	0.0%	0.0%	68.6%	0.0%	0.0%
	915R	S-D-Arch-0911-a-A-20	GTGCTCCCCCGCCAATTCCT						
2	349F	S-D-Arch-0349-a-S-17	GYGCASCAGKCGMGAAW	71.8%	0.0%	0.0%	87.8%	0.0%	0.0%
	915R	S-D-Arch-0911-a-A-20	GTGCTCCCCCGCCAATTCCT						
3	344F	S-D-Arch-0344-a-S-20	ACGGGGYGCAGCAGGCGCGA	51.5%	0.0%	0.0%	73.0%	0.0%	0.0%
	1041R	S-D-Arch-1041-a-A-18	GGCCATGCACCWCCTCTC						
4	349F	S-D-Arch-0349-a-S-17	GYGCASCAGKCGMGAAW	71.2%	0.0%	0.0%	90.0%	0.0%	0.0%
	1041R	S-D-Arch-1041-a-A-18	GGCCATGCACCWCCTCTC						
5	519F	S-D-Arch-0519-a-S-15	CAGCMGCCGCGGTAA	79.3%	0.0%	0.0%	93.7%	0.0%	0.0%
	1041R	S-D-Arch-1041-a-A-18	GGCCATGCACCWCCTCTC						
6	344F	S-D-Arch-0344-a-S-20	ACGGGGYGCAGCAGGCGCGA	48.3%	0.0%	0.0%	71.3%	0.0%	0.0%
	806R	S-D-Arch-0786-a-A-20	GGACTACVSGGGTATCTAAT						
7	349F	S-D-Arch-0349-a-S-17	GYGCASCAGKCGMGAAW	75.2%	0.0%	0.0%	91.1%	0.0%	0.0%
	806R	S-D-Arch-0786-a-A-20	GGACTACVSGGGTATCTAAT						
8	519F	S-D-Arch-0519-a-S-15	CAGCMGCCGCGGTAA	85.6%	6.8%	0.0%	95.2%	90.9	0.1%
	806R	S-D-Arch-0786-a-A-20	GGACTACVSGGGTATCTAAT						
9	349F	S-D-Arch-0349-a-S-17	GYGCASCAGKCGMGAAW	79.3%	0.0%	0.0%	92.8%	0.0%	0.1%
	519R	S-D-Arch-0519-a-A-16	TTACCGCGGCKGCTG						
10	**519F**	S-D-Arch-0519-a-S-15	CAGCMGCCGCGGTAA	88.9%	88.8%	0.6%	95.3%	95.4%	1.2%
	785R	S-D-Bact-0785-b-A-18	TACNVGGGTATCTAATCC						
11	**515F**	515F-original	GTGCCAGCMGCCGCGGTAA	52.9%	86.8%	0.0%	94.6%	95.0%	0.3%
	806uR	806R-original	GGACTACHVGGGTWTCTAAT						
12	**515FB**	515F-modified	GTGYCAGCMGCCGCGGTAA	85.7%	87.7%	0.0%	95.4%	95.1%	1.4%
	806RB	806R-modified	GGACTACNVGGGTWTCTAAT						

*according to (110)

Table 11. *In silico* analysis of the coverage of specific archaeal taxa of interest for the selected primer pairs. For primer full names and sequences, please refer to Table 10. The table is taken from (60).

		Euryarchaeota				Thaumarchaeota		Nanoarchaeota
primer pair	**Name**	**total**	***Methanobrevibacter***	***Methanosphaera***	***Methanomassiliicoccus***	**total**	***Nitrososphaera***	***(Woesearchaeota)***
1	344F 915R	89.80%	94.90%	81.00%	100%	20.40%	87.10%	55.80%
2	349F 915R	89.70%	95.00%	83.00%	100%	91.2%	89.30%	70.30%
3	344F 1041R	89.90%	94.30%	78.20%	100%	20.60%	89.00%	56.60%
4	349F 1041R	90.20%	94.40%	78.60%	100%	95.80%	92.30%	73.40%
5	519F 1041R	94.60%	97.40%	84.60%	92.90%	96.50%	90.60%	82.40%
6	344F 806R	91.50%	95.20%	82.20%	100%	23.20%	88%	55.00%
7	349F 806R	91.40%	95.30%	84.20%	100%	96.10%	90.10%	72.60%
8	519F 806R	96.30%	98.60%	90%	95%	96.50%	89.50%	82.90%
9	349F 519R	91.90%	95.60%	90.30%	95%	97.50%	94.40%	83.10%
10	519F 785R	96.20%	98.40%	89.60%	95%	96.00%	86.90%	87.30%
11	515F 806R	95.90%	98.30%	89.60%	95%	94.60%	86.90%	89.10%
12	515FB 806RB	95.90%	98.30%	89.60%	95%	96.40%	89%	89.10%

We were interested to understand why some combinations of primers had lower coverage for some groups and why one primer combination had a high coverage for the Bacteria and Eukarya domain. Therefore, we further tested two primers, the forward primer 344F and 519F, using TestProbe 3.0 (110) and the SILVA database SSU132 (114). The archaeal primer 344F has often been used for detecting archaea in a variety of environmental samples (148,149), although primer pairs including this primer have a low performance for targeting the Thaumarchaeota phylum. The results of the test revealed that the primer had a 73.2% coverage of the archaeal domain, with high coverage for the Euryarchaeota phylum (93.8%) and the genera within, especially for

Methanobrevibacter with 96.1% coverage, *Methanosphaera* with 89.9% coverage and *Methanomassiliicoccus* with 100% coverage. It also revealed a good coverage for Woesearchaeota with 74.6%, but showed, despite a high coverage for the genus *Nitrososphaera* (93.6%), generally low coverage of the Thaumarchaeota phylum with only 24%, indicating a potentially low capacity for studies with thaumarchaeotal diversity in focus.

The second primer analysed through TestPrime 3.0, namely primer 519F, also known as S-D-Arch-0519-a-S-15, showed a high coverage of over 90% for all three domains of life. Furthermore, the sequence of this primer (5' - CAGCMGCCGCGGTAA - 3') overlaps with the sequence of the "universal" primer S-*-Univ-0519-a-S-18 (5' - CAGCMGCCGCGGTAATWC - 3'). When the two primers were compared for their coverage in silico, both performed similarly, having good coverage for all three domains of life. Considering our *in silico* results, the primer S-D-Arch-0519-a-S-15 cannot be used to target archaea specifically and should be re-named to S-D-Univ-0519-a-S-15.

As most selected archaea-targeting primers revealed an excellent coverage of the archaeal domain in general, all primer pairs were used for subsequent wet-lab experiments.

III.3.2.2. Archaeal community composition in different body sites

After analysing the *in silico* results of the tested primers, we were interested in determining which of these primer pairs performed best in wet-lab experiments with the intent to identify an optimal primer pair for amplicon sequencing to characterise the archaeal communities in human samples. For our study, we selected five representative sample types from different body sites: upper nasal cavity, oral (subgingival sites), stool and appendix specimens, and skin (back) (sample set 1). The stool sample represented positive control, as the donor showed high amounts of methane in the breath test.

Next-generation sequencing was performed after a two-step nested PCR (for archaea) or a single-step PCR ("universal" target). The nested PCR approach was selected based on the reasons given in the Materials and Methods section. In brief, the first PCR was intended to select the archaeal community of interest, the second to further amplify the archaeal signal.

The results obtained after amplification, NGS and data analysis based on DADA2 algorithm (61,111) are summarized in Appendix 2, which includes the number of reads and observed ribosomal sequence variants (RSVs) obtained for all samples covering the three domains, Archaea, Bacteria, and Eukarya (plus unclassified taxa). The results for the Eukarya are not included in Appendix 2, but will be provided on the compact disk attached to the thesis.

The use of universal primers (primer pair 515F-806uR, 515FB-806RB and 519F-785R) resulted in reads that were mainly classified within the bacterial domain, and only a small percentage of the reads were classified within the archaea domain, thus confirming our previous observations that universal primers are not optimal to characterise the diversity of the archaeal communities in human samples (61). When the two universal primer pairs (515F-806uR original and 515FB-806RB) were compared regarding the archaeal domain, only primer pair 515F-806uR allowed the detection of only one RSV being classified within the archaea and from only one sample, the stool sample.

Universal primer pair 519F-785R yielded slightly better results compared with the other two universal primer pairs, allowing the detection of three different archaeal RSVs from two different samples. *Methanobrevibacter* and *Methanosphaera* were detected in the stool sample, and one RSV which was classified within the Thaumarchaeota phylum was obtained from the nasal sample. Similar results were observed from primer pair 519F-806R, which was originally described to be archaea-specific, but revealed wide coverage *in silico* for the Bacteria and Eukarya domain. By using the primer pair 519F-806R, we observed similar methanoarchaeal signatures in the stool sample to those identified by the primer pair 519F-785R, and we detected an additional RSV from the oral sample belonging to Thaumarchaeota phylum.

A phylogenetic tree was constructed with the RSVs obtained by using the universal primer pairs: 515F-806uR, 515FB-806RB, 519F-785R and the archaeal primer pair 519F-806R to identify whether the universal primer pairs allowed the detection of the same RSVs or closely related RSVs in the analysed samples. To these RSVs, the sequences obtained from the archaeal primer combination 344F-1041R/519F-806R were also added for comparison (Fig. 31). This approach based on the archaeal primer combination 344F-1041R/519F-806R allowed the detection of 20 RSVs in the nasal cavity, 19 RSVs in the oral, one RSV in the appendix, 3 RSVs in the stool, and 39 RSVs in the skin sample. For the stool sample, the RSVs obtained from the universal and archaeal specific approach grouped together, either within *Methanobrevibacter* or *Methanosphaera* clade (Fig. 31), whereas the RSVs from nasal and oral samples obtained through either universal or archaeal specific approach diversified.

Overall, 10 out of 23 primer pair combinations allowed the detection of archaeal signatures in all analysed samples. All 23 primer pair combinations were able to detect archaeal reads in at least one of the sample types analysed, for example, all primer pair combinations detected archaeal

RSVs in the stool sample; the number of RSVs, however, varied according to the used primer pair combination.

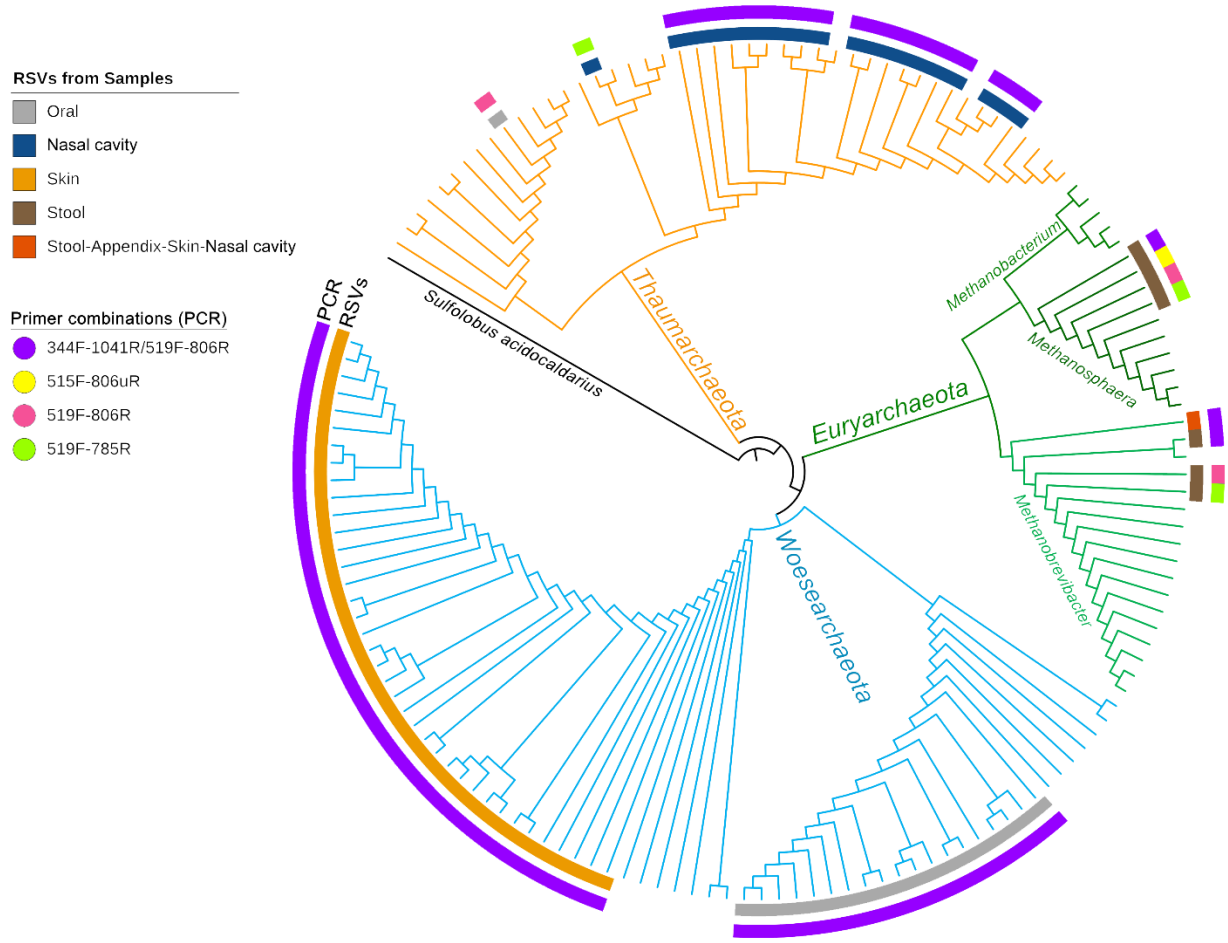

Figure 31. Phylogenetic tree based on the retrieved RSVs from the universal approach based on the primer pairs 515F-806uR, 515FB-806RB and 519F-785R, archaeal approach with primer 519F-806R or from the PCR based on the primer pair combination 344F-1041R/519F-806R as indicated in colours as an outer circle (legend “Primer combinations (PCR)”). The inner-circle represents the body site from where the RSVs were identified (see legend). Reference sequences from the SILVA database are shown without a label. The branches of the tree were coloured according to the phyla, blue: Woesearchaeota, green: Euryarchaeota, and orange: Thaumarchaeota. The figure is taken from (60).

Depending on the used primer pair combination, the archaeal community composition was found to be highly variable (Fig. 32). We observed that the detected variation in the archaeal composition was due to the used primer pair in the first PCR, the primer pair used to select the communities, while the second PCR and primer pair enhanced the signal of the first PCR (Fig. 32). It shall be mentioned that for the second PCR, only three different primer pairs have been used, namely

primer pair 349F-519R, 519F-785R and 519F-806R. The first two primer pairs had been used before to explore archaeal communities in human samples (61) and confined habitats (98).

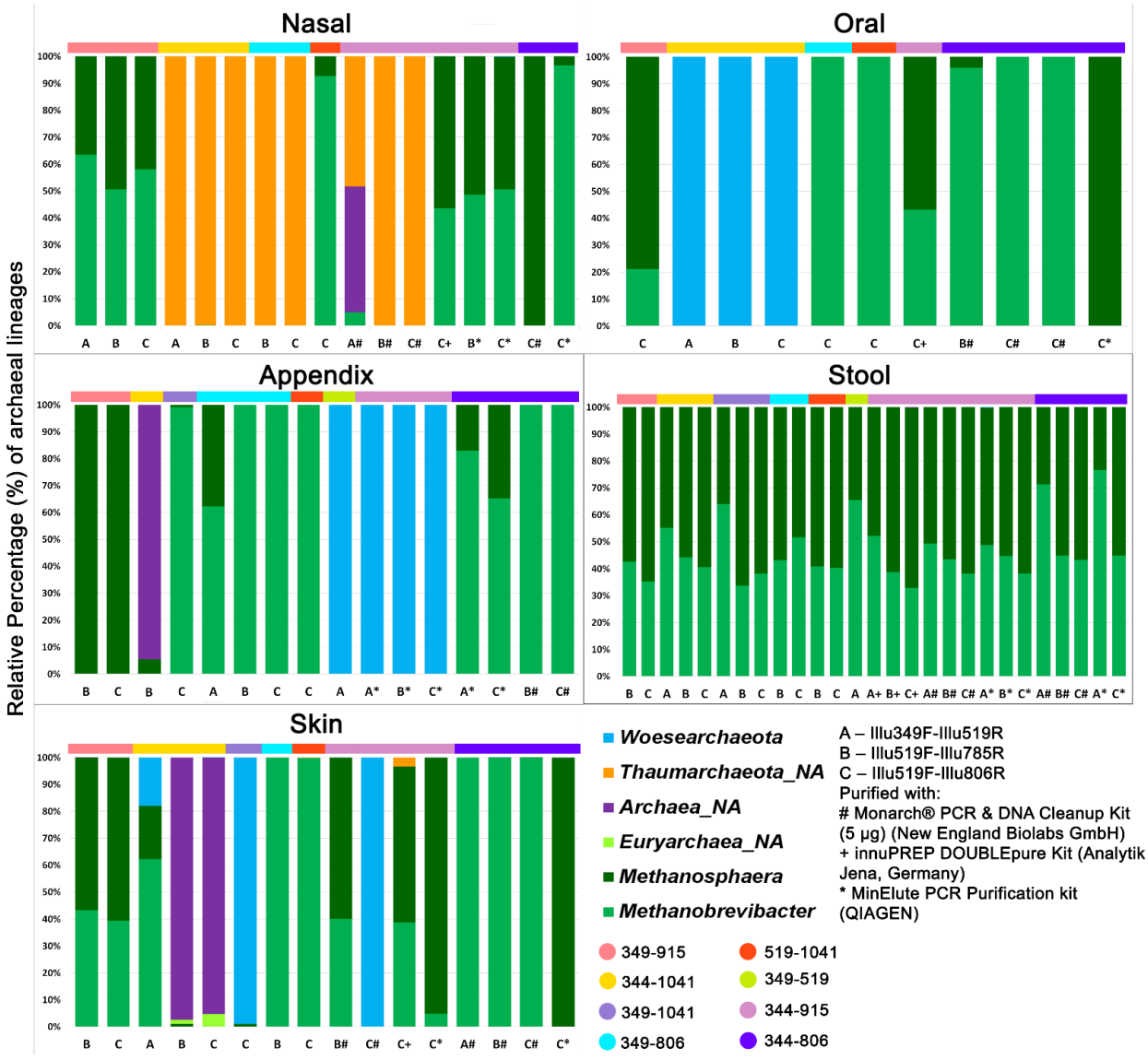

Figure 32. Bar plots illustrate the diversity of archaeal communities dependent on the primer pair combinations used. Primer combinations are presented via the coloured bars above the columns (first primer pair), and by letters below the columns (second primer pair). Also, information is given on the different purification kits that have been used between the two steps of the nested PCR. If no character is given next to the letter, then MinElute PCR Purification kit (QIAGEN) has been used to purify the PCR product. The figure is taken from (60).

Next, we were interested in studying the influence of the primer pair selection on the archaeal diversity. Therefore, we explored the alpha diversity based on the Shannon index for the primer

pair combinations tested (Fig. 33). For this analysis, we excluded the results obtained from the primer pair combinations which contained the primer pair 349F-519R as most samples herein, except for the stool samples, yielded less than 500 reads.

The highest archaeal diversity was detected with the primer combination 344F-1041R/519F-806R (PCR34); this result was found to be significant ($p<0.05$) compared to PCR 33 (344F-1041R/519F-785R), PCR Q7 (344F-806R/519F-806R) and PCR M7 (344F-806R/519F-806R; Table 2 and Fig. 33), whereas no other significant differences could be observed.

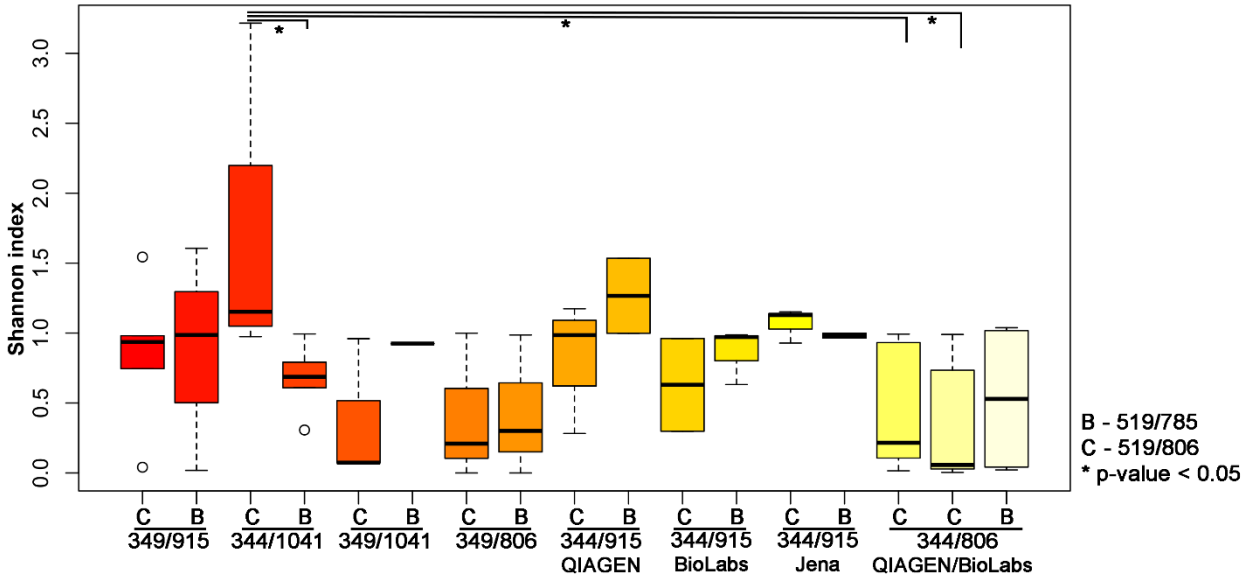

Figure 33. Shannon index showing the archaeal diversity obtained from different PCR approaches. The results have been plotted and grouped according to the first PCR used. The statistical significance (p-value <0.05; Wilcoxon Rank Test) is indicated by *. The figure is taken from (60).

Based on the alpha diversity comparison, we recommend the use of the nested approach with the primer pair 344F-1041R in the first PCR, followed by a second PCR with the primers 519F-806R for studying and exploring the archaeal communities in human samples. Besides, the use of this primer pair combination resulted in a lower number of bacteria/eukarya identified additionally to the archaeal signatures, as showed in the summarised tables (Appendix 2).

The use of the different purification kits between the first and the second PCR resulted in no significant results based on the alpha diversity (Shannon index) comparison using the Wilcoxon Rank Test (p-value >0.05; Fig. 33). Due to visible bands on the gel electrophoresis for the results obtained after the purification with the Monarch® PCR & DNA Cleanup Kit (5 µg) (New England Biolabs GmbH; Ipswich, USA) we decided to use this kit for the purification step further.

III.3.3. Archaeal diversity in stool, appendix, nasal, oral and skin samples

To further test and validate the use of the primer pair combination 344F-1041R/519F-806R for studying the archaeal communities within human samples, we selected additional samples from the same body sites: nasal cavity (n=5), oral (n=6), appendix (n=5), stool (n=5), and skin (n=7) (sample set 2).

Our selected PCR approach allowed the detection of archaea in all samples investigated with an average of 102,366 reads and 8 observed RSVs for the nasal samples, 56,480 reads and 35 observed RSVs for oral, 46,022 reads and 8 observed RSVs for the appendix, 93,948 reads and 4 observed RSVs for the stool samples, and 76,001 reads and 30 observed RSVs for the skin samples. The results were plotted to illustrate the archaeal community composition present in the analysed samples (Fig. 34).

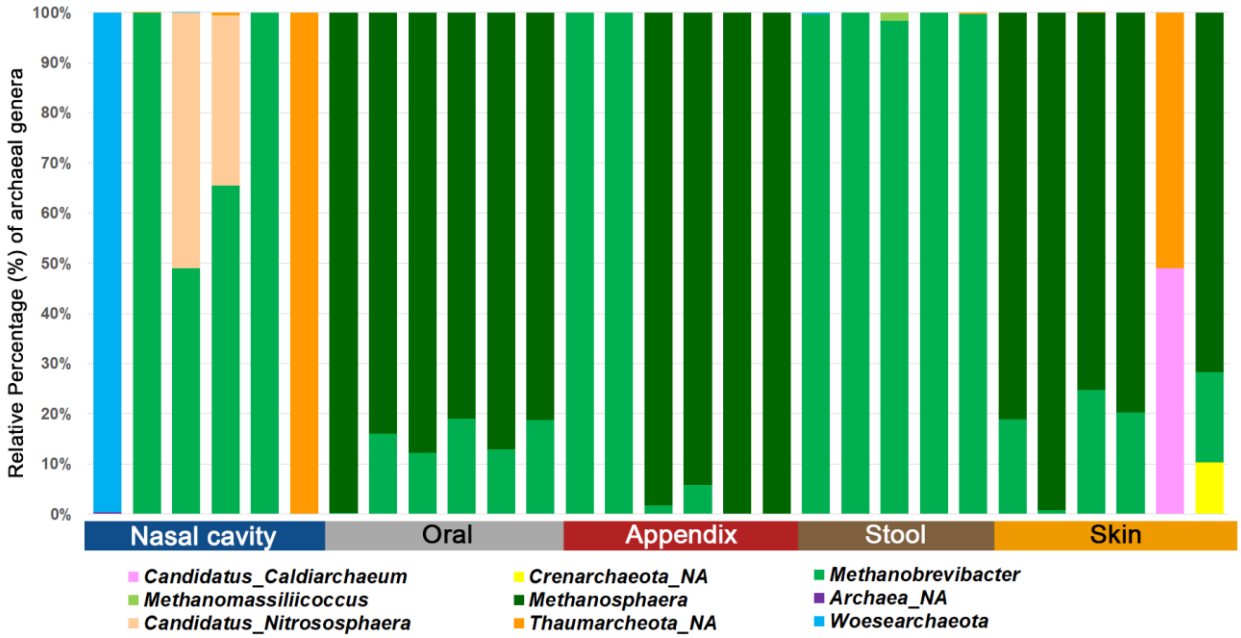

Figure 34. Bar plot displaying the different archaeal taxa detected in different human samples using the primer combination 344F-1041R/519F-806R. The figure is taken from (60).

We further characterised the archaeal community information for alpha and beta diversity. Depending on the body site, a significant difference (p-value < 0.05) could be shown for alpha diversity (Shannon index and richness). In our study, the highest diversity and richness was observed in the oral and skin samples. The beta diversity (PCoA and RDA) analysis indicated that the archaeal communities are body site-specific (Fig. 35), therefore confirming our previous results (61).

Notably, the stool samples revealed the overall lowest diversity of archaea, with only 3-5 identified archaeal RSVs, while skin and oral samples contained a higher diversity, with 5 to 49 RSVs found in the skin samples and 14 to 49 RSVs in the oral samples.

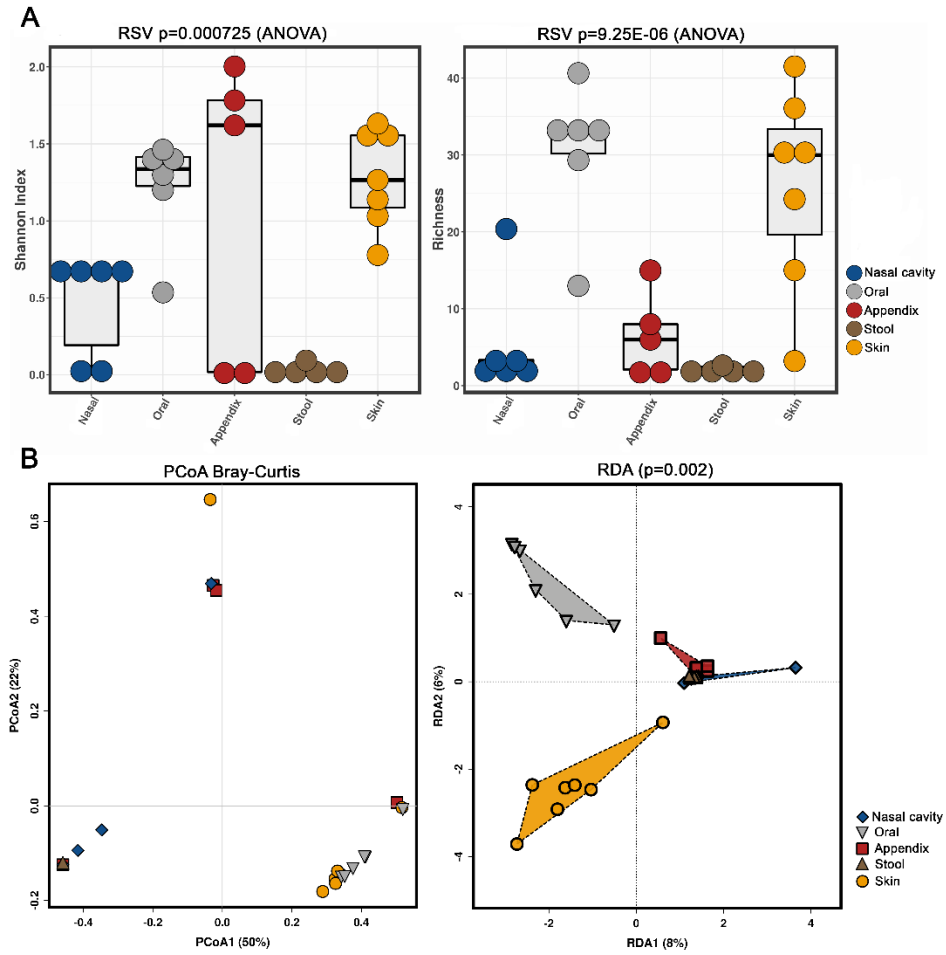

Figure 35. Alpha (A) and beta diversity (B) analyses of the obtained archaeal community information, based on primer combination 344F-1041R/519F-806R. The figure is taken from (60).

III.4. Human Archaeome II

III.4.1. Overview

After we established a new protocol for studying the archaeal communities in the human body, we collected samples from different body sites to explore the diversity and composition of the archaea further. In total, 282 samples have been analysed using the newly developed protocol described in the previous chapters. The samples were obtained from different body sites: lungs (59

bronchoalveolar lavage (BAL) samples), nasal cavity (n=36), oral cavity (n=45), gastrointestinal tract (45 stool samples), skin (n=26), urinary tract (45 urine samples), and vagina (n=26).

After analysing the obtained data and removing bioinformatically the sequences classified within the Bacteria and Eukarya domain, archaeal signatures were detected in only 179 out of 282 samples. The remaining 179 samples were: 20 BAL, 28 oral, 28 nasal samples, 38 stool, 7 skin, 42 urine, and 16 vaginal samples. The number of archaeal reads in these 179 samples varied considerably; for example, the number of archaeal reads identified for the urine samples varied between 14 reads to 101913 reads. Similar results were observed for all body sites studied.

III.4.2. Archaeal community composition

Archaeal signatures were identified from 5 different phyla. Most signatures were classified within the Euryarchaeota phylum (98.82% BAL, 96.45% nasal samples, 87.37% oral, 4.71% skin, 99.98% stool, 82.82% urine, and 99.99% vagina). Thaumarchaeota signatures were found mainly in the skin samples (95.29%) and some in urine (8.84%), BAL (1.18%), nasal samples (3.55%), oral (0.05%), and stool (0.02%). No Thaumarchaeota signatures were observed in any of the vaginal samples. Other detected phyla were Nanoarchaeota (12.53% oral, 3.62% urine, 0.01% vagina), Diapherotrites (3.92% urine), and Crenarchaeota (0.79% urine, 0.04% oral) (Fig. 36).

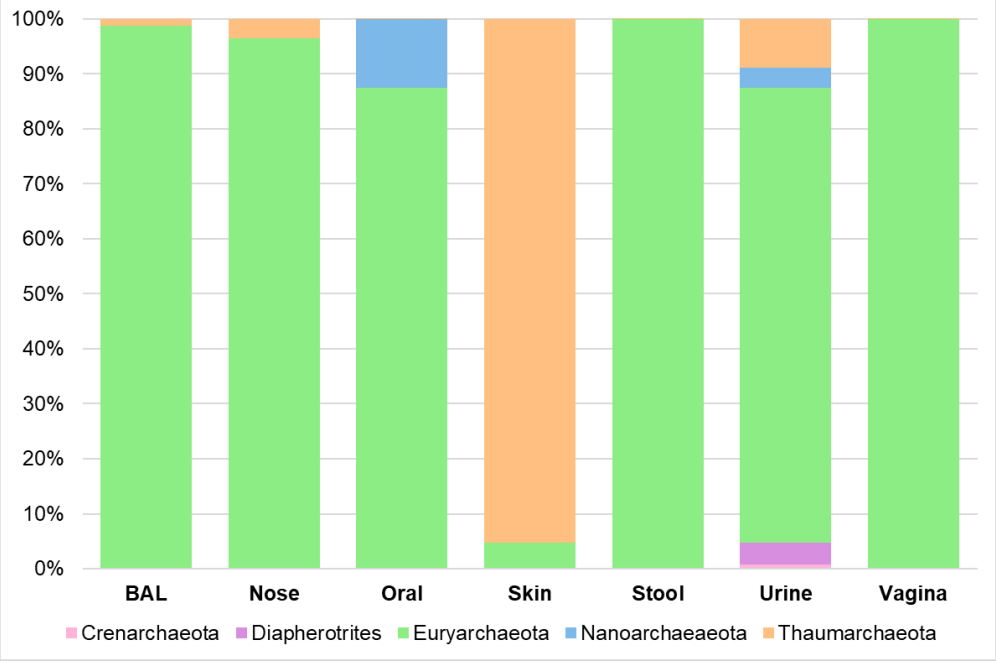

Figure 36. Box plot of the relative abundance of archaeal communities at the phylum level in the analysed body sites.

At the genus level, most identified sequences were classified within *Methanobrevibacter*, *Methanobacterium*, and *Methanosphaera* except for the skin samples which were dominated by Candidatus *Nitrosocosmicus* and other unclassified genera belonging to *Nitrososphaeraceae* family.

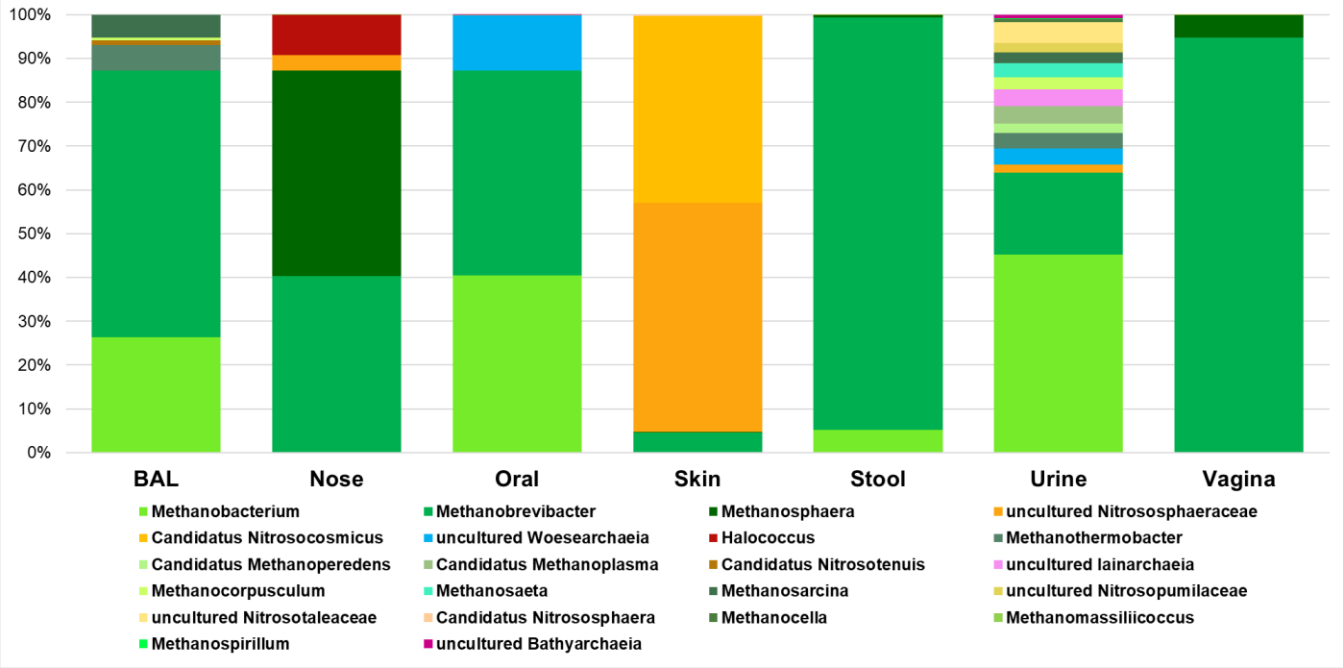

Figure 37. Box plot of the archaeal communities at the genus level in the analysed body sites.

III.4.3. Archaeal communities present in the different body sites

To confirm our previous studies, we explored the effect of the body site location on the archaeal communities. Alpha diversity and richness were measured based on Shannon, Inverse Simpson's and Chao1 indices. Only samples with more than 500 reads were included for the alpha diversity measurements. The results showed that the nasal archaeal community had the highest diversity and richness (Fig.38). Next, we were interested in determining the influence of the body location on the archaeal composition; therefore we performed PCoA based on Bray-Curtis and performed an Adonis test, which showed that the archaeal composition is influenced by body location as previously shown in our study (61). This result was confirmed through an RDA plot which showed that archaea are body site-specific, although some locations do share similarities in their composition (Fig. 39). Oral and urine samples share a similar archaeal composition, while some nasal, stool and vagina samples share similar archaea.

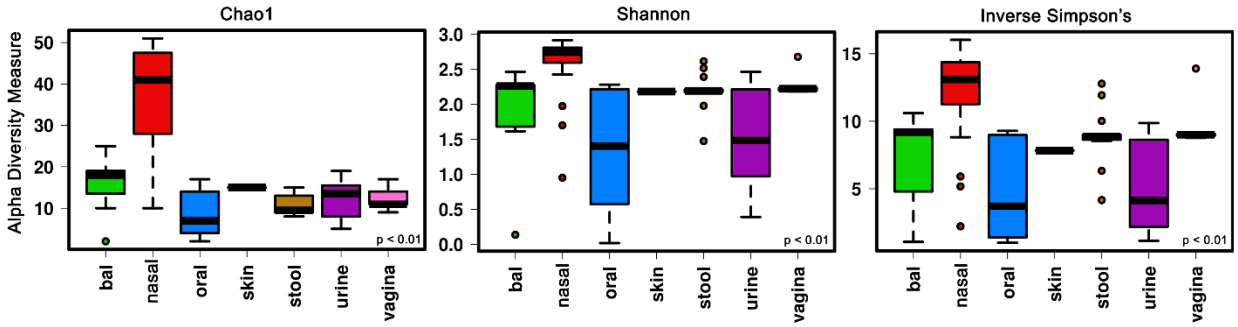

Figure 38. Alpha diversity as measured by using the Chao1, Shannon and Inverse Simpson's indices for different body sites.

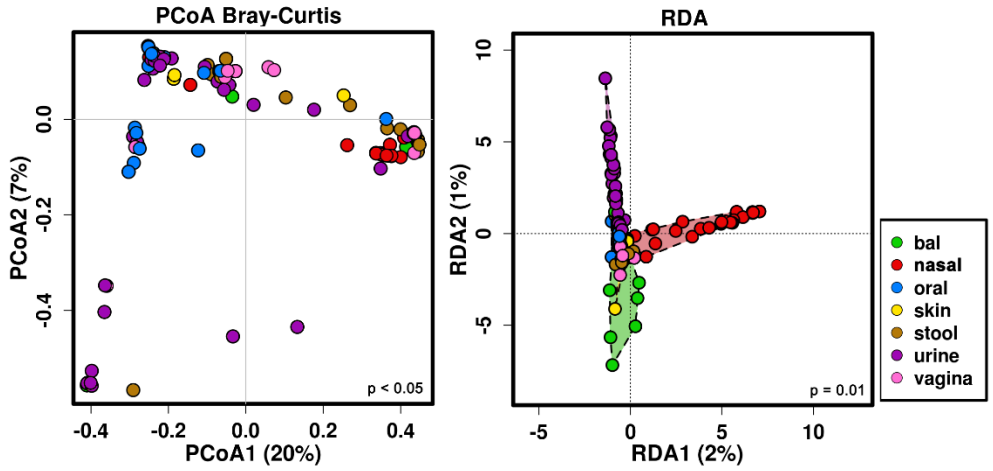

Figure 39. Principal Component Analysis (PCoA) based on Bray-Curtis similarities and RDA plot showing the differences in the microbial composition between different body sites.

We then explored the abundance of specific taxa in the different body sites analysed. The results showed that *Halococcus* was highly abundant in some nasal samples, but has not been identified in any other body sites. *Methanobacterium* was highly abundant in most urine samples, and samples from the BAL, several oral and stool samples and was detected in one nasal and vaginal sample (Fig. 40). The most abundant genus, *Methanobrevibacter*, has been identified in all body sites studied, being highly abundant in stool, BAL and vaginal samples (Fig. 40). Most *Methanosphaera* sequences were observed in the nasal samples, and some in the skin, stool, urine and vaginal samples, but at a lower abundance (Fig. 40). Signatures classified within the Nitrososphaeraceae family were detected in some nasal samples, skin and urine samples, and Woesearchaeia signatures were detected mainly in the oral and urine samples, and only one BAL and one vaginal sample contained sequences classified within this class (Fig. 40).

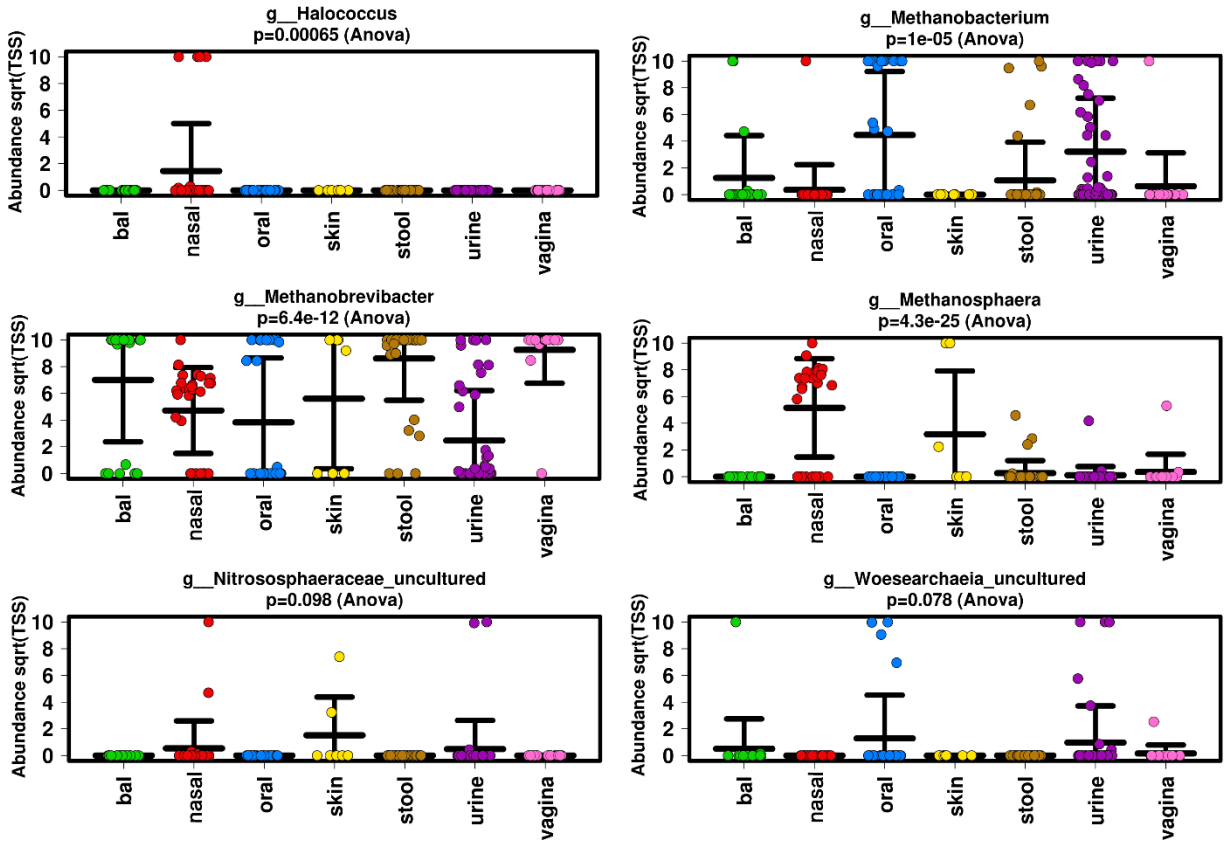

Figure 40. Differences in the relative abundance of archaeal taxa in different body sites.

To understand which archaeal taxa are shared between different body sites, we performed a network analysis. The results indicated that most shared taxa between different body sites are methanogens, and especially *Methanobrevibacter*, *Methanobacterium* and *Methanosphaera* (Fig. 41). In total, 63 RSVs classified within the *Methanobrevibacter* genus were shared between all body sites, 6 *Methanobacterium* RSVs were shared between most body sites except for skin, and 10 *Methanosphaera* RSVs were shared only between nasal, oral, stool, urine and vagina. The majority of the identified RSVs were not shared, the nasal cavity had 342 unshared RSVs, followed by BAL with 186 unshared RSVs and urine with 176 unshared RSVs. However, the network also shows the biogeographic pattern of the human archaeome, which is similar to bacteria, some taxa are shared between different body sites, while some are present only in specific locations within the human body.

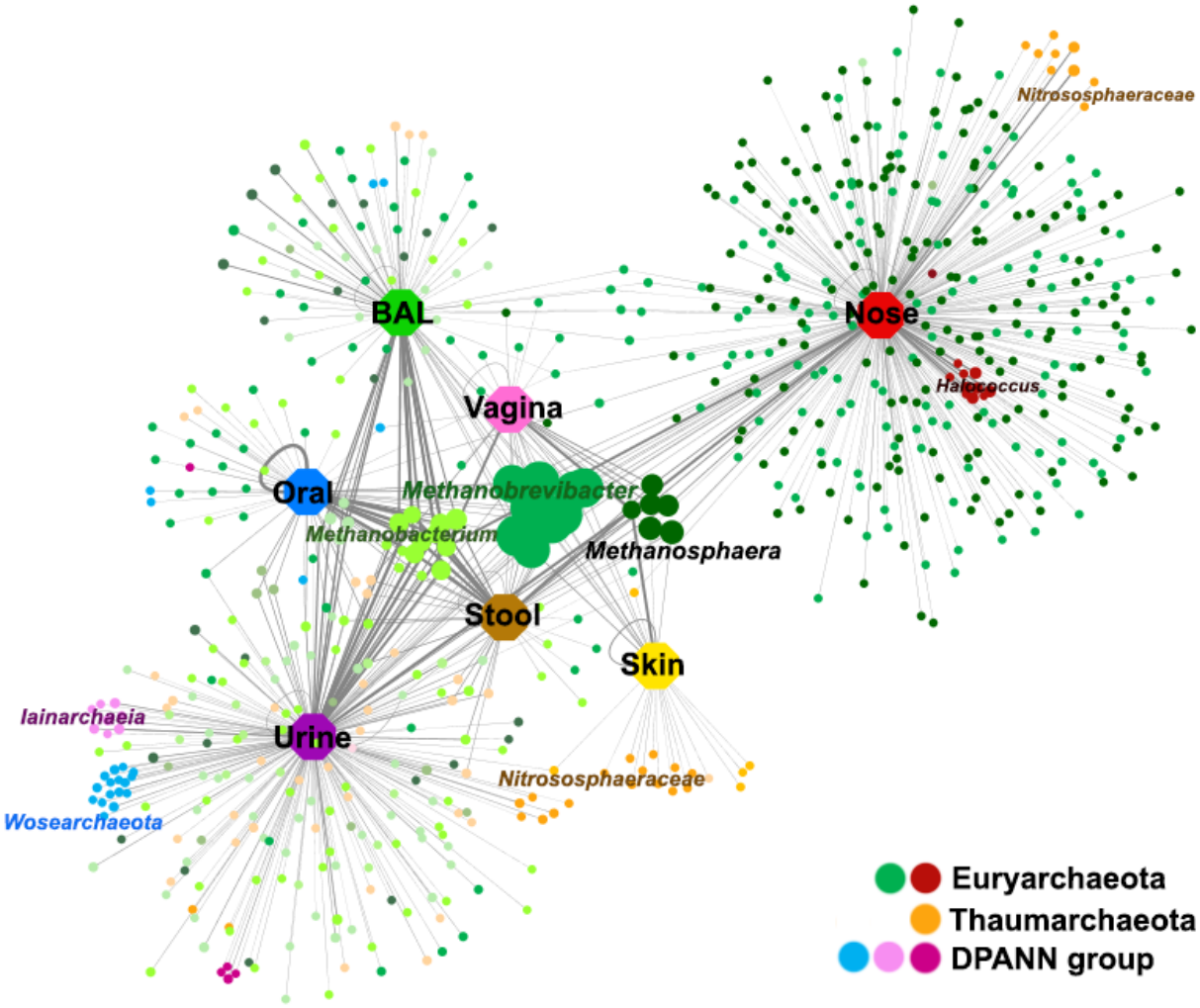

Figure 41. Network showing the biogeography of the human-associated archaeal communities.

III.4.4. The effect of gender and age on the archaeal community

From bacterial studies, it is well known that the microbiome is influenced by gender and age. We were interested in determining if the gender and age have any effect on the archaeal communities in our study; therefore, we performed LEfSe and ANOVA analysis on each body site. For the gender effect, we observed only a few associations between some *Methanobrevibacter* RSVs and female gender in nasal and urine samples (Fig. 42).

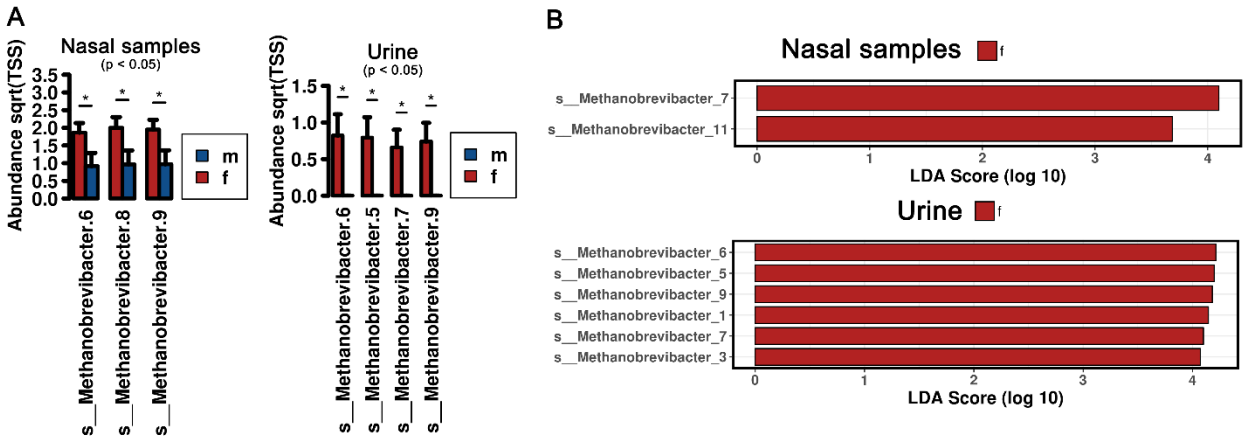

Figure 42. (A) Barplots are showing the different relative abundance of specific RSVs between male and female gender. (B) LEfSe (Linear discriminant analysis effect size) of nasal and urinary archaeaome in relation to gender.

To study the age effect on our identified archaeal community, we grouped the participants in different age groups. For example, the age of the participants from whom we collected nasal, oral, stool, vagina and skin samples ranged from 19 to 43 years old. These were grouped either in the under 30 years old group or over 30 years old group. BAL samples were obtained from older participants; therefore, these were grouped in under 60 years old and over 60 years old. When we analysed the data, we observed similar effects to those of gender, only a few RSVs were identified as being different between the analysed groups. The results showed that a specific *Methanosphaera* RSV identified in nasal samples was associated with individuals who are over 30 years old, while a *Methanobacterium* RSV identified in oral samples was associated with individuals younger than 30 years old. For stool samples, two RSVs belonging to *Methanobrevibacter* genus were associated with individuals over 30 years old (Fig. 43)

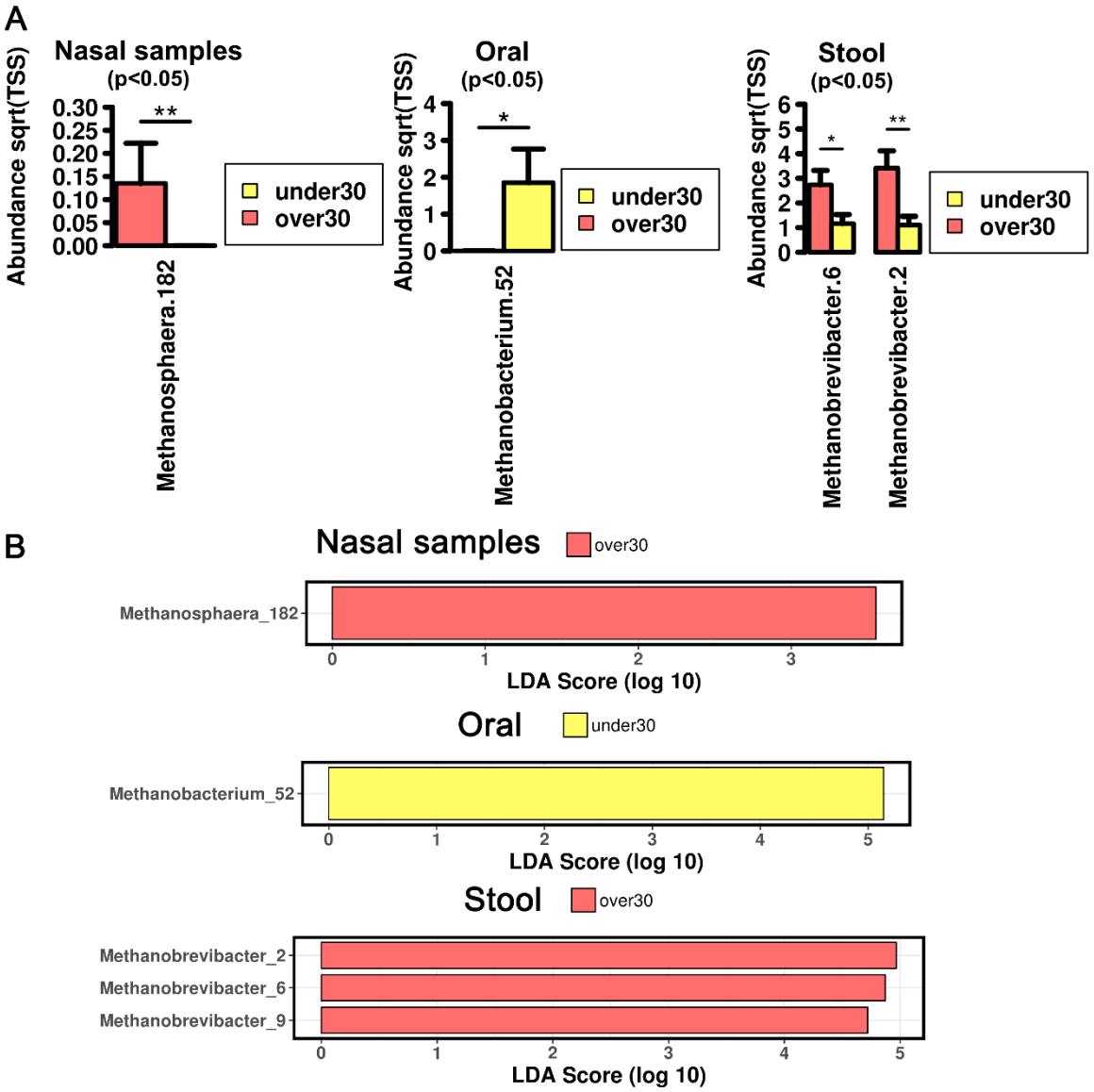

Figure 43. (A) Barplots are showing the different relative abundance of specific RSVs between different age groups in nasal, oral and stool samples. (B) LEfSe (Linear discriminant analysis effect size) of nasal, oral and stool archaeaomes in relation to age.

III.4.5. Metagenomics results

Based on the amplicon sequencing results, 20 samples were further investigated through shotgun metagenomics. In total, 74 draft genomes were obtained. The completeness of the draft genomes varied from 100% to 0.31%, and it was higher for the bacterial draft genomes. 29 draft archaeal genomes were obtained with completeness varying between 33.39% to 0.31% and contamination between 14.79% to 0%. Although the completeness of the archaeal draft genomes is low

compared to the bacterial ones, we were able to reconfirm the presence of archaeal signatures through a different method other than amplicon sequencing. Most archaeal draft genomes were obtained from nasal samples (16 archaeal draft genomes), followed by BAL (7 archaeal draft genomes), oral (3 archaeal draft genomes), vaginal (2 archaeal draft genomes) and urine (1 archaeal draft genome). A list of all identified archaeal draft genomes can be found in Table 12. The results indicate that deeper sequencing is needed for human samples to obtain archaeal draft genomes through shotgun metagenomics at a high quality and completeness. Due to time constraints, the analysis of the metagenomic data was not completed.

Table. 12. CheckM results of the archaeal draft genomes obtained.

Bin Id	Marker lineage	Completeness	Contamination	Strain heterogeneity
Nasal24.contigs..001	k__Archaea (UID2)	33.39	14.79	4.35
Nasal20.contigs..001	k__Archaea (UID2)	24.92	8.41	6.06
Nasal20.contigs..002	k__Archaea (UID2)	23.12	12.68	10.34
BAL1189.contigs..001	k__Archaea (UID2)	22.03	10.83	0
BAL1059.contigs..001	k__Archaea (UID2)	16.73	10.2	3.7
Oral15-Tra.contigs..004	k__Archaea (UID2)	15.73	5.84	33.33
Vaginal23.contigs..011	k__Archaea (UID2)	15.65	3.12	0
BAL1189.contigs..002	k__Archaea (UID2)	15.56	7.01	0
Nasal22.contigs..004	k__Archaea (UID2)	13.85	5.61	8.33
Nasal26.contigs..001	k__Archaea (UID2)	12.76	4.21	0
Nasal22.contigs..001	k__Archaea (UID2)	12.75	1.87	0
Nasal22.contigs..002	k__Archaea (UID2)	12.61	3.74	8.33
Nasal60.contigs..002	k__Archaea (UID2)	11.68	3.58	16.67
Urine4-A.contigs..001	k__Archaea (UID2)	11.29	1.87	0
BAL1190.contigs..002	k__Archaea (UID2)	10.51	0.93	0
Nasal26.contigs..002	k__Archaea (UID2)	10.12	4.67	16.67
BAL1059.contigs..002	k__Archaea (UID2)	9.33	0	0
Nasal26.contigs..003	k__Archaea (UID2)	8.9	2.43	0
Nasal22.contigs..003	k__Archaea (UID2)	8.41	0	0
BAL1190.contigs..001	k__Archaea (UID2)	8.17	0.31	0
Vaginal23.contigs..010	k__Archaea (UID2)	7.17	2.18	0
Nasal22.contigs..005	k__Archaea (UID2)	6	0	0
BAL1059.contigs..003	k__Archaea (UID2)	5.22	0.62	0
Oral3-Tra.contigs..016	k__Archaea (UID2)	3.58	1.87	50
Nasal60.contigs..001	k__Archaea (UID2)	3.27	0	0
Nasal22.contigs..008	k__Archaea (UID2)	1.25	0	0
Nasal22.contigs..007	k__Archaea (UID2)	0.93	0	0
Oral3-Tra.contigs..017	k__Archaea (UID2)	0.62	0	0
Nasal22.contigs..009	k__Archaea (UID2)	0.31	0	0

IV. Discussions

IV.1. House Microbiome

Exploring the microbial communities in indoor environments is important as recent studies show that exposure to indoor microorganisms can have an impact on human health (150,151). As more countries become industrialised and people are moving from the countryside to the cities, studies estimate that in general people spend almost 90% of their time in indoor environments, be it at home, in the office, or at school. Studies have shown that human-associated microorganisms dominate the indoor environment (150,152–154); a single person can emit 10^6 microorganisms per hour into their environment (155,156). Recent studies show how features of the built environment such as allergens, pollutants and especially microbial diversity can contribute to the development of asthma, rhinitis and atopic dermatitis in children (44). Children who live near forests and farmland develop fewer allergies than children who live in cities (157,158). Another interesting aspect is the early indoor exposure on the development of the infant's gut microbiome, as not much is known aside from hospitals studies and preterm born infants (44,159,160).

In our study, we were interested in exploring if the indoor microbiome, with particular focus on the bathroom area, harbours anaerobic microorganisms, therefore being a source of human-associated anaerobic microorganisms. To distinguish between dead cells and intact cells, we treated one of the samples collected from the same house with propidium monozide (PMA). PMA is a photoreactive dye which can enter a cell only when the membrane or the cellular wall is broken. Upon light activation, the PMA binds irreversibly to the DNA; therefore, the DNA bound by PMA can no longer be amplified by the polymerase (161). The focus of our study is not only the bacterial community but also the archaeal community found in the indoor environment.

Most dominant archaeal and bacterial communities identified in our samples were previously associated with human skin, gastrointestinal tract and urogenital tract. The dominant taxa were *Staphylococcus*, *Enhydrobacter*, *Finegoldia*, *Corynebacterium*, and Thaumarchaeota. These taxa have been previously associated with human skin (8,63,162,163). Besides skin-associated microorganisms, we also identified gastrointestinal and urogenital associated taxa such as *Lactobacillus*, *Anaerococcus*, *Bacteroides*, and different methanogens, for example, *Methanobrevibacter*, *Methanomassiliicoccus*, and *Methanobacterium* (13,69,144,146,164–166). Similar results were reported in previous studies focusing on the restroom microbiome, although in our study the dominant bacterial phylum was Firmicutes, followed by Actinobacteria,

Proteobacteria and Bacteroides, while others have reported Actinobacteria as being the dominant phylum (167–170). The differences could be either explained by different geographical locations of the studies, or by the fact that most studies focused on the indoor environment are performed in public spaces used by more than one individual. Our samples were mainly collected from family houses with no more than 4-5 individuals; therefore, the amount of skin microbiome shed is restricted to a smaller group compared to public spaces.

Interestingly in our study, one house had a high abundance of signatures of halophilic archaea, especially genera *Halococcus* and *Halomarina*, but after PMA treatment, only the genus *Halococcus* was still identified in this specific environment. *Halococcus* signatures have been identified before in a clean room facility and as part of the microbiome found to deteriorate cultural heritage (171,172). *Halococcus* thrives in salty-rich environments; unfortunately, we did not measure any chemical parameters of the sampled surfaces; therefore, we could not confirm if this specific sampled location had a higher amount of salts compared to all the other sampled surfaces.

The PMA treatment did not have a strong influence on the diversity and richness of the bacterial and archaeal communities. Still, it influenced the bacterial composition, although the PMA treated and non-treated samples for most houses grouped in the PCoA plot (Fig. 4A). The PMA affected the copies number of bacterial and archaeal 16S rRNA gene, as expected since only the DNA found within intact cells was further used in the following steps after the PMA treatment (173,174). PMA treatment has been previously shown to be useful for indoor microbiome studies, especially as the numbers of microorganisms found in the indoor environment is lower compared to other environments (172,175,176). We observed a decrease in the relative abundance of specific anaerobic microorganisms in the PMA treated samples such as *Lactobacillus*, *Akkermansia*, *Bacteroides*, *Faecalibacterium*, *Prevotella*, *Ruminococcus*, *Methanobrevibacter*, and *Methanobacterium*, although for most taxa, the difference was not significant, and signatures of these taxa were still identified in the PMA treated samples. Most of the DNA of these anaerobic taxa comes from damaged cells as suggested by their increased relative abundance in the nonPMA treated samples, but their presence in the PMA treated samples indicates that some of these cells can survive in the fully aerated indoor environment for a certain period, although we speculate they have no metabolic activity. Therefore, some anaerobic microorganisms can survive the exposure to oxygen for a certain amount of time.

The phenotypes predicted through BugBase analysis (141) showed that the microbial communities present in the PMA treated samples are both aerobic and anaerobic microorganisms.

Also, with the help of BugBase, we identified a high relative abundance of facultative anaerobes present in the PMA treated samples, and as the analysis indicates these microbial communities are better equipped for dealing with specific stresses.

As a next step, we were interested if the methanogens we identified are of human origin or if they are environment-related microorganisms. Our analysis indicated that for genera *Methanobrevibacter*, all signatures identified in the sampled areas were either associated with the gastrointestinal tract or oral cavity. On the other hand, for *Methanomassiliicoccus* and *Methanobacterium,* it was unclear. Previous studies have shown that *Methanomassiliicoccus* can be divided into two clades, one host-associated and one environment-associated. *Methanomassiliicoccus* species associated with both clades have been isolated and identified in the human body (142). For *Methanobacterium*, only one previous study has shown the presence of *Methanobacterium* in human-associated samples (61), unfortunately, using different primer pairs than in our study, making comparison analysis unfeasible.

Methanogens are known to be strictly anaerobic microorganisms. Still, the sensitivity to oxygen varies among different species of methanogens, being dependent on the repertoire of enzymes involved in the oxidative stress, such as catalase, peroxiredoxin, rubrerythrin, superoxide dismutase, superoxide reductase and many more (177,178). Several studies have shown that methanogens can tolerate oxygen exposure (179,180). To test if human-associated methanogens can survive in an aerobic environment, we developed a method to expose methanogens to aerobic conditions for several time points after aerobic washing with 1% sterile PBS. One previous study has shown that aerobic washing with buffer solution has little impact on methane production (181). Our test showed that human-associated methanogens, namely *Methanobrevibacter smithii*, *Methanosphaera stadtmanae* and a new isolate of *Methanobrevibacter* genus, could survive exposure to aerobic environment at least 6h, but not longer than 24h. How they respond to oxygen exposure we do not know; it could be that the methanogens on the top are forming a protective layer for the cells underneath. Further studies are needed to understand which mechanisms are activated within anaerobes after exposure to oxygen in order to cope with the oxidative stress. For example, metagenomics analysis could be used to explore which microbial genes are present, and the use of transcriptomics analysis would help to further investigate which of these genes are being activated.

To conclude, we could confirm one of our hypothesis that human-associated anaerobes are persisting in the indoor environment, although most probably in an inactive state. However, more

studies are needed to explore if the indoor environment can act as a source of these microorganisms and if it plays a role in the seeding and development of the gastrointestinal tract in infants in the first year of life, especially for the archaeome.

IV.2. UMIC project

In our study, we wanted to identify potential changes in the vaginal and urinary microbiome of women at risk of preterm birth (PTB), and to explore the connection between human milk oligosaccharides and the microbial communities present at certain body sites. Therefore, our study combined the comparative analysis of vaginal and urinary microbiome, serum and urinary HMOs in pregnancies at risk for spontaneous PTB. We identified key HMOs and microbial signatures associated with a higher risk of PTB and postulated an essential role of HMOs in the regulation of a healthy, term pregnancy.

Few studies have investigated changes in HMO profiles in human milk as a consequence of PTB (182,183), while their potential causal role in PTB has not been explored so far. Recently, Jantscher-Krenn et al. have shown that HMOs in serum increase during pregnancy, raising the question of whether HMOs are associated with preterm labour and/or PTB (184). An early study reporting on HMOs in the urine of pregnant women speculated about the importance of HMOs in maintaining a healthy pregnancy (185). Herein, we were able to confirm this hypothesis, as specific HMOs indicated a risk for preterm birth. Furthermore, we report for the first time, associations of serum and urinary HMOs with specific taxa of the urinary and vaginal microbiome.

In this study, the sampling window was between 23 to 34 weeks of gestation, and the secretor active HMOs strongly varied as the abundance of 2'FL increases with gestational age according to previous studies (184). The assigned secretor status matched between serum and urine samples in all women and was based on the relative abundance of 2'FL. A negative secretor status was identified in 16% of all women, which was comparable to previous findings (137). Correcting for the time of sampling and excluding the secretor negative women, we found a high concentration of serum 3'SLN in women with a short cervix and high 3'SL associated with women who later delivered preterm. Interestingly, only 3'SLN and 3'SL in serum, but not in urine, were associated with the short cervix or preterm birth. While the reason for the discrepancies between serum and urine could be multiple, e.g. attributed to the relatively small sample size, or errors in the normalization of urine, this might also indicate a more systemic mechanism.

Our finding that sialylated HMOs in serum are associated with a short cervix and preterm birth are in accordance with a study reporting increased total serum sialic acid concentration in PTB (186).

In human milk, HMOs were also found to be different in sialylation after preterm birth. These findings might support the hypothesis that sialyation, in general, is altered in preterm labour mediated by (sterile) inflammatory processes. This pathway would not necessarily be dependent on changes in the urinary and vaginal microbiome and would explain why the composition of urinary HMOs are not associated with preterm birth in our cohort. Besides, studies have shown an increase in sialic acid in plasma in inflammatory pathologies such as cancer, cardiovascular diseases and type 2 diabetes (187).

Similarly, inflammation was shown to be associated with altered glycosylation due to extrinsic sialylation via extracellular sialyltransferases (188). Whether increased sialylation is a general response to inflammatory conditions in pregnancy, and if these changes in sialylation have ameliorating or deteriorating effects on the pregnancy remains to be elucidated. Larger cohorts studies, including over 1000 pregnant women at risk of preterm birth are needed to explore whether these sialylated HMOs in serum might have a potential as predictive markers for PTB. A limitation of such a study would be the recruitment of a larger ethnic heterogeneous group of pregnant women at high risk of preterm birth, especially as previous studies have shown that women of African origin have an increased risk of preterm birth in comparison with women of others ethnic backgrounds (189).

Our study aimed to investigate medically unexplained preterm labour. Thus, we excluded women who were diagnosed with or had previously been treated for urinary tract infections, which are often linked to preterm labour and PTB. Our results indicate that HMOs in urine and serum shape the microbial community, supporting, in particular, those that can feed on them, such as *Gardnerella*. Like bifidobacteria, *Gardnerella vaginalis* possess the fructose-6-phosphate phosphoketolase (F6PPK), the key enzyme of the so-called “bifid shunt”, a specific pathway for carbohydrate metabolism (190,191). This indicates that *Gardnerella* is capable of utilising HMOs, in particular, those that are associated with positive secretor status. The results represent a potential adaptation and growth advantage of *Gardnerella* in secretor positive, pregnant women, therefore, opening up new questions on longitudinal associations with gestation and increasing HMO concentrations.

Previous studies have reported a decrease in vaginal microbial diversity and richness between first and second trimester in women with PTB (54), while other studies could not identify a change in the microbial diversity and richness (55,57,145). Recently, one study has shown a significant difference in the alpha diversity of the vaginal microbiome between women delivering term to those

who delivered preterm (192). In our study, we did not observe significant changes in diversity, richness or bacterial load in women at increased risk of preterm birth.

We identified CST V, dominated by *Lactobacillus jensenii,* mainly in women who were at high risk of PTB and received tocolytic treatment, and especially in women who had a short cervix. Interestingly, none of the women who delivered preterm had CST V at the time of sampling. Additionally, we observed an increase in the relative abundance in specific taxa, such as *Lactobacillus jensenii* and *Ureaplasma urealyticum,* in women who received tocolytic treatment and in women with a short cervix. In our study, we also found signatures of *Gardnerella* associated with preterm labour and short cervix. Others have shown associations between either specific taxa or vaginal CSTs and preterm birth (56,57,193). The observed associations of CST IV with preterm birth might be explained by a higher risk for bacterial vaginosis in these women compared to women with a vaginal microbiome dominated by *Lactobacillus* species.

Kindinger et al. (55) showed associations between *Lactobacillus iners*, short cervix and PTB. In our study, *Lactobacillus iners* was not associated with short cervix or PTB. In contrast, more women who had a normal cervix, delivered term and received no tocolytic treatment had a vaginal microbiome dominated by *Lactobacillus iners*. This discrepancy might be explained by differences in the methodology used and the ethnicity of the cohorts. The study cohort of Kindinger et al. included a higher proportion of women of African Americans than ours, and 32% of the preterm deliveries were of mothers of African origin. In our study, we had only two (3.33%) women of African origin, none of whom delivered preterm. Furthermore, it has previously been shown that vaginal microbiome of African American women is more likely to be colonised by *L. iners* (CST III), *Anaerococcus* or *Mycoplasma* (CST IV); while European women are more likely to be colonised by *L. crispatus*, *L. jensenii* and *L. gasseri* (194). Therefore, the association found in previous studies between *L. iners* and PTB might be cohort-specific.

Recently, a newer study including different metaomics techniques showed that women with a significantly lower abundance of *Lactobacillus crispatus* and an increased abundance in BVAB1, *Sneathia aminii*, *Prevotella* cluster 2, *Megasphaera* type 1, TM7-H1 in the vaginal microbiome delivered preterm. The cohort analysed in this study was also predominantly comprised of women of African ancestry (around 78%). Interestingly, four out of the nine cytokines which they measured in vaginal samples were significantly increased in preterm birth, namely eotaxin, IL-1β, IL-6 and MIP-1β. In our study, we did not identify any changes in the taxa which have been associated with

preterm birth in Fettweis et al. (189). The differences between our study and the study of Fettweis et al. (189) could also be due to different ethnicities present in the analysed cohorts.

Our findings that *L. jensenii* and *Gardnerella* abundance in the vaginal microbiome was associated with a higher risk of PTB are following previous studies (57,195). However, the observation that vaginal microbiome dominated by *L. crispatus* might be protective against PTB (55,57,189,195,196) was not confirmed in our study. In contrast, seven out of eleven women who delivered preterm had a vaginal microbiome dominated by *L. crispatus*. We are aware that the number of women who delivered preterm in our cohort is relatively small compared to the other studies, and a large cohort of patients delivering preterm is needed to validate previous results.

Our results on the urinary microbiome are consistent with a previous study, where no distinct changes were observed in the alpha and beta diversity of urinary microbiome between women who delivered preterm compared to women who delivered term (197). However, we observed enriched taxa between the groups; for example, *L. crispatus* was highly abundant in pregnant women who did not receive tocolytic treatment and especially in those who delivered term. *L. gasseri, L. jensenii* and species of *Finegoldia* were enriched in women with a short cervix, while the relative abundance of *Ureaplasma urealyticum* was increased in women delivering preterm. We also observed strong correlations between PTB, short cervix and species of *Gardnerella*. As none of the women showed symptoms of urinary infections at the time of sampling, the increase in *Ureaplasma urealyticum* in the PTB group could indicate asymptomatic infections, which could be monitored as a risk factor. Different to our study, Ollberding et al. have shown an increase in the relative abundance of *Prevotella*, *Sutterella*, *L. iners*, *Blautia*, *Kocuria*, *Lachnospiraceae* and *S. marcescens*, which might be due to the different ethnic groups recruited (197).

In conclusion, our results indicate correlations between preterm labour, short cervix and PTB with several microbial signatures in both, the vaginal and urinary microbiome and concentrations of specific HMOs. In the vaginal microbiome, we identified *Gardnerella sp*. associated with preterm labour, short cervix and preterm birth. *Ureaplasma sp*. correlated with a short cervix and PTB. *L. jensenii*, *L. gasseri* and *Flavobacterium* were associated with preterm labour, while *L. crispatus* was correlated with preterm birth. In the urinary microbiome, we detected changes in similar taxa. *Gardnerella* was the only taxa associated with preterm labour, short cervix and PTB, while other microbial signatures were associated only with preterm birth; for example, *Ureaplasma urealyticum* was associated with PTB, while *Finegoldia* and *L. gasseri were associated with short cervix*, and *L. jensenii* and *Staphylococcus* were correlated with both preterm labour and short

cervix. We observed strong correlations between sialylated HMOs, especially 3'SL, and short cervix, preterm delivery and inflammation.

To sum up, our observations point at two different mechanisms that could lead to preterm labour and preterm birth (Fig. 28). One seems to be driven by sterile inflammation, characterised by increased sialylated HMO concentrations in serum, and the second mechanism appears to be indirect and microbiome-mediated, which could, however, be driven by secretor-associated HMOs. Our initial hypothesis was confirmed, although no major changes were observed in the vaginal and urinary microbiome of women at high risk of preterm birth, we still identified changes in specific taxa in these body sites associated with preterm labour and preterm birth. Furthermore, our results indicate that the HMOs influence the vaginal and urinary microbiota, although the mechanism behind still needs to be elucidated.

Our results in identifying key HMOs and key microbial taxa associated with preterm birth will guide current efforts to better predict the risk for PTB in seemingly healthy pregnant women, and also provide appropriate preventive strategies by modulating the microbiome or tackling the inflammation during pregnancy. Preventive treatments for women at high risk of preterm birth could include prebiotics, such as commercially produced HMOs, and probiotics, for example, *Lactobacillus crispatus* which has been associated with term birth.

IV.3. Human Archaeome

Human-associated archaea remain understudied in the field of microbiome research, although numerous studies have indicated the potential of archaea (in particular methanogens) to represent keystone species, as they can be significant drivers of the gut metabolic processes, especially in keeping the hydrogen pressure low and supporting fermentation (86,91). Unfortunately, studies on human archaeome are scarce compared to the bacteriome. Most of the archaea studies in human samples are associated with gastrointestinal tract, oral diseases such as periodontitis, peri-implantitis and recently focused on anaerobic abscesses (69,88,95,198–201).

The lack of more human archaea oriented research could be due to several factors. Some of these factors are: the unfavourable ratios between human DNA, bacterial DNA and archaeal DNA; insufficient cell lysis or inefficient DNA extractions, many commercial DNA extraction kits offering only enzymatic cell lysis with enzymes which are unable to break archaeal cell wall; the primer mismatch or the use of only universal primers, which should target both archaea and bacteria according to *in silico* analysis of these primers, but fail to do so; incomplete databases, only a

small fraction of genomic information data on archaea is available on the level of complete genomes; another issue, is the lack of archaeal isolates, to this date, only a few representatives of human-associated isolates are available (91).

To gain a first glance on the overall human archaeome, we used our optimised protocol, based on the primer pair combination 344F-915R/349F-519R, in a proof-of-principle experiment, screening for the archaeal diversity in samples from the gastrointestinal tract (biopsies), skin, nasal cavity and lung (bronchoalveolar lavage) (61). Overall, the retrieved amount of information on the human-associated archaeal community was highly unexpected, as we were, for instance, able to show, that the human archaeome is body site-specific and that it includes a much broader diversity than previously thought. For the first time, we were able to retrieve *Methanobacterium* signatures from the human gut, as well as a broad variety of DPANN superphylum associated sequences from diverse samples. Besides, we could show that not only skin (62,63) and gut (69), but also nasal cavity and lung harbour a unique archaeal community.

Network and phylogenetic analysis confirmed archaeal community-type associations to be strongest within a body region as already observed earlier for microbial communities in general (202). Costello et al. reported a predictable biogeographic bacterial pattern for body sites and even within different parts of the body sites (203), which is also true for archaeal communities. In particular, on phylum level, the archaeal biogeographic pattern can be divided into an (i) **thaumarchaeal** skin landscape, (ii) (methano)**euryarchaeal** GI, (iii) a **mixed** skin/GIT landscape for the nasal cavity, and (iv) a **woesearchaeal** lung landscape (61).

The dominance of thaumarchaeal signatures on human skin has already been observed earlier (62,63), reflecting an oxygenated, ammonia/urea-rich environment. In the anoxic gastrointestinal tract, methanogenic archaea are predominant (69,165). Our study confirmed the presence of methanogenic archaea throughout the entire gastrointestinal tract, from the stomach to anus. The question remains if and how these methanogenic archaea colonise the tissues or whether they interact with the host, similarly to mucosa-associated bacteria. Previous studies have hypothesised an attachment of these strains to the intestinal epithelia due to their capability to form biofilms (73). Furthermore, genomic studies of the strains *M. smithii* and *M. stadtmanae* showed that both strains express adhesion-like proteins similar to the bacterial adhesins (72).

As a consequence of our first human archaeome specific study, we decided to explore the diversity of archaea within the human body further using the same amplicon sequencing protocol based on the nested approach with the primer pairs 344F-915R/349F-519R. Unfortunately, the gel

electrophoresis revealed that the primer pair 349F-519R was leading to the formation of strong primer dimers, and no positive bands were observed for most samples. Thus, we were determined to look for a different approach which would yield not only better results but also provide a longer sequencing fragment for improved resolution, and to be able to compare it with the bacterial analysis. To address this problem, we tested 12 different primers previously described in the literature (110), in 27 primer pair combinations and evaluated their performance using *in silico* and experimental approaches on five different human sample types. In our study, we also tested three previously described primers able to amplify both archaea and bacteria, namely, the universal primers 515F-806uR, 515FB-806RB and 519F-785R (60).

Despite their overall good *in silico* results, the three universal primer pairs tested failed to assess the archaeal diversity in the experiments. Two of these primer pairs represent the most-used universal primers for amplicon sequencing methods (140,147), resulting in the detection of one (515F-806uR) or zero archaeal RSVs (515FB-806RB) in the five sample types that evidentially possessed a variety of archaeal signatures. The reasons for the failure of the universal primers to detect archaea are unclear; however, it seems that bacterial signatures outcompete archaeal signatures, just due to slightly better primer matches, depending on the diversity within the sample.

Furthermore, an archaeal primer pair (519F-806R) that has been used before for amplicon sequencing (204) detected only a small proportion of the archaeal diversity in the analysed samples. Still, the same primer pair performed better when used in a nested PCR together with the primer pair 344F-1041R for the first PCR.

Nested PCR has been shown to improve sensitivity and specificity and are useful for suboptimal DNA samples (205,206). Based on our experience in the past (61), other reports (207), and because all attempts to use directly Illumina-tagged archaeal primers to identify archaeal 16S rRNA genes in human samples failed, we kept this approach for the archaeal diversity assessment. However, we are aware that nested PCR introduces biases into the detected microbial community composition and diversity. Although we detected the largest number of archaeal RSVs using the primer pair combination 344F-1041R/519F-806R, we most likely also missed taxa due to the double selection by consecutive amplifications with two different primer pairs. In general, nested PCR method is valuable when samples with very low DNA concentration need to be amplified, or when inhibitors hinder the reaction with a larger quantity of starting material. However, the bias is more substantial with communities with higher diversity, and when

more cycles are employed in the first PCR round and should be applied when standard PCR is not successful (208).

In our study, we used a combination of an archaea-specific first PCR (9 different primer combinations) and two archaeal specific and one universal primer pair in the second PCR, resulting in 23 different approaches. Ten out of the 23 different primer combinations allowed the detection of archaeal signatures in all analysed samples (sample set 1). The results of two of the primer pair combinations were outstanding regarding the number of reads and observed RSVs identified in each sample, namely primer pair 344F-1041R/519F-806R and 344F-1041R/519F-785R. The comparison of the alpha diversity (based on Shannon index) indicated that the archaeal diversity uncovered with the primer pair 344F-1041R/519F-806R was significantly higher than the one obtained with the primer pair combination 344F-1041R/519F-785R, which was thus considered superior.

To further test and validate the use of the primer pair 344F-1041R/519F-806R, we selected 29 samples from different body sites (nose, oral, appendix, stool, skin; sample set 2), resulting in overall 85 archaeal RSVs from 6 different phyla. We were able to confirm body site-specificity through PCoA and RDA analysis (61). Euryarchaeal communities dominated the gastrointestinal tract (stool and appendix samples). Also, the oral samples were dominated by archaeal communities from the Euryarchaeota phylum, but different from the ones found in the gastrointestinal tract. The nasal archaeal community was mainly comprised of taxa belonging to Euryarchaeota and Thaumarchaeota signatures. The skin revealed a mixture of Euryarchaeota, Thaumarchaeota, Aenigmarchaeota, and, in very low amounts, also Crenarchaeota, confirming previous results (61,62,209).

According to the obtained results we recommend the use of the primer pair combination 344F-1041R/519F-806R to identify and characterize archaeal communities within human samples, even though the second primer pair 519F-806R performs like a universal primer pair according to the *in silico* results. Although this approach will lead to the retrieval of not only archaeal reads but also reads classified within Bacteria and Eukarya, which had to be filtered bioinformatically, this procedure proved superior to all the other primer pairs tested in identifying archaeal signatures in the analysed samples.

With the established protocol to study archaea in human samples, we explored the archaeal diversity and composition in 282 samples from 7 different body locations: lungs (59 bronchoalveolar lavage (BAL) samples), nasal cavity (n=36), oral cavity (n=45), gastrointestinal

tract (45 stool samples), skin (n=26), urinary tract (45 urine samples), and vagina (n=26). We could not identify archaea in all 282 analysed samples; only 179 samples were positive for archaea. Methanogens were present in all studied body sites and were the dominant taxa in 6 out of 7 body sites. The skin was dominated by taxa belonging to the Thaumarchaeota, as previously shown (62,63). The most dominant taxa which we identified associated with the human skin was the Candidatus *Nitrosocosmicus*. One species of the Candidatus *Nicrosocosmicus* has been isolated before from water waste treatment system (210) and was previously identified as part of the plant microbiome (211). We reconfirmed the body site-specificity of the human archaeome (61). Although, in this study, the bronchoalveolar lavage was mainly composed of methanogens, especially signatures belonging to *Methanobrevibacter* and *Methanobacterium* genera. We identified *Methanobacterium* in 4 different body sites, namely lungs, oral, stool, and urine. The nasal microbiome showed an interesting composition, having a high number of *Methanobrevibacter* and *Methanosphaera* taxa. Signatures belonging to Thaumarchaeota and *Halococcus* have also been identified in the nasal samples. In our previous studies, we mainly detected *Methanobrevibacter* and Thaumarchaeota, as well as signatures belonging to Woesearchaeota (61). In this study, the nasal archaeal community showed the highest diversity and richness in comparison with any other body site. We also identified rare taxa belonging to Diapherotrites and Crenarchaeota in urine and oral samples, but in lesser amounts, less than 4% for Diapherotrites and less than 1% for Crenarchaeota. Most notable was the presence of the signatures belonging to Woesearchaeota in the lung, oral, urine and vaginal samples. In our previous studies, Woesearchaeota signatures were detected in the bronchoalveolar lavage, oral, gastrointestinal tract and skin samples (61). However, presence of Woesearchaeota signatures has been reported previously in human-associated environments such as door handles (212), and in the dust of the International Space Station (98), as well as in other environmental samples (213,214). Moreover, Woesearchaeota associated signatures were detected in the oral microbiome of dolphins (215). Information is sparse on this clade of archaea, except speculations on a potential parasitic/symbiotic lifestyle, based on the observation of a small, reduced genome. Recent studies suggest a close syntrophic relationship to methanogens, but their role in the environment is still unclear (216–218).

Interestingly, we reconfirm the presence of methanogens in the vaginal microbiome. In 1990, Belay et al. (74) were able to cultivate methanogens from vaginal samples in two women with bacterial vaginosis. Since then, no reports mentioned the presence of archaea or methanogens in the healthy vaginal microbiome. Recently, Grine et al. (219) have shown the presence of

Methanobrevibacter smithii in vaginal samples, but only in women with bacterial vaginosis. In our study, we show the presence of methanogens, especially *Methanobrevibacter* in 16 out of 26 women through amplicon sequencing approach. None of the women recruited in our study had bacterial vaginosis at the time of sampling.

Our studies on human archaeome bring new information into the diversity and composition of archaea within the human body. Within this study, it was possible to confirm our hypothesis that archaeal communities show a body site-specificity and are present in all body sites investigated. The Euryarchaeota phylum was the dominant taxa in most body sites investigated, except skin, which was dominated by Thaumarchaeota as previously shown (62,63). The dominant Euryarchaeota taxa present in all body sites were the methanogens, mainly three dominant genera *Methanobrevibacter*, *Methanosphaera* and *Methanobacterium*. Other methanogens have also been identified in specific body sites, for example in urine and bronchoalveolar lavage. We reconfirmed the presence of archaeal signatures in different human samples through metagenomics. Unfortunately, the obtained archaeal draft genomes were less than 40% complete, making further analysis unfeasible for a genomic-centric approach. Nevertheless, we plan to explore further the archaeal genes identified through metagenomics and add new information to the Human Archaeome topic.

Although our studies based on amplicon sequencing offer information on the archaeal diversity and composition within the human body, unfortunately, no information could be provided on the roles of these communities in these body sites and how they interact with the other components of the microbiome. Further studies are needed to address the function of human-associated archaea and their interaction with the host and other microorganisms. For future metagenomics oriented studies, deep sequencing should be considered to increase the chance for obtaining archaeal draft genomes with a higher level of completeness and low level of contamination. Although some studies claim that archaea are pathogens (198), we believe this should be treated with cautious as previous studies have shown low immunogenicity of methanogens *in vitro* analysis (220,221). The presence of methanogens in diseased tissues or even infections does not indicate that these microorganisms are the cause of the disease or infection.

IV.4. Conclusions

With the help of the fast development of high-throughput sequencing in the last decade, our knowledge of microbiome has increased rapidly. In the last couple of years, we discovered the composition of the human microbiome and how these microorganisms interact with us and

influence our lives. However, we are only at the beginning of understanding how the microbiome affects us and what roles it plays in health and disease.

Microorganisms surround us, and we are in a constant exchange of microbes with the environment. Although many studies have focused on the indoor environment and tried to understand how the microbial communities form in these environments, especially in hospitals, we are still far from defining what a healthy indoor microbiome is and how we could promote it. Until now, no study has shown if microorganisms found in our houses can form stable colonisation within the human body and more studies are needed to understand how these microorganisms influence the development of our microbiome in the first years of life. In our study, we explored both the bacterial and archaeal community in the indoor environment. We showed for the first time the composition of the archaeal communities present in our houses. Furthermore, we detected human-associated anaerobic microorganisms in the indoor environment and revealed that human-associated methanogens could cope with oxygen and survive for specific periods in the aerobic environment.

Besides the constant exchange of microorganisms with the surrounding environment, we now know that the human microbiome is quite stable over time; however, specific changes in our environment or body can lead to dysbiosis and disease. One topic related to dysbiosis is that specific changes in the microbiome during pregnancy, especially in the vaginal microbiome, could lead to preterm birth. Spontaneous preterm birth is a multifactorial process, often accompanied by infections, inflammation, uteroplacental ischemia, stress or other immunological mediated processes (222). Therefore, different strategies would be needed to prevent preterm birth, modulating the microbiome of the mother could be one of these strategies. As a first step, we need to understand how the microbiome plays a role in preterm delivery. To tackle this topic, we investigated the vaginal and urinary microbiome of women at high risk of preterm birth. Our results suggested, two possible mechanisms leading to preterm birth, one linked to sterile inflammation and a certain HMO, namely 3'-sialyllactose (3’SL), and one mediated by certain microbial taxa and their connection with specific human milk oligosaccharides. Further studies are needed to confirm our results and develop new prevention methods. Future research focused on preterm birth should include besides the microbiome of the mother, additional experiments focused on the inflammation status and the composition of human milk oligosaccharides to achieve a comprehensive picture of changes which occur during pregnancy in women at high risk of preterm birth.

Discussions

In the last couple of years, microbiome studies have become an essential field of research for finding potential and novel methods for disease diagnosis and treatment. As most studies mainly focus on bacteria, we strongly suggest the use of several approaches to target all components of the microbiome to answer questions related to health and disease and find solutions for diseases linked to dysbiosis. Therefore, studies should also include the archaeal, fungal and viral communities besides the bacterial communities to answer questions related to microbiome and health. In this regard, our studies have tried to explore the composition of the archaeal communities within the human body. We have shown that archaea are body site-specific like bacteria and that the archaeal communities vary based on the location, although most archaeal communities are being dominated by species belonging to Euryarchaeota. The study of the human archaeome is still in infancy; future studies should focus on exploring the role of the archaeal communities in the microbiome, health and disease.

V. References

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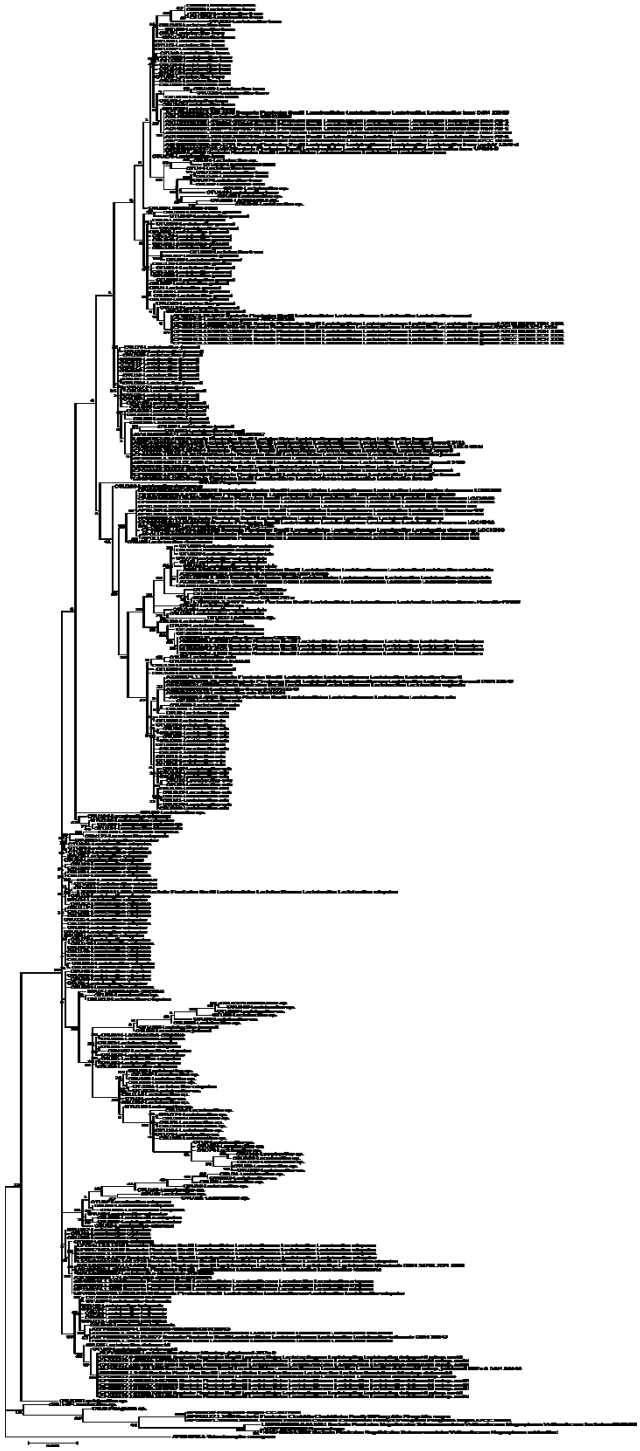
VI. Appendices

Appendix 1: Phylogenetic tree, displaying the *Lactobacillus* genus.



Appendix 2: Reads and RSVs obtained by each primer pair combination for archaea and bacteria.

The tables were taken from (60)

Archaea			Oral		Appendix		Nose		Stool		Skin	
			Reads	RSVs observed	Reads	RSVs observed	Reads	RSVs observed	Reads	RSVs observed	Reads	RSVs observed
PCR21	349F-915R	Illu 349F-Illu519R	12	1	24	2	987	4	21	2	11	1
PCR22		Illu 519F-Illu785R	62	2	92914	5	259	2	82231	3	106285	9
PCR23		Illu 519F-Illu806R	580	2	119864	6	4107	2	74979	2	93479	7
PCR31	344F-1041R	Illu 349F-Illu519R	177	1	0	0	167	1	155228	4	572	5
PCR33		Illu 519F-Illu785R	55699	19	582	2	77481	13	107383	5	29938	14
PCR34		Illu 519F-Illu806R	102909	19	23	1	10655	19	80596	3	162723	39
PCR41	349F-1041R	Illu 349F-Illu519R	0	0	6	1	0	0	67580	4	0	0
PCR42		Illu 519F-Illu785R	6	2	0	0	48	1	117171	7	15	1
PCR43		Illu 519F-Illu806R	229	1	702	2	11	1	50183	3	1011	2
PCR61	349F-806R	Illu 349F-Illu519R	0	0	260	4	0	0	37	2	0	0
PCR62		Illu 519F-Illu785R	0	0	157	1	35338	7	1496	5	631	1
PCR63		Illu 519F-Illu806R	0	0	1302	1	28177	4	2416	2	0	0
PCR71	519F-1041R	Illu 519F-Illu785R	3	1	0	0	0	0	56082	5	0	0
PCR72		Illu 519F-Illu806R	35817	6	1302	1	2010	2	86018	3	1032	2
PCR81	519F-806R	Illu 519F-Illu785R	0	0	0	0	0	0	60	1	0	0
PCR82		Illu 519F-Illu806R	0	0	0	0	0	0	86	2	0	0
PCR91	344F-519R	Illu 349F-Illu519R	121	1	128	1	68	1	92987	4	0	0
PCRQ1	344F-915R	Illu 349F-Illu519R	47	2	129	1	68	2	85556	6	0	0
PCRQ3	(QIAGEN)	Illu 519F-Illu785R	0	0	119649	45	670	2	76957	5	68	2
PCRQ4		Illu 519F-Illu806R	0	0	104755	28	14943	4	55396	4	475	2
PCRM1	344F-915R	Illu 349F-Illu519R	24	1	0	0	752	3	83949	4	0	0
PCRM3	(Monarch)	Illu 519F-Illu785R	0	0	0	0	119032	22	83187	2	650	2
PCRM4		Illu 519F-Illu806R	0	0	0	0	111738	8	74511	3	125	2
PCRA1	344F-915R (Analytikjen	Illu 349F-Illu519R	10	1	7	1	30	1	104882	4	0	0
PCRA3	a)	Illu 519F-Illu785R	0	0	0	0	116	2	86896	2	65	3
PCRA4		Illu 519F-Illu806R	160	2	41	3	4945	3	80898	4	475	3
PCRQ5	344F-806R	Illu 349F-Illu519R	0	0	112	2	79	1	111922	4	11	1
PCRQ6	(QIAGEN)	Illu 519F-Illu785R	0	0	3	1	37	2	7	1	0	0
PCRQ7		Illu 519F-Illu806R	111440	4	99011	3	95582	3	90887	2	127939	5
PCRM5	344F-806R	Illu 349F-Illu519R	147	4	81	1	55	1	90932	4	240	3
PCRM6	(Moarch)	Illu 519F-Illu785R	116479	21	120377	3	0	0	74819	6	130588	4
PCRM7		Illu 519F-Illu806R	129876	12	51549	3	73879	5	94749	5	112419	4
PCR8-Uni		Illu515F-Illu806uR	0	0	0	0	0	0	30	1	0	0
PCR9-Uni		Illu515FB-Illu806RB	0	0	0	0	0	0	0	0	0	0
PCR10-Uni		Illu 519F-Illu785R	0	0	0	0	83	1	184	2	0	0
PCR11-Uni		Illu 519F-Illu806R	22	1	0	0	0	0	690	2	0	0

Bacteria			Oral		Appendix		Nose		Stool		Skin	
			Reads	RSVs observed	Reads	RSVs observed	Reads	RSVs observed	Reads	RSVs observed	Reads	RSVs observed
PCR21	349F-915R	Illu 349F-Illu519R	0	0	0	0	0	0	0	0	0	0
PCR22		Illu 519F-Illu785R	366	4	222	6	2538	17	1298	8	58	3
PCR23		Illu 519F-Illu806R	735	15	10	3	2373	17	2626	2	0	0
PCR31	344F-1041R	Illu 349F-Illu519R	0	0	0	0	0	0	0	0	0	0
PCR33		Illu 519F-Illu785R	53	1	64	1	107	3	9	1	99	2
PCR34		Illu 519F-Illu806R	30	5	6	3	45	1	6	2	251	7
PCR41	349F-1041R	Illu 349F-Illu519R	0	0	0	0	0	0	0	0	0	0
PCR42		Illu 519F-Illu785R	45	1	9	2	102	4	264	18	14	1
PCR43		Illu 519F-Illu806R	1724	26	498	12	2427	23	33	2	2149	24
PCR61	349F-806R	Illu 349F-Illu519R	0	0	0	0	0	0	0	0	0	0
PCR62		Illu 519F-Illu785R	19515	60	6496	33	92043	30	97344	386	6444	31
PCR63		Illu 519F-Illu806R	78892	72	9420	31	91310	23	104674	308	11290	35
PCR71	519F-1041R	Illu 519F-Illu785R	4251	21	586	6	13224	22	72060	302	525	6
PCR72		Illu 519F-Illu806R	35371	67	2668	27	69269	78	3400	65	5121	46
PCR81	519F-806R	Illu 519F-Illu785R	71062	104	43963	55	78739	244	67666	639	76340	125
PCR82		Illu 519F-Illu806R	46708	90	12616	39	61178	161	66084	286	16449	96
PCR91	344F-519R	Illu 349F-Illu519R	0	0	0	0	0	0	42	1	0	0
PCRQ1	344F-915R	Illu 349F-Illu519R	0	0	0	0	0	0	0	0	0	0
PCRQ3	(QIAGEN)	Illu 519F-Illu785R	275	9	68	5	2155	17	14	2	941	8
PCRQ4		Illu 519F-Illu806R	320	7	68	7	2797	20	0	0	849	17
PCRM1	344F-915R	Illu 349F-Illu519R	0	0	0	0	0	0	0	0	0	0
PCRM3	(Monarch)	Illu 519F-Illu785R	2750	23	1406	17	82	6	13	3	1371	42
PCRM4		Illu 519F-Illu806R	5296	34	1626	20	0	0	18	4	2092	31
PCRA1	344F-915R	Illu 349F-Illu519R	0	0	0	0	0	0	0	0	0	0
PCRA3	(Analytikjena)	Illu 519F-Illu785R	180	2	291	5	342	5	0	0	13	2
PCRA4		Illu 519F-Illu806R	106	7	94	5	233	8	0	0	105	6
PCRQ5	344F-806R	Illu 349F-Illu519R	0	0	0	0	0	0	54	2	0	0
PCRQ6	(QIAGEN)	Illu 519F-Illu785R	0	0	0	0	0	0	0	0	0	0
PCRQ7		Illu 519F-Illu806R	146	5	0	0	6171	4	2016	16	169	9
PCRM5	344F-806R	Illu 349F-Illu519R	0	0	0	0	0	0	58	1	0	0
PCRM6	(Moarch)	Illu 519F-Illu785R	9253	16	121	7	0	0	11451	58	2469	9
PCRM7		Illu 519F-Illu806R	154	6	0	0	555	4	2402	23	27	4
PCR8-Uni		Illu515F-Illu806uR	36483	67	13554	42	28838	121	36554	208	33527	90
PCR9-Uni		Illu515FB-	46186	58	15743	26	35855	122	35134	171	51762	74
PCR10-Uni		Illu 519F-Illu785R	7736	34	1124	11	40565	68	70997	224	15346	46
PCR11-Uni		Illu 519F-Illu806R	22667	32	3459	13	6313	63	18703	42	21158	53

Appendix 3: Detailed list of all used media and ingredients

In this thesis, only two media have been used: **MS medium** for *Methanobrevibacter* strains and a **special medium (MpT1)** for *Methanosphaera stadtmanae*.

MS medium

$(NH_4)_2\ Ni(SO_4)_2$	0,002 g
$(NH_4)_2SO_4$	0,225 g
$CaCl_2$ x 2 H_2O	0,06 g
$FeSO_4$ x 7 H_2O	0,002 g
K_2HPO_4 x 3 H_2O	0,3 g
KH_2PO_4	0,225 g
$MgSO_4$ x 7 H_2O	0,10 g
NaCl	0,450 g
$NaHCO_3$	5,00 g
Wolfe's mineral solution 10x	1,0 ml
Wolfe's vitamine solution 10x	1,0 ml
Na_2S x 7-9 H_2O	0,5 g
alternativ:	
Na_2S x 2 H_2O	0,25 g
H_2O bidest	ad 1000,0 ml

After autoclaving add:
NaAcetate (10%) and yeast extract (10%) to a final concentration of 0,1%.

Wolfe's mineral solution 10x

$MgSO_4$ x 7 H_2O	30,0 g
$MnSO_4$ x H_2O	5,0 g
NaCl	10,0 g
$FeSO_4$ x 7 H_2O	1,0 g
$CoSO_4$ x 7 H_2O	1,8 g
$CaCl_2$ x 2 H_2O	1,0 g
$ZnSO_4$ x 7 H_2O	1,8 g
$CuSO_4$ x 5 H_2O	0,1 g
$KAl\ (SO_4)_2$ x 12 H_2O	0,18 g
H_3BO_3	0,1 g
Na_2MoO_4 x 2 H_2O	0,1 g
$(NH_4)_2Ni(SO_4)_2$ x 6 H_2O	2,80 g
Na_2WO_4 x 2 H_2O	0,1 g
Na_2SeO_4	0,1 g
H_2O bidest, ad	1000,0 ml

Wolfe's vitamin solution 10x

Biotin	20 mg
Folic acid	20 mg
Pyridoxamine dihydrochloride	100 mg
Thiamine hydrochloride	50 mg
Riboflavin	50 mg
Niacin	50 mg
D-Calcium pantothenate	50 mg
Cyanocobalamin	1 mg
4-Aminobenzoic acid	50 mg
DL-alpha-lipoic acid	50 mg
H_2O bidest, ad	1000 ml

Selenite-tungstate solution

NaOH	0.5 g
Na_2SeO_3 x 5 H_2O	3.0 mg
Na_2WO_4 x 2 H_2O	4.0 mg
Distilled water	1000.0 ml

MpT1 Medium

(Components liter^{-1}):

NaCl	1 g
KCl	0.5 g
$MgCl_2 \cdot 6H_2O$	0.4 g
$CaCl_2 \cdot 2H_2O$	0.1 g
NH_4Cl	0.3 g
KH_2PO_4	0.2 g
Na_2SO_4	0.15 g
Casaminoacids	2 g (Casein Hydrolysate) (0.2%)
yeast extract	2 g (0.2%)
trace element solution	1 mL (same as for the master thesis)
selenite-tungstate solution	20 µL (2 g NaOH, 0.01 g Na_2SeO_3, 0.017 g $Na_2WO_4 \cdot 2H_2O$, in 50 mL) MQ)
Na-acetate	0.082 g (1mM)
H_2O	990 mL (= 1000 mL final volume → subtract the mL which are added after autoclaving (and 1mL trace elements) the medium)

→ deoxygenate the medium (flushing it with N_2 + constant stirring; 20-30 minutes)

$NaHCO_3$	2.52 g (= 30mM; add to anoxic medium; add in anaerobic tent)

→ autoclave the medium

add after autoclaving from sterile stocks kept under N_2 atmosphere (all together: 9 mL):

vitamin solution	1 mL (same as for the master thesis)
methanol	2 mL (2,025 mL = 50mM)
dithiothreitol (DTT)	2 mL stock solution (1.54 g / 20 mL) = 0.154 g (1mM) L^{-1}
Cysteine	2 mL stock solution (2.4 g / 20 mL) = 0.24 g (2mM) L^{-1}
Na-formate	1 mL stock solution (0.68 g / 20 mL) = 0.034 g (0.5mM) L^{-1}
Na-coenzym M	1 mL stock solution (0.1 g /10 mL) = 0.01 g (0.001%) L^{-1}

→ Adjust the pH to 7 - 7.2

Modified Non-chelated trace element mixture:

Distilled H_2O	987 mL	
HCl (~12.5M)	8 mL	(100 mM)
H_3BO_3	30 mg	(0.5 mM)
$MnCl_2$ $4H_2O$	100 mg	(0.5 mM)
$CoCl_2$ $6H_2O$	190 mg	(0.8 mM)
$NiCl_2$ $6H_2O$	24 mg	(0.1 mM)
$CuCl_2$ $2H_2O$	2 mg	(0.01 mM)
$ZnSO_4$ $7H_2O$	144 mg	(0.5 mM)
Na_2MoO_4 $2H_2O$	36 mg	(0.15 mM)

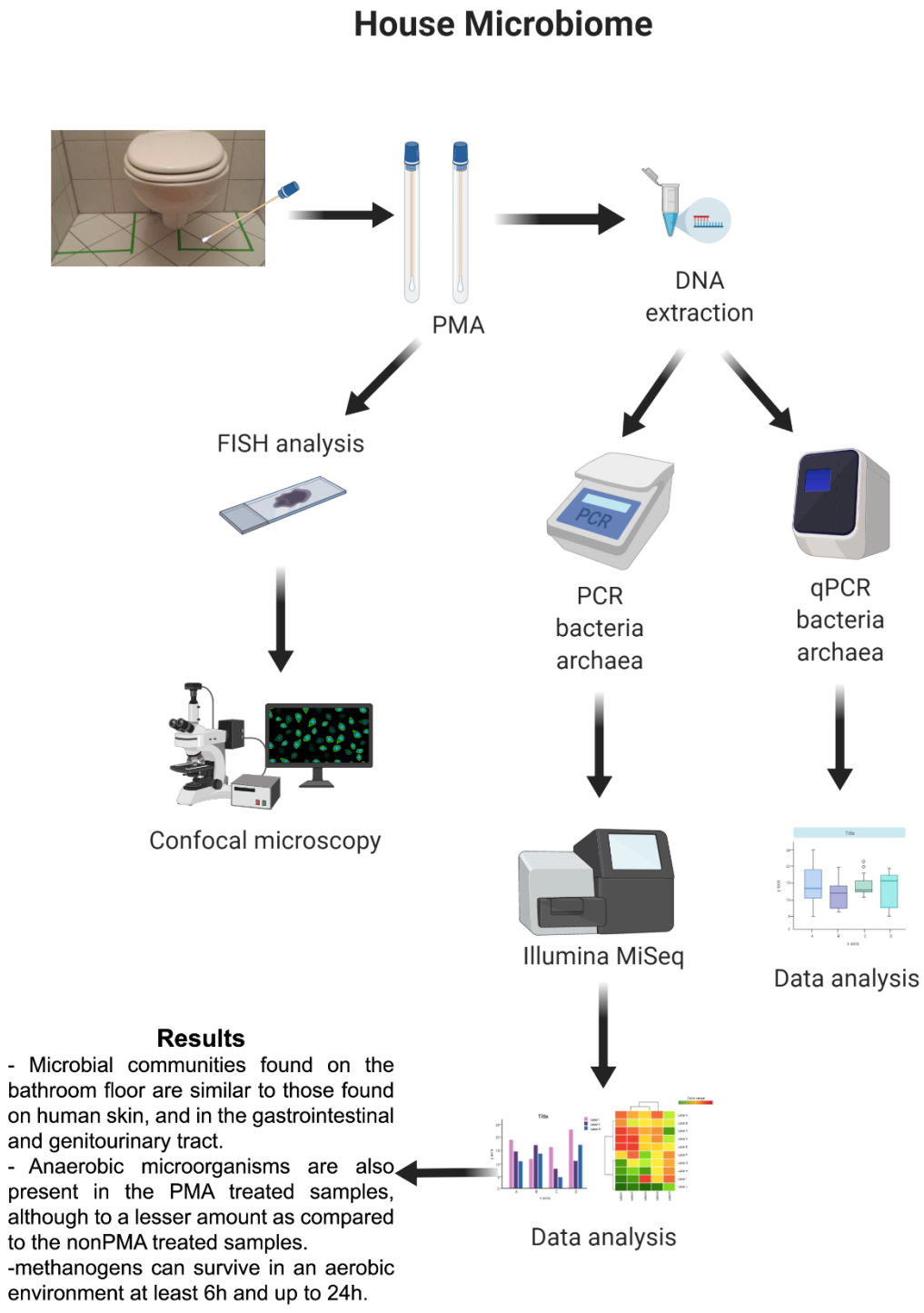
The trace element mixture is autoclaved in bottles tightly closed with rubber-fitted screw caps or fixed stoppers; a headspace of approximately 1/3 of the volume must be left. Store dark at 4°C.

Vitamin solution (per L)

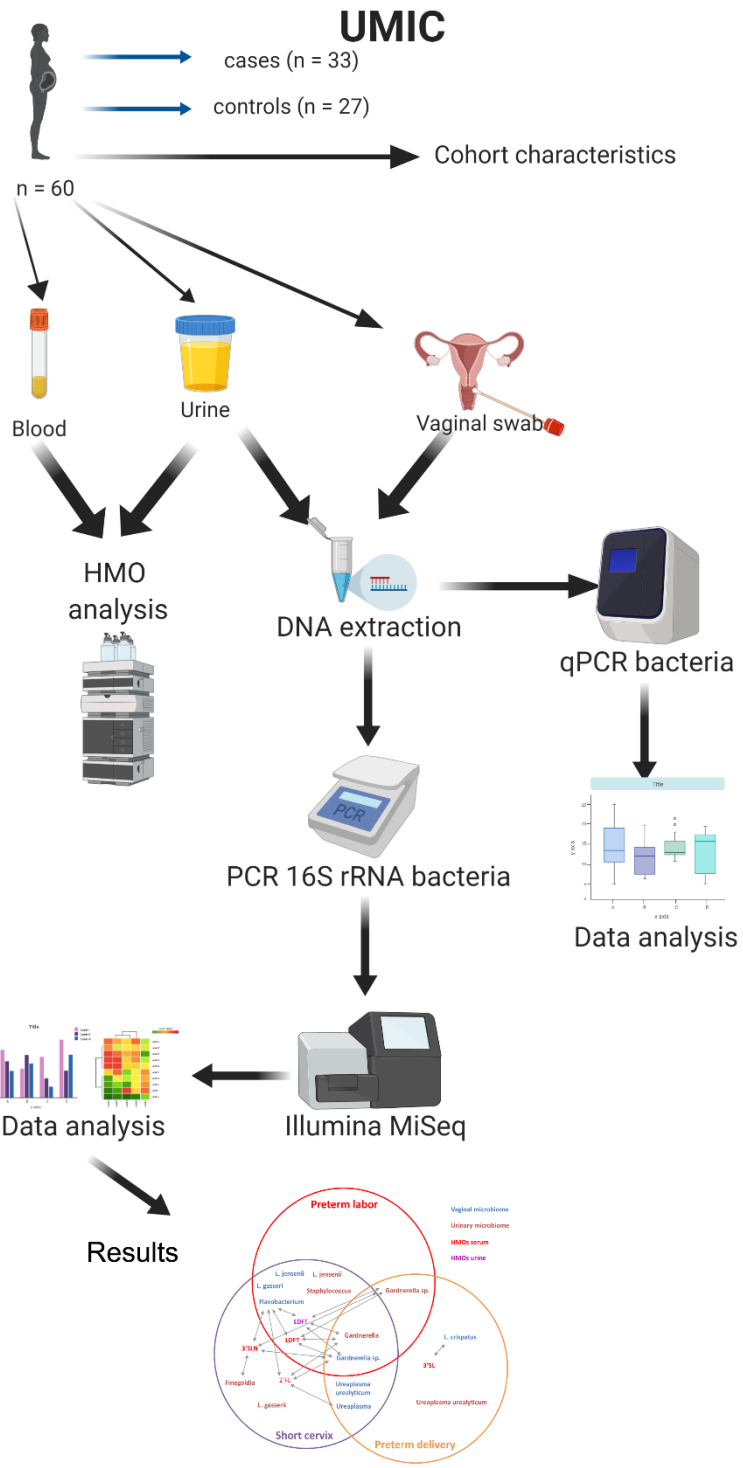
Biotin	0.02 g
Folic acid	0.02 g
Pyridoxine HCl	0.10 g
Thiamine HCl	0.05 g
Riboflavin	0.05 g
Nicotinic acid	0.05 g
DL Pantothenic acid	0.05 g
P Aminobenzoic acid	0.05 g
Choline Chloride	2.00 g
Vitamin B12	0.01 g

Adjust to pH 7 with KOH. Sterilize by filtration and store at 4°C.

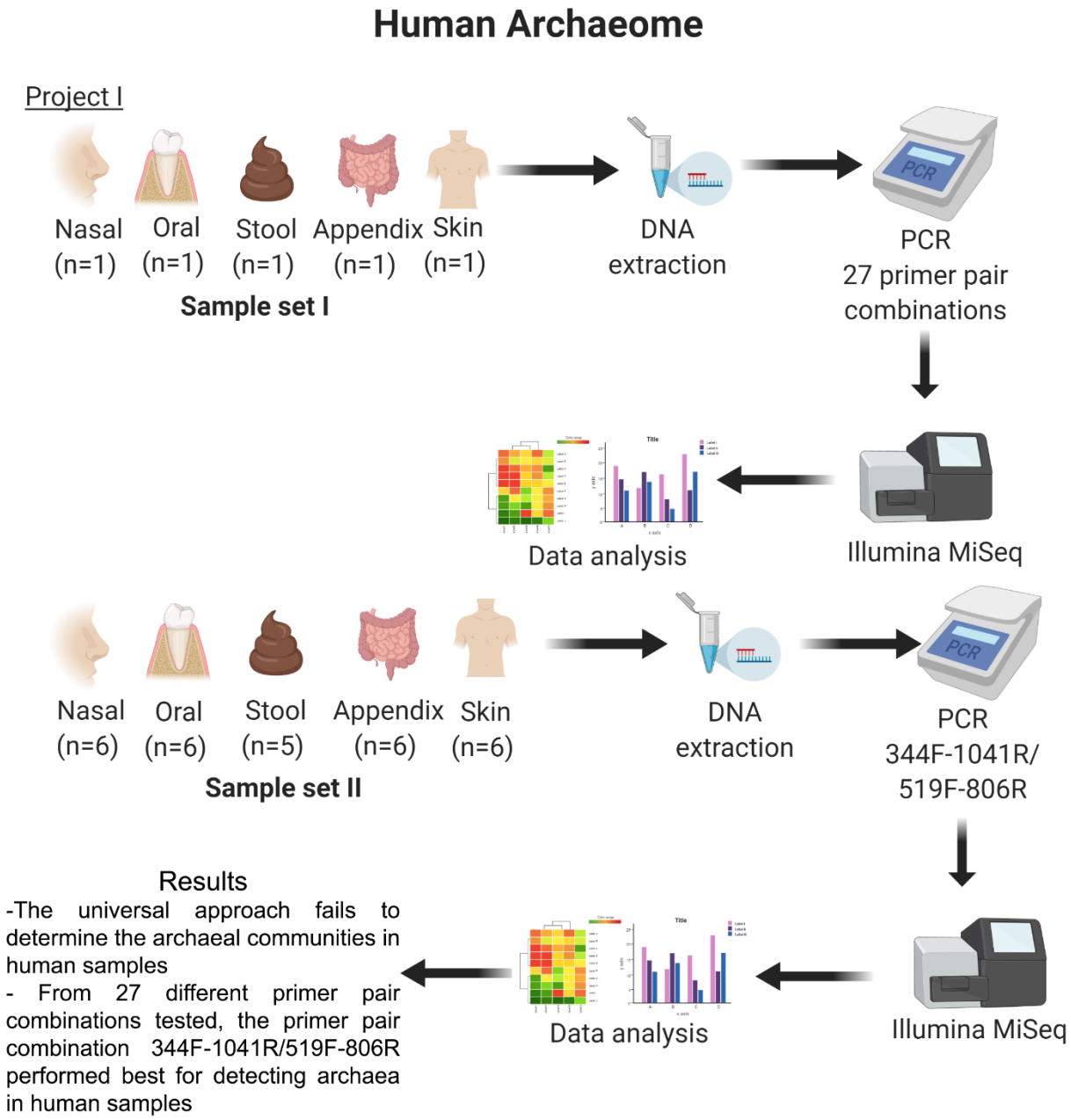
Appendix 4: Schematic overview of the House Microbiome Project



Appendix 5: Schematic overview of the UMIC project



Appendix 6: Schematic overview of the Human Archaeome I Project



Appendix 7: Schematic overview of the Human Archaeome II Project