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Characterising the maternal vaginal microbiome during pregnancy and its vertical transmission

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List of abbreviations

BMI	Body Mass Index
BV	Bacterial vaginosis
CFU	Colony forming units
CI	Confidence interval
C-section	Caesarean section
CST	Community state type
D-value	Inverse Simpson's diversity index
D	Daughter
G	Group
GA	Gestational age
GBS	Group B *Streptococcus*
GM	Grandmother
HMP	Human microbiome project
IBD	Inflammatory bowel disease
IVF	*In vitro* fertilisation
LAB	Lactic acid bacteria
LAMB	Laboratory of Applied Microbiology and Biotechnology
LBG	Lactobacillary grade
M	Mother
OD	Optical density
P	Pair
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
ProPreB	Probiotics for Preterm Birth prevention
PTB	Preterm birth
RZ T	RZ Heilig Hart Tienen
SNP	Single nucleotide polymorphism
Spp.	Species
STI	Sexually transmitted infection
UTI	Urinary tract infection
UZA	University Hospital Antwerp
VALENCIA	Vaginal community state type nearest centroid classifier
VM	Vaginal microbiome
WGS	Whole genome sequencing

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Abstract

The vaginal microbiome (VM) is of major importance for reproductive health as vaginal dysbiosis during pregnancy has been linked to adverse pregnancy outcomes such as preterm birth (PTB). PTB is a global health issue, resulting in an urgent need for prevention strategies and therapies. A better understanding of the VM dynamics during pregnancy in the general population could elucidate factors that contribute to a dysbiotic VM and PTB. Therefore, this Master's thesis aimed to further reveal the dynamics of the VM during pregnancy by investigating the VM composition of 92 participants from the Probiotics for Preterm Birth prevention (ProPreB) trial control group at three time points (each trimester) throughout pregnancy. It was demonstrated that lactobacilli dominated the VM of 82 women in this study cohort at all time points, while a VM dominated by anaerobes was rarely observed. Moreover, a VM dominated by *Lactobacillus crispatus* showed the highest stability throughout pregnancy. As a recommendation for future research, it might be of interest to study both the pre- and postpartum period to further expand knowledge on the changes in the VM composition at the onset of pregnancy and after delivery. In addition, more frequently collecting samples would be recommended to get more detailed insights into the VM dynamics throughout pregnancy.

Besides vaginal dysbiosis during pregnancy, a series of clinical factors, including a limited cervical length, smoking behaviour, advanced maternal age, as well as high and low maternal Body Mass Index (BMI), have been previously associated with an increased risk for PTB. However, many of these associations remain to be further explored. Therefore, associations between PTB and a series of clinical factors were examined using clinical data collected from 413 women participating in control groups of the ProPreB trial. A weak positive correlation was found between gestational age at delivery and cervical length in the second trimester. This observation supports the currently used measurement of cervical length as a screening method for PTB risk. However, further research is required to determine an appropriate cut-off value for women at risk for PTB. In addition, an association was observed between the presence of Group B *Streptococcus* (GBS) in the third trimester and PTB. This finding stimulated further investigation of the VM composition of women who had a GBS positive status. Here, an association was found between the vaginal community state type in the second trimester of pregnancy and GBS status. However, more research is necessary to further investigate the relation between the VM during pregnancy and the presence of GBS. Surprisingly, no correlations with PTB were found for the presence of bacterial vaginosis, vaginal pH, maternal age, maternal BMI and smoking behaviour. Nevertheless, these factors should be further investigated since previous studies have shown significant associations with PTB.

The VM does not only play an important role during pregnancy, it is also crucial for the initial colonisation of newborns. Some studies have already shown that certain members of the maternal microbiota are vertically transmitted towards the offspring's gut. However, a knowledge gap remains regarding the origin of the VM. Some research suggest that the VM is seeded at birth by vertical transmission of maternal vaginal microbiota members. However, up to date, limited research was performed to explore vertical transmission of vaginal lactobacilli, especially in combination with a study of lifelong persistence of these transferred bacteria. Other studies suggest that the VM originates from external sources such as the environment and diet. If the hypothesis of vertical transmission of the VM from mother to daughter would be confirmed, this might implicate that the VM during pregnancy does not only impacts mother's health, but also the daughter at later age. This Master's thesis aimed to explore the hypothesis of vertical transmission of vaginal microbiota members from mother to daughter and whether the transmitted bacteria persist in the vagina until the daughter reaches reproductive-age. In short, a culturing campaign was set up and successfully tailored towards the isolation of vaginal lactobacilli from vaginal swabs by introducing a subculture phase. In total, 258 vaginal lactobacilli were isolated from 21 vaginal swabs collected from seven pairs and two groups of mothers and adult daughters from the Isala project. Based on phylogenetic analyses, three *L. crispatus* isolates from one mother-daughter pair (including one maternal isolate and two daughter isolates) were determined to be very similar, as these clustered within the same clade on the tree with a single nucleotide polymorphism (SNP) count of 489 and 493 between mother and daughter sequences. Notably, this daughter was delivered by Caesarean section. Further investigation is required, including an analysis of the genome-based SNP locations, to confirm that these daughter strains originate from vertical transmission at birth. Expanding research in this relatively new field is certainly recommended to further elucidate the origin of the VM.

1 Introduction

1.1 Introducing the human microbiome

The human body is colonised by billions of microorganisms, such as bacteria, archaea, and fungi (1). These microorganisms occupy several niches in and on the human body, including the skin and mucous surfaces of cavities exposed to the exterior, such as the gastrointestinal, urogenital and respiratory tract, and secretory glands such as the sebaceous, biliary and mammary glands (2). All living microorganisms residing in a distinct niche are collectively called the microbiota. The microbiota form together with their genomes and other non-living elements within the environment (i.e., viruses, phages, signalling molecules, structural DNA/RNA) the human microbiome (3). Since the human host and inhabiting microorganisms co-evolved, microbiota members are adapted to the different niches they occupy (4, 5), resulting in a unique set of microorganisms per niche (6). Nevertheless, multiple niches are connected to each other. The gut and vaginal microbiota, for instance, interact with each other, resulting in local and systemic immune responses and an overall effect on host physiology (7).

1.1.1 The human microbiome in health and disease

Within a microbial community, several types of community members can be found, such as commensals, pathogens and pathobionts (8). These share a unique biological relationship with their host, referred to as symbiosis. Symbiosis is a broad term to describe “different organisms living together”, and encompasses several biological interactions, such as mutualism (benefit for all organisms involved) and commensalism (benefit for one of the organisms involved) (9). Commensals are non-pathogenic microbiota members that frequently play an essential role in many host functions and thus benefit the human host (8). For example, in the intestines, beneficial gut bacteria aid in the digestion of food and absorption of nutrients, produce vitamins such as biotin (vitamin B8) and vitamin K, regulate the immune system, and facilitate barrier integrity by interfering with the colonisation of pathogenic microorganisms (10). On the other hand, these resident microbiota members can benefit from food-derived nutrients present in the intestines. Despite their designation as commensals, both the host and these beneficial microbiota members clearly benefit from this mutualistic interaction (8, 10, 11).

Nevertheless, under certain circumstances some commensals can become pathogenic, then referred to as pathobionts. Normally, pathobionts are present in low abundances and do not cause health problems, yet, outgrowth of these microorganisms can disturb the beneficial symbiotic relationship between host and microbiota, and thus contribute to disease (12, 13). As an example, a high abundance of *Clostridium difficile* in the gut can cause mild to severe pseudomembranous colitis. However, a “healthy” microbial community provides resistance to this pathology by preventing *C. difficile* proliferation (14). In general, imbalances in indigenous microbial composition are reflected in dysbiotic states which can shift the microbiome from being optimal to non-optimal to the human host. Several host factors can cause these dysbiotic states, such as genetic background and presence of disease. Additionally, environmental factors and lifestyle habits can be involved, such as diet, use of antibiotics and other medication, and hygiene practices (15). Dysbiosis can have significant causes and implications, including (i) an expansion of pathobionts, (ii) a reduction in commensals, and (iii) a decrease in overall microbial diversity (12, 16). It has been documented that dysbiosis plays a role in various disease states such as inflammatory bowel disease (IBD) (17), depression (18), asthma (19), bacterial vaginosis (20), and others. Nevertheless, as many individuals with dysbiosis remain asymptomatic, it is controversial to use the terms “healthy” and “unhealthy” when referring to the composition of the human microbiome. In this regard, the use of the terms optimal and non-optimal is rather preferred (21-23). Overall, to further elucidate the underlying causes of dysbiosis as well as the role of the microbiome in disease predisposition and pathogenesis, an increased understanding of the microbiome composition under optimal conditions is first needed (24).

1.1.2 Studying the human microbiome

For a long time, scientists could only rely on culture-based techniques to study the microbiome composition. Even so, it is well established that only approximately 1% of microorganisms living on Earth can be readily cultivated *in vitro* (25). Some of the main reasons for culture failure include the absence of host factors (e.g., interactions with the immune system), missing nutrients and other community members, inappropriate temperature and atmospheric gas composition, and accumulation of toxic waste products in culture (26). As a result, the emergence of culture-independent technologies, such as metagenomics (i.e., the study of genomes extracted from a complex mixture of microorganisms), introduced a rapid evolution in microbiome research and clinical applications (27-29). These new insights and advances in techniques have changed the perspective on microorganisms, moving from a pathogen-oriented perspective towards a broader view in which microorganisms play a significant role in maintaining human health (22, 27). Therefore, during the past decades, studies have shifted towards defining the microbiome in the general population, rather than focussing on disease states only (4, 27). The Human Microbiome Project (HMP) was one of the first large-scale studies to characterise the human microbiome in the general population when setup in 2007 (30). Additionally, by establishing the HMP researchers aimed to determine possible associations between changes in the microbiome and several medical conditions (30). In the first phase of the project, the microbiome was characterised by sampling five body sites, including the skin, nasal cavity, mouth, gastrointestinal tract and vagina. A second phase started in 2014 to focus on the dynamics of the microbiome for three different medical conditions, namely preterm birth, IBD and diabetes. Overall, the entire project has been a major help in accelerating microbiome research and was finished in 2016 (21, 22, 30).

Large-scale studies, such as the HMP, revealed that the microbiome composition of the general population is extremely variable across different body sites (i.e., intra-individual variation) (21, 23). Besides, a high amount of inter-individual variation exists for the microbiome of a particular body site. For instance, the human gut harbours approximately 1000 different bacterial species of which only 160 species are commonly shared across individuals, while others are variable between individuals (31). Nevertheless, the presence of a 'core microbiome' has been suggested by numerous studies (21, 32, 33). This core microbiome refers to a set of features, such as the presence of certain microbiota members and functions at the level of genes or pathways, that are consistent across host populations (4, 21, 34, 35). Of particular interest are so-called keystone species which are microbiota members with a crucial role in maintaining the structure and functions of a microbial community. Consequently, loss of such keystone species can dramatically change the microbial community, possibly resulting in significant clinical implications. Therefore, further understanding of the core microbiome and characterising keystone species could help to identify causes of dysbiosis and develop treatments for microbiome-related disease states (35). Here, a variety of challenges are encountered because of the intra- and inter-individual variation and limited understanding of the exact interactions between the microbiome and the human host (21-23, 35). As microbiome research is advancing, it becomes clear that understanding the roles of the human microbiome in human health is crucial. While previous microbiome studies have mainly focussed on the gut microbiome, the oral, skin and vaginal microbiome are gaining increasing attention to date (4, 27). In particular, the vaginal microbiome is considered of major importance for women's health and reproduction, thus stressing the need for evidence-based research in this area (20, 36).

1.2 Introducing the vaginal microbiome

Similar to the gut, the vagina contains numerous microbiota members, including bacteria, algae, and fungi, but also non-living elements such as viruses and phages. The vaginal microbiome (VM) thus refers to the collection of indigenous microorganisms (i.e., the vaginal microbiota), their structural elements (i.e., nucleic acids, proteins, lipids, polysaccharides), metabolites, and other non-living elements (i.e., viruses and phages) inhabiting the vaginal microenvironment (3, 20, 36), and showing a mutualistic interaction with the host in this niche (37).

1.2.1 The vaginal microenvironment

The vaginal microenvironment is characterised by the resident vaginal microbiota, a mucosal epithelial barrier, and endocrine regulatory mechanisms (38, 39). The vaginal mucosa is composed of a multi-layered stratified squamous epithelial cells. The top layer (i.e., the *stratum corneum*) consists of approximately 28 cell layers containing flattened, cornified cells, covered by a cervicovaginal fluid layer, thus offering a physical protective barrier (40, 41). Those epithelial cells are filled with glycogen (i.e., a polysaccharide composed of multiple glucose monomeric units). When epithelial cells exfoliate, they disintegrate and release their glycogen content into the vaginal lumen (41, 42). This is subsequently followed by depolymerization of glycogen into maltose, maltotriose and glucose by the human α-amylase enzyme (43). The resulting carbohydrates can serve as fermentation substrates for some vaginal microbiota members. Epithelial proliferation, glycogen accumulation and release, as well as thickness of the mucus layer are influenced by hormonal fluctuations, with key roles for oestrogen and progesterone (40, 44, 45). Oxygen, additional glucose and other nutrients are supplied from underlying submucosal layers through diffusion via a limited blood supply, making the vaginal microenvironment a micro-aerophilic niche (20, 41).

1.2.2 The vaginal microbiota

Given these specific factors that characterise the vaginal microenvironment, micro-aerophilic and fermenting *Lactobacillus* species (spp.) naturally tend to dominate the VM of most reproductive-age women (46-48). This vaginal lactobacilli dominance was first reported in 1892 by Albert Döderlein, who described the vaginal microbial community as a homogenous group of Gram-positive bacilli, called Döderlein's bacilli (49, 50). Their high abundance is a unique characteristic of the human VM while lactobacilli rarely comprise more than 1% of the microbiota in the vagina of other mammals (51). Importantly, lactobacilli play a key role in maintaining an optimal vaginal environment. Firstly, these bacteria can adhere to the vaginal epithelium, thus preventing colonisation of pathogens (52, 53). Secondly, lactobacilli are lactic acid bacteria (LAB) that are capable of fermenting depolymerised glycogen into organic acids (e.g., lactic acid), thereby acidifying the vaginal environment to a typical pH below 4.5, which limits the growth of certain pathogenic microorganisms (54). Lactic acid also modulates the immune system, creating a non-inflammatory environment (55, 56). In addition, lactobacilli can produce other compounds, such as hydrogen peroxide (H_2O_2) and bacteriocins (i.e., antimicrobial peptides) called lactocins, that can also suppress pathogenic growth (57, 58). However, it is important to highlight that the VM is not exclusively composed of *Lactobacillus* spp. (46).

Over the last decades, increased research is being conducted to reveal the taxonomic composition of the VM in the general population (46, 59). More specifically, advances in molecular sequencing techniques have enabled high-throughput characterisation of the VM composition. In this regard, bacterial 16S ribosomal RNA (rRNA) sequencing has greatly increased the identification of VM members that have never been cultured before (44, 46). Reproductive-age women in the general population, often have a VM dominated by one or more of four *Lactobacillus* spp., namely *Lactobacillus crispatus, Lactobacillus iners, Lactobacillus gasseri*, and *Lactobacillus jensenii* (46, 47). These four species are often designated as the 'big four' in VM research (59). Some women however lack these high proportions of vaginal lactobacilli and instead have a VM that is dominated by strict anaerobes such as *Gardnerella vaginalis, Prevotella, Atopobium, Dialister, Sneathia, Peptoniphilus*, and *Megasphaera* (46, 47). Overall, the taxonomic composition of the VM can be approached by a limited number of configurations called community state types (CSTs) (44). The CST concept was first introduced in 2011 by Ravel *et al.*, who defined five of them based on the VM of 396 reproductive-age North American women. More specifically, CST I, CST II, CST III, and CST V are dominated by *L. crispatus*, *L. gasseri, L. iners*, and *L. jensenii,* respectively, whereas CST IV is more diverse, and characterised by higher proportions of different anaerobic bacteria (46). In 2020, a nearest centroid-based tool called VALENCIA (Vaginal community state type nearest centroid classifier) was developed based on a reference data set of 13,160 taxonomic profiles from 1,975 women in the USA. This tool further expanded the five original CSTs and allows for robust and reproducible assignments of vaginal samples to CSTs (60). Based on this tool, VM compositions could be assigned to one out of seven CSTs,

which are even further divided into thirteen sub-CSTs (Table 1) (60). The assignment of CSTs to VM compositions allows better comparison between different datasets. In addition, it allows the use of standard statistical tests to study associations between CSTs and certain host factors (i.e., ethnicity, age), clinical factors (i.e., vaginal pH) and presence or absence of diseases (i.e., sexually transmitted infections, bacterial vaginosis) (60).

Table 1: Vaginal microbiome composition based on the thirteen sub-CSTs according to the VALENCIA algorithm (60). *Abbreviations: CST = Community state type, spp. = species.*

Sub-CST	Vaginal microbiome composition
CST I-A	Community dominated by *L. crispatus*
CST I-B	Community with less *L. crispatus* than CST I-A, but still majority
CST II	Community dominated by *L. gasseri*
CST III-A	Community dominated by *L. iners*
CST III-B	Community with less *L. iners* than CST III-A, but still majority
CST IV-A	Community contains a high to moderate relative abundance of *Candidatus Lachnocurva vaginae* and *G. vaginalis*
CST IV-B	Community contains a high to moderate relative abundance of *G. vaginalis* and *Atopobium vaginae*
CST IV-C0	Community contains low relative abundances of *G. vaginalis, Candidatus Lachnocurva vaginae,* and *Lactobacillus* spp., is an even community, with moderate amount of *Prevotella*
CST IV-C1	Community contains low relative abundances of *G. vaginalis, Candidatus Lachnocurva vaginae,* and *Lactobacillus* spp., and is dominated by *Staphylococcus* spp.
CST IV-C2	Community contains low relative abundances of *G. vaginalis, Candidatus Lachnocurva vaginae,* and *Lactobacillus* spp., and is dominated by *Enterococcus* spp.
CST IV-C3	Community contains low relative abundances of *G. vaginalis, Candidatus Lachnocurva vaginae,* and *Lactobacillus* spp. and is dominated by *Bifidobacterium* spp.
CST IV-C4	Community contains low relative abundances of *G. vaginalis, Candidatus Lachnocurva vaginae,* and *Lactobacillus* spp., and is dominated by *Staphylococcus* spp.
CST V	Community dominated by *L. jensenii*

1.2.3 Vaginal dysbiosis and its impact on health

The vaginal environment is exposed to many disturbances, for example during intercourse, menstruation and pregnancy. Therefore, it is important that the interactions between the host and the VM are balanced, in order to maintain vaginal homeostasis (i.e., eubiosis) (Figure 1A) (40, 55). When a balanced VM gets disrupted, vaginal dysbiosis can succeed. Vaginal dysbiosis is characterised by the absence of vaginal lactobacilli dominance, increase of microbial diversity, and overgrowth of opportunistic pathogens (61-63). This reduction in *Lactobacillus* spp. increases the vaginal pH, creating a favourable environment for colonisation by various pathogenic microorganisms. One of the most common vaginal conditions associated with vaginal dysbiosis is bacterial vaginosis (BV), afflicting 23-29% of women worldwide, which results in an annual global economic burden of US $4.8 billion for treating symptomatic BV (62, 64).

BV is characterised by a loss or sharp decline in the total number of vaginal lactobacilli, increased species diversity and a high abundance of anaerobic bacteria (i.e.,*Gardnerella, Prevotella, Atopobium* and *Mobiluncus*) (20). Those anaerobic species produce short chain fatty acids, which increase the vaginal pH and generates a pro-inflammatory environment (Figure 1B) (55). BV can increase susceptibility for infections, for instance, urinary tract infections (UTIs) are more common among women with lower abundance of vaginal lactobacilli (65).

Moreover, increased abundances of anaerobic bacteria are linked to a higher risk for sexually transmitted infections (STIs), such as human immune deficiency virus (HIV) (66), gonorrhoea and chlamydia (38, 67). Additionally, BV is the most common cause of (malodourous) vaginal discharge. Other clinical manifestations include burning sensation during urination and itching surrounding the vagina (38, 68). Moreover, some studies have linked BV to adverse pregnancy outcomes, including PTB, miscarriage and low birth weight (69). It should however be noted that approximately half of women with BV have no overt symptoms (i.e., asymptomatic BV) (70). Currently, the pathogenesis of asymptomatic BV remains underexplored and its treatment remains controversial. Since asymptomatic women have been infrequently studied, it remains unknown whether a treatment can prevent adverse outcomes such as increased infection risk and averse pregnancy outcomes (71). Therefore it is important that more asymptomatic women are examined, however it is more difficult to recruit asymptomatic women outside a clinical setting (59).

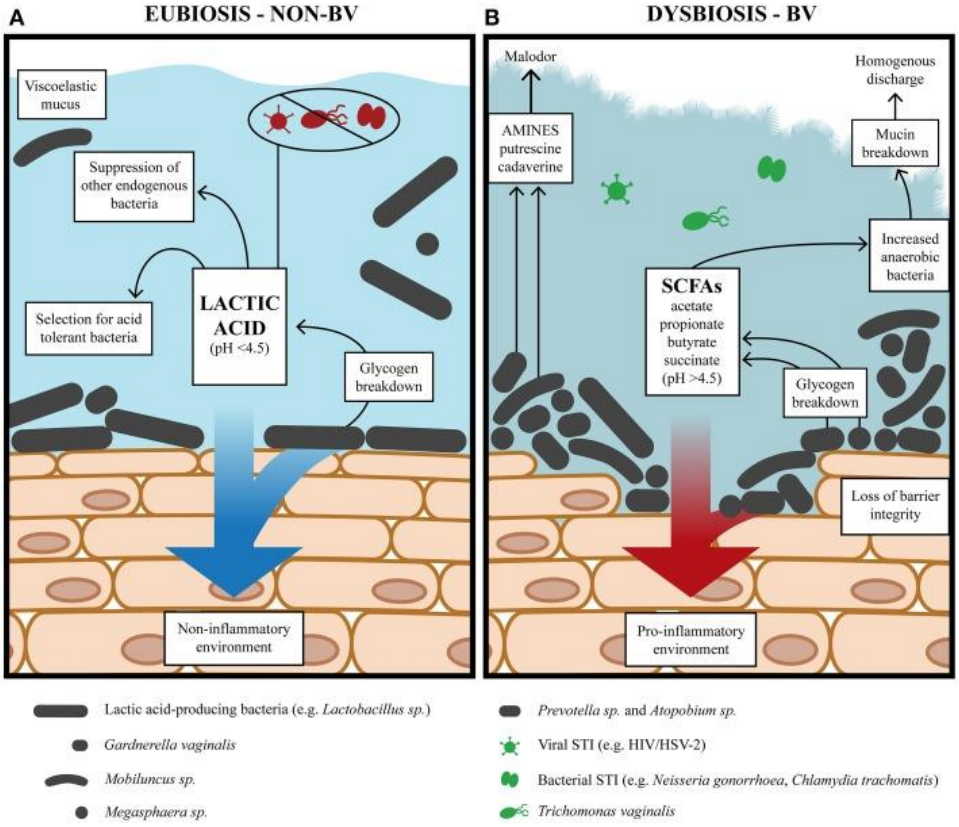

Figure 1: The vaginal microenvironment *(55). (A) A balanced VM (i.e., eubiosis), lactic acid produced by lactic acid bacteria creates a non-inflammatory environment. In addition, lactic acid acidifies the vaginal milieu to pH below 4.5. Lactic acid inactivates pathogens causing STIs and suppresses growth of other endogenous bacteria. (B) A dysbiotic VM, short chain fatty acids produced by anaerobic bacteria increase the vaginal pH. The diverse anaerobic bacteria also generate a pro-inflammatory environment, and increase risk for infections (55). Abbreviations: BV = bacterial vaginosis, SCFAs = short chain fatty acids, VM = vaginal microbiome.*

1.2.4 Characterising the vaginal microbiome in the general population: The Isala project

To further explore the composition, ecology and determinants of the VM in the general population, outside of the hospital setting, a large-scale citizen-science project was recently set up by the Laboratory of Applied Microbiology and Biotechnology (LAMB; ENdEMIC; University of Antwerp), led by Prof. Sarah Lebeer (59). The project was named Isala (https://isala.be/en), in honour of Isala Van Diest (1842-1916), the first female doctor in Belgium. The main research goal of the Isala project was to map the VM in a large study cohort of healthy women, and how their VM was associated with several life-course and lifestyle factors (59). Moreover, they aimed to establish a large biobank with vaginal isolates, which could be used for further fundamental and applied research (59). Additionally, Isala also had a societal goal to break certain taboos around female and vaginal health and thus make these topics more accessible for discussion. Therefore, the Isala research team provided clear science communication towards all participants and the general public.

In March 2020 a call for 200 participants was launched. Astonishingly, within ten days 6,007 Flemish participants registered through the website. All registrants were invited to fill out an extensive questionnaire, questioning their general health, reproductive history, hygiene practices, sexual habits, dietary habits, birth mode, living situation and other lifestyle parameters. A total of 4,682 registrants completed this questionnaire and received a self-sampling kit at home via national postal service (59). 3,345 women ranging from 18 to 98 years old provided two vaginal swabs, including one for microbiome profiling based on 16S rRNA amplicon sequencing and one for culturing purposes (59). Based on the large set of vaginal samples and detailed questionnaire data, the Isala team analysed which factors are influencing the VM. A total of 166 covariates were assessed, including reproduction, lifestyle, health status, hygiene practices, environmental and dietary information (59).

As mentioned before, the VM composition can be reflected in five CSTs (cfr. '1.2.2 The vaginal microbiota') (46). The Isala research team adopted a different approach and determined four main modules of interacting microbes by investigating the correlations between taxa abundances. The modules are centred around *L. crispatus*, *Bacteroides*, *Gardnerella*, and *Prevotella,* respectively. These modules comprises microbiota community members that seem to be functionally connected, as they are frequently found in close association with one another. The *L. crispatus* module, comprising *L. crispatus, L. jensenii*, and *Limosilactobacillus* taxa*,* probably reflects a common healthy homeostatic state, and is negatively associated with the number of vaginal complaints, while a reduction of this module was associated with different vaginal complaints reported by the participants (e.g., vaginal discharge and malodour) (59). Those findings are not surprising, considering that LAB are known to create an anti-inflammatory environment (55). By grouping the VM in modules, the importance of low-abundance taxa, such as *Limosilactobacillus*, becomes more clear. The Isala data thus suggests a keystone role for *Limosilactobacillus* spp. in the VM (59). More specifically, *Limosilactobacillus* spp. show a low average relative abundance of 0.4%, while being prevalent among the Isala cohort (47.9%). Moreover, the positive correlations found between *Limosilactobacillus* spp. and *L. crispatus* and *L. jensenii* in the *L. crispatus* module*,* suggest a potential role of *Limosilactobacillus* spp. in supporting their dominance (59).

1.2.5 Diversity in the vaginal microbiome

Both the Isala project (59) as well as the study of Ravel *et al.*(46) demonstrated the heterogeneity of the VM composition among individuals (i.e., inter-individual variability). In general, vaginal lactobacilli dominance appears to be a good biomarker for an optimal vaginal ecosystem, because of the protective functions of these bacteria. Nevertheless, a non-*Lactobacillus* dominated VM does not necessarily cause symptoms (i.e., malodourous discharge) (72). Up until now, it is not yet fully understood why in most asymptomatic women the VM is dominated by lactobacilli while others are not (73). When investigating underlying causes for this inter-individual variability, several studies have already shown that the composition of the VM varies among ethnic groups (46, 48, 74). For example, a study of Zhou *et al*. (2007) demonstrated that a VM dominated by *Lactobacillus* spp. was more common in Caucasian women, whereas the VM of black women was mainly dominated by anaerobes (48). The aforementioned CST-revealing study of Ravel *et al.* (2011), including 396 asymptomatic reproductive-age women with different ethnic backgrounds (i.e., white, Asian, black, and Hispanic), revealed that the proportions of CSTs varied significantly among these four ethnic groups (46). More specifically, *Lactobacillus* spp. were more abundant among white and Asian women, while anaerobic species dominated in black and Hispanic women in their study (46). In addition, the composition of the VM is known to be influenced not only by human behaviour, including personal hygiene, diet, contraceptive use, and sexual practices (46, 75), but also by environmental factors such as socioeconomic status (for example, household income and access to healthcare) (76). Nevertheless, it remains to be further explored to what extent these VM differences along ethnic groups are reflections of host genetics, environmental factors or a combination of both (77).

1.3 The vaginal microbiome throughout life

Apart from inter-individual variability, the VM is a dynamic ecosystem of which the composition changes throughout a woman's life. Therefore, the VM of every woman can vary over time (78). Several factors can influence the VM composition throughout a woman's life, including the natural menstrual cycle (accompanied by hormonal fluctuations), contraceptive use, pregnancy, as well as behavioural factors (75, 79, 80).

1.3.1 Oestrogen shapes the composition of the vaginal microbiome throughout life

The VM composition is shaped throughout a woman's life by fluctuating oestrogen levels, which vary from childhood to onset of puberty, during reproductive-age and pregnancy, as well as during menopause (Figure 2) (42). Oestrogen acts directly on vaginal epithelial cells by promoting cell proliferation and accumulation and deposition of glycogen (40, 42, 80). Directly after birth, the vaginal environment of newborns is influenced by residual maternal oestrogens that are temporarily present in the newborn's blood. The presence of these oestrogen molecules induce thickening of the vaginal epithelium and glycogen deposit in the epithelial cells. As a result, within the first 24h after birth, the vaginal mucosa is rich in glycogen which favours colonisation by LAB, including lactobacilli. Subsequently, by the fourth post-natal week, the residual maternal oestrogen levels drop, leading to a thinning of the vaginal mucosa and decreased epithelial glycogen levels. In this way, the decreased availability of fermentation substrates for LAB lead to a more neutral to alkaline vaginal pH (42, 80, 81). During childhood and pre-menarche, the vaginal pH remains neutral, resulting in a highly diverse vaginal community, consisting of Gram-positive anaerobic bacteria (i.e., A*ctinomyces, Bifidobacterium, Peptococcus, Peptostreptococcus*, and *Propionibacterium*), Gram-negative anaerobic bacteria (i.e., *Bacteroides*, *Fusobacterium*, and *Veillonella*), as well as aerobic bacteria (i.e., *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans,* and *Enterococcus faecalis*) (42).

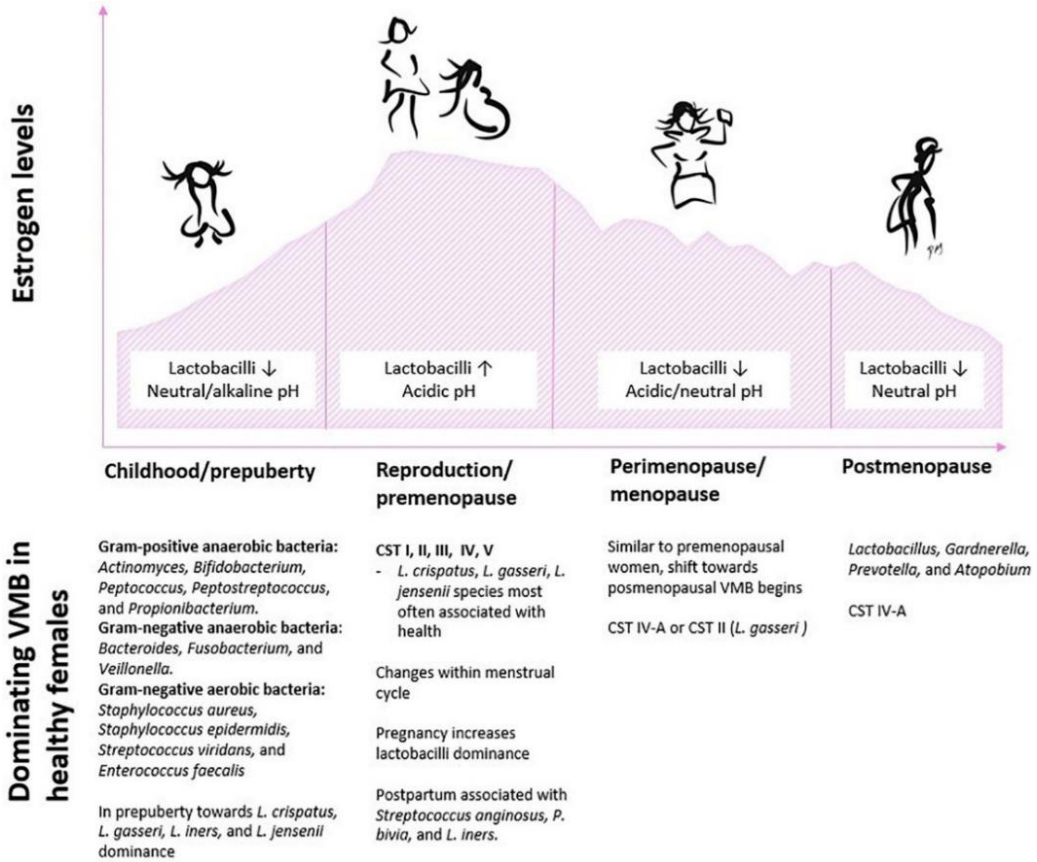

Figure 2: A general overview of the dominant vaginal microbiota members and fluctuations in oestrogen levels across the female life span *(42). Abbreviations: CST = community state type, VMB = vaginal microbiota.*

At the onset of puberty, oestrogen levels will increase, thus triggering the transition from low to high glycogen deposits in epithelial cells available for *Lactobacillus* spp. This results in a shift towards vaginal lactobacilli dominance and a subsequent decrease in vaginal pH during reproductive-age (80). Importantly, such lactobacilli-dominated VMs are not necessarily stable over time. Changes in the microbial community can occur, for example over the course of the menstrual cycle or after sexual intercourse (78, 82). This was demonstrated by a longitudinal study of Srinivasan *et al.* (2010) in fourteen healthy women, who self-collected vaginal swabs at several time points over a three-week period. This study showed a decrease in relative abundances of *L. crispatus* and *L. jensenii*, and increase in *G. vaginalis* and *L. iners* at the onset of and during menses. After menses, the opposite was observed (42, 82). The increase in *G. vaginalis* can be potentially explained by the presence of iron in the menstrual blood, since iron is known as an essential growth factor for most bacteria and enhances replication of certain pathogens (82, 83). Moreover, experiments evaluating growth of *G. vaginalis* have shown that this species is unable to grow in iron-limiting conditions (84). As coping strategy, *G. vaginalis* has a well-adapted ability to harvest iron from the environment, thus clarifying its increased growth when menstrual blood is present (82, 84). Another longitudinal study of Gajer *et al.* (2012) further explored the temporal dynamics of the VM by analysing 32 reproductive-age women over a 16-week period (including 4-5 menstrual cycles) (78). During this period, fluctuations in the VM composition were observed for all women in this study, however being variable across them in terms of how their VM changed over time. For instance, women with a VM dominated by anaerobic bacteria (CST-IV) most often transitioned to a *L. iners*-dominated (CST-III) VM. Likewise, their findings showed that women with a *L. crispatus*-dominated (CST-I) VM most often transitioned to a *L. iners*-dominated (CST-III) VM. Women with a VM dominated by *L. gasseri* (CST-II), on the other hand, rarely underwent VM transitions. While the fluctuations in composition were correlated with the timepoint in the menstrual cycle, as well as bacterial community composition, and to a certain extent with sexual activity, other (unknown) factors are certainly at play as well (78). Overall, these findings suggest that VM compositions determined in cross-sectional studies should be interpreted with care as the VM varies over time, thereby stressing the importance of longitudinal studies in reproductive-age women.

At the end of their fertile period, most women enter menopause between the ages of 49 and 52. Menopause is defined as a cessation of menstruation of twelve consecutive months. Due to decreased ovary functions in this life-stage, levels of circulating oestrogens are lower, possibly resulting in various symptoms such as hot flashes, night sweats, decreased cognitive functions and mood changes (85, 86). As women approach menopause, the drop in oestrogen results in a decrease of vaginal lactobacilli, accompanied by a subsequent rise in pH (42, 80). While reproductive-age women mainly have a VM dominated by *L. crispatus* (CST-I) and *L. iners* (CST-III) (87), the VM of perimenopausal women often contains a proportion of *L. iners* and strict anaerobes (CST-IV) or *L. gasseri* (CST-II), whereas the VM of postmenopausal women is typically categorised as CST-IV (42, 87). Due to the increased pH, the postmenopausal stage is associated with vaginal symptoms such as vulvovaginal atrophy, dryness and dyspareunia (i.e., painful intercourse) (80, 87, 88).

1.3.2 The vaginal microbiome during pregnancy

Another life event that will significantly alter the composition of the VM, besides the menopause, is pregnancy. If a woman becomes pregnant, fluctuations in hormone levels result in behavioural changes and modifications of the physio-chemical properties of the vaginal mucosa, making the VM of a pregnant woman different from a non-pregnant one (89). The dynamics of the VM during pregnancy have been investigated in a few longitudinal studies (76, 90-96). These studies have shown that the VM is reshaped during an uncomplicated pregnancy, towards reduced alpha diversity (i.e., diversity at a local site, within one community) and thus increased stability of bacterial communities, characterised by higher abundances of *Lactobacillus* spp. and lower abundances of bacterial species associated with BV (e.g., *Gardnerella* and *Prevotella*) (76, 90-97). It is not yet completely understood why the VM of pregnant women undergoes these changes, but they have been linked to increased oestrogen levels during pregnancy, as well as increased endocrine stability, and the absence of menstrual bleeding, all favouring vaginal lactobacilli dominance (89, 98).

When examining the impact of ethnicity on the VM, a recent study of Serrano *et al.* (2019) showed that the VM composition of women of African and non-African ancestry responded differently during pregnancy (76). Here, a shift towards *Lactobacillus* spp. dominance occurred early in pregnancy, and was observed predominantly in women of African ancestry. This finding is consistent with previous research, which has revealed that the VM of women of African ancestry is more often dominated by non-*Lactobacillus* spp. during reproductive-age, compared to during pregnancy. In contrast, in women of non-African ancestry, a non-*Lactobacillus*-dominated VM is uncommon in pregnant as well as in non-pregnant women. It is suggested that both genomic and environmental factors are likely to contribute to these differences (76).

Fewer studies have been focussing on the VM in the postpartum period (92, 94, 99, 100). These studies report that after delivery, a decline in *Lactobacillus* spp. abundance was observed, as well as a sharp increase in alpha diversity and anaerobe species such as *G. vaginalis, Streptococcus anginosus* and *Prevotella bivia* (82, 84, 89, 90). The decreased lactobacilli abundance could be explained by the massive drop in oestrogen following delivery (99, 101). However, further research is needed to gain insight in how this postpartum changes have an influence on perinatal outcomes and infections risks. Nonetheless, the VM need to restore after pregnancy to create again an optimal environment (89, 100, 102).

1.4 The importance of the maternal microbiome

Up until now, the vital role of an optimal maternal microbiome during and after pregnancy is increasingly emphasised (103-105). Vaginal dysbiosis during pregnancy has been linked with many adverse health outcomes such as preterm birth (PTB) (98, 106) and miscarriages (107). In addition, numerous evidence suggests that vaginal dysbiosis also influences fertility and can potentially impair the success of natural conception (108) and assisted reproductive treatments such as *in vitro* fertilisation (IVF) (109, 110). By consequence, vaginal dysbiosis does not only have adverse effects on women, but also on their partners, as this can manifest as anxiety, stress, depression, among other consequences (111). Moreover, the maternal microbiome is assumed to be of major importance for initial colonisation of a newborn (103, 112-114). Shared microbial strains have already been detected among mothers and their newborn, suggesting vertical transmission of maternal microbiota members (97). However, the extent of this maternal microbiota transmission from different body sites, the mechanism by which these microorganisms are selected, the factors shaping this selection process and the mechanisms by which they can persist throughout life remain largely unclear (115). Moreover, emerging evidence suggest that early microbial colonisers play a key role in the establishment and maturation of several metabolic and developmental pathways, such as stimulating immune development after delivery, thus re-emphasising the importance of an optimal maternal microbiome (115, 116).

1.4.1 Vaginal microbiome and preterm birth

Several studies in pregnant women have suggested that the composition of the VM during pregnancy has an impact on the risk for PTB (93, 95, 117). The World Health Organization defines PTB as birth before 37 weeks of gestational age (GA) (118). PTB is a global health concern, affecting 7-15% of all pregnancies and resulting in approximately 15 million PTBs annually (119). This makes PTB one of the major causes for neonatal mortality, together with childbirth-related complications, infections (e.g., meningitis or sepsis) and birth defects (120, 121). In high-income countries, about 50% of preterm infants born at 24 weeks survive the first 28 days of life. In general, of those born preterm, more than 1 million dies before the age of 5 years (122). Besides these high numbers of neonatal mortality, PTB also causes long-term morbidities, such as respiratory distress syndrome, cerebral palsy and developmental delay (123). Moreover, the care of preterm infants is expensive and creates a financial burden for both the family and the healthcare system (124). About 70% of PTBs follow spontaneous onset of labour with intact membranes (45%) or premature rupture of the membranes (25%). For the remaining 30% labour is induced, or the infant is delivered by pre-labour Caesarean section (C-section), due to maternal or foetal indications (e.g. pre-eclampsia, eclampsia, and intrauterine growth restriction) (125, 126). Several pathological mechanisms are suggested to contribute to spontaneous PTB, including stress, uterine

overdistension, cervical disease, breakdown of maternal-foetal tolerance, vascular disease and infections (126). Most of these factors only seem to be associated with PTB, while the relationship between invasion of microorganisms in the amniotic cavity and PTB is likely to be causal (125, 127, 128). Microorganisms can enter the amniotic cavity via ascension from the lower reproductive tract, however also hematogenous dissemination with transplacental passage can occur (126). Some examples of microorganisms that are known to cause infection during pregnancy are pathogens (e.g., *Neisseria gonorrhoeae, Trichomonas vaginalis, Chlamydia trachomatis*), Group B *Streptococcus* (GBS), entero-pharyngeal organisms (e.g., *Escherichia coli, Enterococcus faecalis*), and microorganisms related to BV (e.g., *G. vaginalis* and other anaerobes) (129).

However, the use of antibiotic prophylaxis does not appear to lower the risk for PTB associated with infections (130-132). Moreover, inappropriate use of antibiotics during pregnancy, such as in women without signs of infection, has been linked to an increased risk for PTB (133). Therefore, the use of probiotics (i.e., live microorganisms that confer a health benefit on the host when consumed or applied to the body (134)) during pregnancy, emerges as a promising alternative to reduce the risk for PTB (135). However, more research is needed to evaluate the efficacy of probiotics during pregnancy. An ongoing trial, called the ProPreB (Probiotics for Preterm Birth prevention) trial, tries to elucidate the effects of probiotics during pregnancy. The ProPreB trial started in 2017 and has three major aims: (i) to investigate the relation of VM alteration during pregnancy and PTB, (ii) to investigate the prophylactic use of probiotics to prevent adverse pregnancy outcomes in women with high vaginal pH (≥ 4.5) and (iii) to investigate whether oral and vaginal probiotics potentiate each other's effect on the VM.

A VM, dominated by lactobacilli, plays a key role in protecting against inflammation and infections, in contrast to a VM with high abundances of anaerobic bacteria as observed in women with vaginal dysbiosis (55, 56). Therefore, throughout the last decade, several studies have been looking for associations between the composition of the VM during pregnancy and risk for spontaneous PTB (93-95, 117, 120, 136, 137). Overall, women with a VM dominated by lactobacilli during pregnancy are assumed to have a lower risk of delivering pre-term compared to women with a dysbiotic VM (98, 105). Nevertheless, geographical and ethnic differences of which the underlying mechanisms are yet to be elucidated remain. However, in previous research, a consistent finding is the association of a VM dominated by *L. crispatus* during pregnancy with a lower risk for PTB (98, 105). Since vaginal dysbiosis affects millions of women, it is important to gain more insights in its relation with PTB (105, 138).

In addition, it has been documented that species diversity of vaginal lactobacilli present during pregnancy also has an impact on the risk for PTB. This was demonstrated by a study of Petricevic *et al*. (2014) investigating 111 pregnant women with a *Lactobacillus*-dominated VM in the first trimester of pregnancy, and without any vaginal complaints (117). The main outcome of the study was the diversity of *Lactobacillus* spp. during full-term pregnancies compared to preterm pregnancies. Interestingly, 85% of women who delivered preterm had a VM dominated by *L. iners*, in contrast to 16% of the women who delivered at-term. This suggests an association between *L. iners*-dominance in early pregnancy and PTB (136). Further research is needed to define which VM compositions are at risk for PTB and to better understand the underlying pathophysiological mechanisms. In this regard, longitudinal studies mapping the changes of the VM during pregnancy will be of use in order to find predictors for PTB (105).

1.4.2 Mother-to-infant transmission in early life

Several maternal niches, including the vagina, are involved the initial microbial colonisation of a newborn (139). Therefore, a non-optimal maternal VM is not only involved in adverse pregnancy outcomes (e.g., PTB), it might negatively affect the initial microbial colonisation of a newborn as well. Apart from the vaginal microbiota, other maternal microbial communities are also involved, including the gut, skin, oral and breast milk microbiota (Figure 3). Vertical transmission of microbial communities from maternal niches promotes the development of the foetus (e.g., immune and brain development) (116, 140, 141), and favours the transmission of beneficial and specific microbial communities to the next generation (116). It has been reported that initial microbial

colonisation primarily occurs during delivery and immediately following birth, apart from an ongoing discussion on *in utero* seeding (112, 142, 143).

To further investigate vertical transmission of the maternal microbiome, studies have focussed on identifying shared strains between mothers and their offspring (144). However, the majority of this research has focussed on the newborn's intestinal, oral and skin microbiome (113, 144, 145). For example, Ferretti *et al.* (2018) conducted a study on the gut microbiome of newborns, revealing that on the day of delivery, 50.7% of the microbiota members in the newborn's gut were derived from the mother's gut, vagina, oral cavity, or skin. The largest contribution was from the mothers' gut (22.1%), followed by the vagina (16.3%), the oral cavity (7.2%), and the skin (5%), respectively. In the weeks following delivery, the abundance of vaginal, oral, and skin microbial species in the newborn's gut decreased. This indicates that these species are only temporary inhabitants of the lower gastrointestinal tract. In contrast, microbial species from the maternal gut seem to be more persistent over time (Figure 3) (97, 104). In addition to vertical transmission, several other factors have an impact on the newborn's microbiome in early stages. The microbiota that are acquired from various maternal body sites are influenced by mode of delivery, GA at birth, the maternal diet, as well as other factors such as antibiotic use and host genetics (Figure 3)(97, 116, 146).

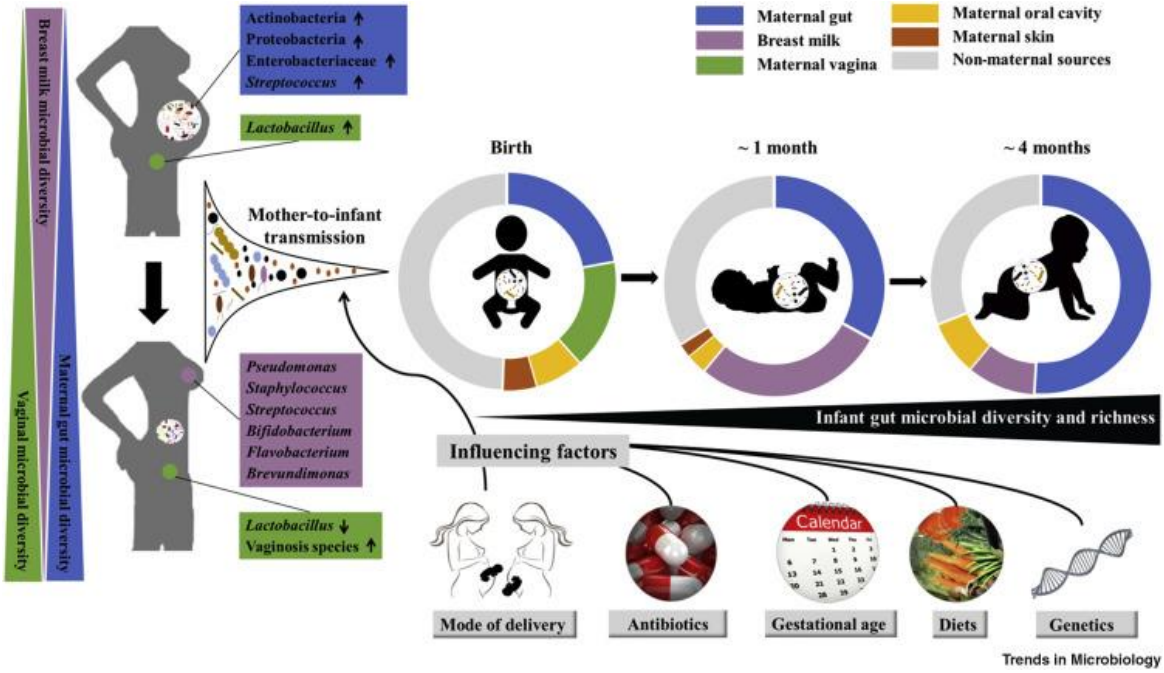

Figure 3: Dynamics of the maternal microbiome (vaginal, breast milk and gut) during and after pregnancy, and to what extent these contribute to mother-to-infant transmission in early life *(97).*

1.4.3 The origin of the vaginal microbiome

To date, most studies on mother-to-infant transmission have been focussing on the infant gut, oral and skin microbiome (139), while less attention has gone towards the VM. Therefore, the origin of the VM remains unknown as well as how its specific community composition is maintained from generation to generation (112, 147). Because of the neutral to alkaline vaginal pH during childhood (148), not all bacteria possibly transmitted at birth may be capable of surviving this period (112). While it is known that hormonal and physiological changes at onset of puberty create an environment which is favourable for lactobacilli (80), it is not clear where those strains originate. On one hand, they might be of maternal origin and might have persisted in a dormant state or at low abundance throughout childhood. On the other hand, it could be that they are acquired later in life through other mechanisms (e.g., diet and environment) (103, 112).

At present, only a limited number of studies investigated vertical transmission of the maternal VM to the female offspring (112, 147). Bassis *et al.* (2022) studied the similarity between the vaginal microbiota members of

thirteen adolescents (ages 15-21) and their mothers based on 16S rRNA gene sequencing in relation to the daughter's birth mode (147). The VM of mother and daughter were more similar if the daughter was born by vaginal delivery rather than C-section. Moreover, *L. crispatus* isolates from one mother-daughter pair, in which the daughter was born by vaginal delivery, had highly similar genome sequences, based on recombination-filtered single nucleotide polymorphisms (SNPs). Therefore, this study provides preliminary evidence that VM members may be transmitted from mother to daughter at birth during vaginal delivery with persistence until adolescence (147). Another recent study showed that a small number of vaginal bacterial strains is shared between mother and daughter, but shared strains were more frequently identified in unrelated individuals (112). However, this finding does not rule out vertical transmission from the maternal VM, as longitudinal studies have shown that the VM of reproductive-age women is not necessarily stable over time (78, 80). The findings of these studies highlight the importance of conducting further research on vertical transmission of the VM and its impact on the future reproductive health of daughters (112, 147).

1.5 Study aims

The preceding literature review has emphasised the crucial role of the VM in women's health, as well as in their partner's and children's health. Up to date, the majority of research, however, has been focussing on the VM in the clinical context of infections and adverse pregnancy outcomes (e.g., PTB) (38, 98). Therefore, it becomes increasingly important to gain more knowledge on the composition and dynamics of the VM in the general population to obtain more evidence-based insights in how the VM influences overall health (59). Moreover, many knowledge gaps remain regarding the dynamics of the VM during pregnancy and how the VM composition during pregnancy can contribute to the risk for PTB. Currently, an urgent need exists for research on predictive factors for PTB being a major health problem worldwide. Nevertheless, assessing the risk for PTB remains challenging, since there are numerous asymptomatic women with a non-*Lactobacillus* dominated VM (46). It became clear that longitudinal studies are needed to further investigate the dynamics of the VM during pregnancy and to identify factors that could contribute to vaginal dysbiosis during pregnancy and PTB. These insights could be used to implement new strategies for treatment of vaginal dysbiosis and prevention of PTB. Another knowledge gap is the origin of the VM, with a possible role for vertical transmission of the maternal VM.

Overall, this Master's thesis aims to provide a better understanding of the dynamics of the VM composition during pregnancy, the associations with PTB and its vertical inheritance. In a first part, the dynamics of the VM during pregnancy will be explored by examining microbiome profiles collected at three different time points (in each trimester) during pregnancy. In addition, the relationship between GA at delivery and several clinical signs and features will be explored by analysing clinical data collected at these three time points. The goal is to unravel the potential presence of clinical signs and/or features of the VM that can predict adverse birth outcomes, more specifically PTB.

The second part aims to examine whether vertical transmission of the maternal VM to the daughter occurs at birth and whether these communities persist until the daughter's reproductive-age. Therefore, vaginal bacteria from mother and daughter pairs will be isolated, followed by a comparison of their genome sequences. One hypothesis is that the genome sequences of those isolates are highly similar between mother and daughter, thus suggesting vertical transmission at birth. When vertical transmission from the maternal VM at birth can be proven, this highlights the importance of a healthy VM during pregnancy, as the daughter may inherit a similar composition.

2 Materials and Methods

The following section provides an overview of the experimental methods implied in this Master's thesis. The first part describes all methods used to analyse the VM dynamics throughout the three trimesters of pregnancy, and to analyse clinical data collected during pregnancy to investigate potential associations with PTB. Subsequently, the second part focusses on the methodology for isolating and identifying bacterial strains from vaginal swabs of healthy mothers and daughters to explore vertical transmission of the VM and persistence of vaginal bacteria.

2.1 Studying the dynamics of the vaginal microbiome during pregnancy and preterm birth

To study the dynamics of the VM composition during pregnancy and possible associations with PTB, clinical and microbiome data from the ongoing ProPreB trial were explored.

2.1.1 The ProPreB trial: study population and data collection

To take part in the ProPreB trial (cfr.'1.4.1 Vaginal microbiome and preterm birth'), pregnant women were recruited by their obstetric staff members (in Antwerp University Hospital (UZA) and RZ Heilig Hart Tienen (RZ T)) when they presented for routine prenatal care between 6 and 13 weeks of GA. Recruitment started in 2017 and is still ongoing to date. At the time of recruitment, all participants lived in Belgium, had a minimum age of 18 years, and signed an informed consent form. Exclusion criteria were as follows: (i) use of local and/or systemic antibiotic and antimycotic treatments and local progesterone treatment two weeks or less prior to examination, (ii) clinical evidence of vulvo-vaginitis or cervicitis requiring treatment, (iii) untreated *Chlamydia* infection, gonorrhoea and/or syphilis at time of recruitment, (iv) serious extra-genital pathologies (e.g., insulin-dependent diabetes mellitus), (v) vaginal bleeding of any cause at the moment of inclusion, and (vi) multiple pregnancy. Women with disorders such as pre-eclampsia and gestational diabetes that originated during pregnancy were not excluded.

Data were collected from the participants at each trimester and at delivery (four visits) (Table 2). During the first trimester visit (i.e., inclusion visit between 6 and 13 weeks of GA), women filled out an intake questionnaire questioning personal information (incl. name, date of birth) and socio-demographics (incl. ethnicity, education, employment). In addition, questions about their medical, obstetric and gynaecological history, as well as smoking and drinking behaviour, and medicine use were asked. Besides the intake questionnaire, clinical data were collected. More specifically, an ultrasound was performed (routine) and four vaginal smears were collected, one to measure vaginal pH and three for microscopic analyses. In more detail, the pH was measured using pH test strips (Macherey-Nagel, pH-Fix 3.6-6.1), and microscopy on fresh vaginal smears was performed to determine the lactobacillary grade (LBG), and to investigate the presence of BV-associated bacteria, *Candida, Trichomonas* and other vaginal morphotypes. Vaginal smears for pH measurements and microscopic analyses were kept at room temperature. Additionally, a vaginal swab was taken for microbiome analyses. After collection, vaginal swabs were stored at -20 °C.

Later during pregnancy, three more medical visits were planned at the second (between 18 and 23 weeks of GA), and third (between 35 and 37 weeks of GA) trimester and at delivery, respectively. At each of these three visits, different types of tests and data collection were performed to monitor pregnancy and assess vaginal parameters. Table 2 provides an overview of the different time points at which the clinical data used for analyses in this Master's thesis were collected.

Table 2: ProPreB trial: examination overview and timing. *Visits were planned at different time points during pregnancy and at delivery. An overview of the performed clinical examinations at four different time points is shown. Abbreviations: GA = gestational age, GBS = Group B Streptococcus.*

	Visit 1 (Inclusion)	**Visit 2**	**Visit 3**	**Visit 4**
Timing	6-13 weeks of GA	18-23 weeks of GA	35-37 weeks of GA	At delivery
Intake questionnaire	X			
Ultrasound (routine)	X	X		
Vaginal smears for pH (1x) and microscopy (3x)	X	X	X	
Vaginal swab for microbiome analyses	X	X	X	
Routine GBS recto-vaginal swab			X	
Perinatal data				X

Microbiome profiling of the collected vaginal swabs was previously performed by the host lab (LAMB; ENdEMIC; University of Antwerp) as previously described (59). Briefly, the V4 hypervariable region of the 16S rRNA gene was amplified and sequenced using Illumina MiSeq. Sequenced reads were processed using a state-of-the-art denoising algorithm (DADA2), resulting in an overview of abundances of amplicon sequence variants for each vaginal swab. Subsequently, an in-house bioinformatic pipeline was used to identify the taxonomic composition of the vaginal microbial communities (59). In general, the obtained sequencing data do not allow identification at species level (59). Therefore, to enhance taxonomic resolution, the *Lactobacillus* genus was further divided into subgenera, including the *L. crispatus*-group, *L. jensenii*-group, *L. iners*-group and *L. gasseri*-group. For instance, the *L. crispatus*-group as subgenus comprises different species, such as *L. helveticus* and *L. kefiranofaciens*. This classification enables to discriminate between *L. crispatus, L. iners, L. jensenii* and *L. gasseri*, often designated as the 'big four' in VM research (59). In addition, all samples were assigned to CSTs and sub-CSTs (cfr. Table 1 in section 1.2.2), using the VALENCIA algorithm (available at https://github.com/ravel-lab/VALENCIA) (60). For each swab, the resulting data, including identified taxa and corresponding relative abundances, were stored in CSV (Comma-Separated Values) files for further data handling in this Master's thesis. At the different study sites (UZA and RZ T), questionnaire and clinical data of all four visits were directly recorded in REDcap. These data were retrieved by the host lab (Excel version 16.43) and prepared for further data analyses in this Master's thesis. More specifically, data was explored, variables of interest were selected and unclear data points as well as duplicate and incomplete data were removed (Table 3).

Table 3: Overview of selected variables from the clinical ProPreB data that were used for further analyses in this Master's thesis. *Data were collected at visit 1 if not stated otherwise. Abbreviations: BMI = Body Mass Index, BV = bacterial vaginosis, GBS = Group B Streptococcus, ID = identity.*

Variable name	**Variable type**	**Meaning**
Participant ID	categorical	Participant ID number
Age	continuous	Participant's age, expressed in years
Last time smoked	categorical	Last time when the participant smoked - Never - <30 days ago or currently smoking - 1-12 months ago - >12 months ago

BMI	continuous	Participant's BMI, expressed in kg/m^2
Vaginal pH (for each visit)	continuous	Participant's vaginal pH measured at visit 1, 2 and 3
Bacterial vaginosis (for each visit)	categorical	Presence of BV related cells - No - Patchy granular; <20% clue cells - Full granular: >20% clue cells
Cervical length	continuous	Participant's cervical length measured at visit 2, expressed in mm
GBS culture	categorical	Result of recto-vaginal GBS swab, taken at visit 3. - GBS positive - GBS negative - Not known/ sample inconclusive - GBS swab not taken
Gestational age at delivery	continuous	The participant's gestational age at delivery, expressed in weeks

2.1.2 Selection of ProPreB participants

Based on personal agreement levels (i.e., informed consent) and vaginal pH evaluated during the first visit, women participating in the ProPreB trial were divided into two control groups. One control group included women with a vaginal pH below 4.5. No study medication was administered since absence of vaginal lactobacilli dominance is not expected in these women. A second control group included women with a vaginal pH ≥ 4.5 who did not give consent to use the study medication. The actual study group included women with a vaginal pH ≥ 4.5 who did give consent to use the study medication. These women were randomly allocated to one of the different treatment groups. Each of those groups receive a specific treatment combination, ranging from the oral active product, vaginal active product, oral placebo and vaginal placebo, resulting in four different possible treatment combinations. Over the entire duration of the trial, both participants and researchers will not be aware of the allocated treatment groups (i.e., double-blind trial). Since the trial is still ongoing, and thus blinded up to date, this Master's thesis will focus on different types of data (incl. questionnaire, clinical and microbiome data) collected from the control groups. In this Master's thesis, control group 1 includes women with a vaginal pH below 4.5 and control group 2 includes women with a vaginal pH ≥ 4.5 without consent to use study medication.

2.1.3 Data analysis and statistical testing

Microbiome data, as well as clinical and questionnaire data, were analysed using the open source program R within the RStudio platform (version 4.2.3). Microbiome data were explored using the "tidyverse" set of R-packages and the in-house developed R-package "tidyamplicons" version 0.2.2 (available at github.com/Swittouck/tidyamplicons). VM profiles were visualised to analyse dynamics throughout pregnancy. In addition, relative abundances of taxa, as well as alpha diversity throughout the three trimesters of pregnancy were examined. As measure for alpha diversity within each sample, the inverse Simpson's diversity index (D-value) was calculated. D-values were compared among women who delivered at term and preterm for each visit using the Wilcoxon rank-sum test. Besides, the Wilcoxon signed-rank test with Bonferroni correction was used to compare D-values between visits for all women. In addition, a Fisher exact test was performed to look for possible associations between GBS status and sub-CSTs at each visit.

Besides exploring the dynamics of the VM during pregnancy, several statistical tests and analyses were performed on the clinical data. The Wilcoxon signed-rank test with Bonferroni correction was used to compare pH values between visits for all women. Subsequently, the Wilcoxon rank-sum test was used to compare the vaginal pH between women who delivered at term and preterm. In addition, the Fisher exact test and Spearman correlation coefficient were used to unravel possible associations between PTB, GA at delivery and clinical parameters such as pH, GBS status, maternal Body Mass Index (BMI), maternal age and cervical length.

2.2 Studying vertical transmission of the vaginal microbiome

To study vertical transmission of the maternal VM and to examine whether vaginal strains remain present until daughter's reproductive-age, vaginal swabs from mother-daughter pairs from the Isala project were analysed.

2.2.1 The Isala project: Isala cohort and citizen-science based study design

The study cohort of the Isala project encompasses 6,007 healthy women aged 18 to 98. All Isala participants signed an informed consent form to analyse the data retrieved from their vaginal swabs, and to collect extensive questionnaire data. Two self-collected vaginal swabs were donated by 3,345 participants, including one for 16S rRNA amplicon sequencing (Copan, eNat®) and one for culturing and metabolomics analyses (Copan, ESwab™) (39). All swabs were transported at room temperature, with an average transport time of 2.9 +- 3.3 days (59). The eNat® swab contains a guanidine thiocyanate based medium as transport buffer, which stabilises DNA. This vaginal swab was used to determine relative taxonomic bacterial abundances, based on 16S rRNA amplicon sequencing of the V4 region of the 16S rRNA gene. After arrival in the host lab, the ESwab™ containing Amies medium was vortexed for 15 sec and separated in two aliquots of 500 µL, for culturomics and metabolomics analyses, respectively. After adding 500 µL glycerol (50%), the aliquot intended for culturomics analyses was stored at -80°C in a 96-tube Micronic rack. In this Master's thesis, the aliquot for metabolomics analyses will not be used.

2.2.2 Selection of Isala participants

Within the Isala cohort, several types of relations were identified, resulting in a total of 115 pairs of two related participants. The relationships within pairs were defined as follows: mother and daughter (31 pairs), partners (4 pairs), sisters (41 pairs), housemates (20 pairs), and other (19 pairs). In addition, nineteen groups with three or more related participants were identified.

To analyse vertical transmission of the VM from mother to daughter, a selection of mother-daughter pairs and groups was made. Pairs with a VM (co-)dominated by the *L. crispatus*-group (including *L. crispatus* and closely related species such as *Lactobacillus gallinarum, L. acidophilus,* and *L. hamsteri*) were selected for further analyses. Bacterial dominance was defined as a relative abundance equal to or higher than 30%. Groups were selected regardless of *L. crispatus*-group dominance in their VM. In parallel, for the selected participants, a set of metadata was retrieved from the extensive questionnaire data, including the participants age, age difference between mother and daughter, cohabitation status, pregnancy history and daughters' birth mode. Cohabitation status was determined based on answers on living situation in the questionnaire (e.g., postal code, number of house mates).

2.2.3 Culturing conditions

To optimise the isolation of vaginal lactobacilli from vaginal ESwabs of the selected participants, different laboratory growth media and growth conditions (micro-aerophilic vs. anaerobic) were tested. The composition of the growth media used in this Master's thesis is shown in Table 4.

Table 4: Medium composition of laboratory growth media used in this Master's thesis. *Ingredients and preparation instructions are shown. Abbreviations are used in following protocol descriptions and thesis parts.*

Medium	Abbreviation	Composition	Company
Difco™ Lactobacilli De Man – Rogosa – Sharpe Broth	MRS broth	- Proteose Peptone No. 3 (10 g/L) - Beef extract (10 g/L) - Yeast extract (5 g/L) - Dextrose (20 g/L) - Polysorbate (80 g/L) - Ammonium Citrate (2 g/L) - Sodium Acetate (5 g/L) - Magnesium Sulfate (0.1 g/L) - Manganese Sulfate (0.05 g/L) - Dipotassium Phosphate (2 g/L) - Agar (15 g/L)	Becton, Dickinson and Company (BD), Difco
	MRS pH 4.6 + Cys broth	- Composition of MRS broth - Supplemented with 0.3 % L-cysteine before autoclaving - Brought to pH 4.6 by adding hydrochloric acid (1M) before autoclaving	BD, Difco Roth
Difco™ Lactobacilli De Man – Rogosa – Sharpe Agar	MRS agar	- Agar (15 g/L) was added to the composition of MRS broth to obtain a solid medium	BD, Difco
	MRS + Cys agar	- Composition MRS agar - Supplemented with 0.3% L-cysteine before autoclaving	BD, Difco Roth
	MRS pH 4.6 agar	- Composition of MRS agar - Brought to pH 4.6 by adding hydrochloric acid (1M) or acetic acid (5M) before autoclaving	BD, Difco
Difco™ Columbia Blood Agar Base EH	CB agar	- Pantone (12 g/L) - Bitone H Plus (6 g/L) - Enzymatic Digest of Animal Tissue (3 g/L) - Starch (1 g/L) - Sodium Chloride (5 g/L) - Agar (12 g/L) - Addition of 5% sheep blood (after autoclaving, right before use)	BD, Difco

2.2.4 Culturing vaginal ESwabs and growth evaluation on different laboratory growth media

Vaginal ESwabs from the selected mothers and daughters were retrieved from the host lab biobank at -80°C. For each swab, a ten-fold dilution series (from 1:1 up to $1:10^7$) in phosphate-buffered saline (PBS, 1X) was prepared in total volumes of 200 µL in a sterile, flat-bottom 96-well microtiter plate. Subsequently, 50 µL of each dilution was spotted on a Petridish (20 mL) containing either CB agar or MRS + Cys agar. Sterile glass beads were used to obtain a homogeneous spread. Subsequently, plates were incubated at 37°C with 5% CO_2. Growth was evaluated after each 24 h period.

In addition, the potential of a subculture phase was explored. Therefore, some frozen swab material was inoculated in 10 mL MRS pH 4.6 + Cys broth, in duplicate. Test tubes were incubated at 37°C with 5% CO_2 directly or in an anaerobic jar with anaerobic gas generating sachets (AnaeroGen™ 2.5 L, Thermo Scientific™). Growth was evaluated after each 24 h period. When microbial growth was observed, the cultured swabs were vortexed whereafter the optical density (OD) at 600 nm of a ten-fold dilution was measured with a spectrophotometer (Genesys 20, Thermo Scientific™). First, sterile MRS pH 4.6 + Cys broth was set as blank to take into account a background signal. After OD measurements, Formula 1 was used to determine an appropriate dilution factor of

the cultured swabs to obtain a countable plate (i.e., between 30 and 300 colony forming units (CFU)) after overnight incubation. Subsequently, a ten-fold dilution series in PBS (1X) was prepared, whereafter 50 µL of the appropriate dilution and of these within a ten-fold difference range were streaked on a Petridish (20 mL), containing MRS agar, MRS + Cys agar, MRS pH 4.6 agar or CB agar. After drying, the agar plates were incubated at 37°C with 5% CO_2. Growth was evaluated each 24 h.

Formula 1: 0,25 OD600nm = 10^9 CFU/mL (dilution 1:10) ↔ OD600nm = 4 x 10^9 CFU/mL (undiluted)

2.2.5 Selection and identification of vaginal isolates from mothers and daughters

Single colonies were picked up from countable agar plates and suspended in Eppendorf PCR tubes containing 15 µL sterile molecular grade water. From this suspension, 10 µL was used for colony polymerase chain reaction (PCR) using universal amplification primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3', Integrated DNA Technologies) and 1492R (5'-GGTTACCTTGTTACGACTT-3', Integrated DNA Technologies), to amplify the complete 16S rRNA gene. A negative control, including 10 µL of sterile molecular grade water, was included. The remaining suspension (5 µL) was inoculated in MRS broth and incubated at 37°C with 5% CO_2 followed by a regular growth check (each 24 h period). After sufficient bacterial growth was observed, 800 µL of each bacterial culture was mixed with 800 µL of glycerol (50%) in cryovials. Cryovials were labelled appropriately and stored in the host lab biobank at -80°C for potential future research purposes.

Subsequently, Eppendorf PCR tubes were heated three times for 1.5 min at 800 W to impair bacterial cell walls and membranes. Additionally, a PCR master mix was prepared, for each sample and a negative control, including the ingredients mentioned in Table 5, with 10% extra to account for pipetting bias. Samples were centrifuged 10 sec at 3,000 rpm before adding 14.9 µL of the PCR master mix. All Eppendorf PCR tubes were placed in a PCR machine (Mastercycler®,Eppendorf) and colony PCR was performed according to the program in Table 6.

Table 5: Master mix ingredients for 16S rRNA gene amplification.

Ingredients	Volume per sample
10X VWR buffer	2.5 µL
dNTPs (10 mM)	0.5 µL
Forward primer (10 µM)	2.5 µL
Reverse primer (10 µM)	2.5 µL
Taq polymerase	0.1 µL
Molecular grade water	6.8 µL

Table 6: Colony PCR program for 16S rRNA gene amplification.

Step	Repetitions	Temperature	Duration
Denaturation	1 cycle	95°C	2 min
Denaturation	30 cycles	95°C	30 sec
Annealing		58.5°C	30 s
Extension		72°C	1 min 30 sec
Final extension	1 cycle	72°C	5 min
Hold		10°C	∞

Gel electrophoresis was performed to check the success rate of the performed colony PCR. First, a 1% agarose gel (10 g/L) was made using 750 mg agarose, 75 mL Tris-acetate EDTA (TAE) buffer (1X) and 7.5 µL GelRed®(Biotium). For each sample and the negative control, 1 µL of loading dye (6X TriTrack, Thermo

Scienctific™) was added to 5 µL of PCR product. Subsequently, samples and negative control were loaded on the agarose gel. Additionally, 3.6 or 6 µL of a reference ladder was loaded (GeneRuler 1 kb, Thermo Scienctific™), depending on the number of wells in the gel (eight and fifteen, respectively). The gel was run at 120 V for 25 min, and afterwards visualised with ultra-violet light (Fisher Bioblock Scientific).

Successful PCR products were prepared for Sanger sequencing of the 16S rRNA gene. First, PCR products were diluted with molecular grade water based on the band brightness after gel electrophoresis (i.e., a proxy for DNA concentration). Diluted PCR products (10 µL) were loaded in duplicate in a 96-well plate. In parallel a primer plate was prepared. Therefore, for each PCR product, 10 µL of the forward primer (27F; final concentration 5 µM) and 10 µL of the reverse primer (1492R; final concentration 5 µM) was added. Sequencing was performed by the Genetic Service Facility of the University of Antwerp. Sequencing data of forward and reverse reads (ab.1 files) were analysed in Geneious Prime (version 2023.0.4). Sequences were trimmed (error probability of 0.1), whereafter reading directions were set. Next, reads were *de novo* assembled. The consensus sequence of each assembly was analysed in EZBioCloud (available via https://www.ezbiocloud.net/) for species-level identification. When the assembly of sequences failed, species-level identification was based on the sequence of one read. The top-hit taxon species identity (%) and completeness of the sequence (%) were retrieved from EZBioCloud and stored in the host lab online inventory. If the obtained sequences could not be identified using EZBioCloud, the NCBI nucleotide BLAST tool was used for species-level identification (available via https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.6 Phylogenetic analysis of 16S rRNA gene sequences

If vaginal isolates from the same species were identified for participants of the same pair or group, these were selected for phylogenetic analysis of their 16S rRNA gene sequences. To select 16S rRNA consensus sequences suitable for phylogenetic tree construction in Geneious Prime (version 2023.0.4), a threshold of 1,200 base pairs was set. Trees were constructed, focussing on the most frequently isolated vaginal species (including *L. crispatus, L. gasseri, L. paragasseri, L. jensenii* and *Limosilactobacillus reuteri*). The reference sequence of the 16S rRNA gene of each selected species was retrieved from the NCBI nucleotide database (available via https://www.ncbi.nlm.nih.gov/nuccore). A summary of the used reference strains is shown in Table 7. To establish homologous positions across the sequences of isolates of a certain species, the Clustal Omega algorithm was used for multiple alignment of all consensus sequences, including the reference strain. The resulting alignment was visually checked, unaligned sequences were removed and uneven ends were trimmed to obtain sequences of equal length. The aligned sequences were then used to build a phylogenetic tree, using the Jukes-Cantor evolutionary model and the neighbour joining algorithm.

Table 7: Reference strains and accompanying 16S rRNA gene sequences for all species included in the phylogenetic trees. *Data were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/nuccore).*

Species	Strain	Reference sequence nr.
Lactobacillus crispatus	ATCC 33820	NR_041800.1
Lactobacillus gasseri	ATCC 33323	NR_07051.2
Lactobacillus paragasseri	JCM 5343	NR_179257
Lactobacillus jensenii	ATCC 25258	NR_025087
Limosilactobacillus reuteri	F275	NR_025911.1

2.2.7 Bacterial DNA extraction and whole genome sequencing

A selection of vaginal isolates was made for whole genome sequencing (WGS) performed by the BGI Genomics Sequencing Service (Shenzhen, China). This selection was made with focus on the most frequently isolated

Lactobacillus spp. (i.e., *L. crispatus, L. jensenii, L. gasseri, L. reuteri*), including isolates from the same species for mother and daughter of the same pair. In preparation of WGS, the selected isolates were streaked on a Petridish (20 mL) containing MRS agar using the pentagon method to check for pure cultures. Petridishes were incubated at 37°C with 5% CO_2. for 48 h. After incubation, a single colony from each agar plate was inoculated in 10 mL of MRS broth and incubated overnight at 37°C with 5% CO_2.. After incubation, genomic DNA was extracted according to an optimised in-house pipeline. First, 1.5 mL overnight culture was transferred to a sterile Eppendorf tube, in duplicate, whereafter 1.5 µL Ampicillin (100 mg/mL) was added to inactivate the bacteria. The mixture was incubated for 1 h at 37°C with 5% CO_2. After incubation, the culture was centrifuged at 12,000 rpm for 5 min. Supernatant was removed and the resulting pellet was washed once with 1 mL NaCl-EDTA (30 mM NaCl, 2 mM ethylene diamine tetra-acetic acid). Subsequently, the two cell pellets were pooled and resuspended in NaCl-EDTA, followed by two additional wash steps. After the last wash step, the cell pellet was resuspended in 100 µL NaCl-EDTA. Next, 100 µL lysozyme (10 mg/mL) and 1 µL RNase (20 mg/mL) were added followed by incubation with periodic shaking (284 rpm) for 1 h at 37°C. Subsequently, 229 µL NaCl-EDTA, 50 µL 10% SDS and 20 µL proteinase K (20 mg/mL) were added followed by incubation with periodic shaking (250 rpm) for 1 h at 55°C. After incubation, 200 µL cold protein precipitation solution (containing kalium acetate, glacial acetic acid, distilled water) was added and Eppendorf tubes were put on ice for 5 min. The obtained mixture was centrifuged for 5 min at 3618 rpm and 4°C, whereafter the supernatant was transferred to a clean Eppendorf tube and centrifuged for 3 min at 3,618 rpm and 4°C. The supernatant was transferred to a clean Eppendorf tube and 600 µL ice-cold isopropanol solution was added to precipitate the DNA, after which the mixture was centrifuged for 3 min at 3,618 rpm and 4°C. The supernatant was discarded, whereafter the pellet was washed with fresh 70% ethanol. The supernatant was again carefully removed and after air-drying, the pellet was dissolved in 50 µL molecular grade water. The final DNA concentration was measured using the Qubit 3.0 Fluorometer and standard kit (Invitrogen™ by Life Technologies) according to the manufacturer's instructions.

Samples with a DNA concentration lower than 10 ng/µL were concentrated, prior to sending them for WGS. Samples were centrifuged for 20 min at 13,500 rpm, supernatant was removed and the resulting pellet was dissolved in 100 µL 70% ethanol. The obtained mixture was centrifuged again for 20 min at 13,500 rpm, supernatant was removed and after air-drying, the pellet was dissolved in molecular grade water to reach a final DNA concentration above 10 ng/µL. The final DNA concentration was confirmed using the Qubit 3.0 Fluorometer (Invitrogen™ by Life Technologies). Samples (50 µL or 25 µL depending on the DNA concentration) were then collected in a 96-well plate and shipped on dry ice. Upon arrival, the sequencing facility checked sample integrity and assigned a quality label ranging from A to C. Level A means that the sample meets the requirements of library construction and sequencing, success rate of sequencing is around 93.59%. Level B indicates that the sample does not totally meet the requirements of library construction and sequencing, resulting in a lower success rate (88.34%).Level C means that the sample does not meet the requirements of library construction and sequencing and the success rate is only 70.53%.

2.2.8 Phylogenetic analysis of whole genome sequences

Successfully obtained genome sequences of isolates identified as *L. crispatus* were processed by the host lab bio-informaticians. More specifically, upon arrival, the overall quality of the sequences was assessed using CheckM. Previously available genome sequences of *L. crispatus* strains of the host lab were included in the following phylogenetic analysis. Here, sequences were classified using the GTDB-Tk software toolkit (available via https://github.com/Ecogenomics/GTDBTk). The pangenome of all sequenced isolates was determined using in-house SCARAP software. Subsequently, all genes with a prevalence of at least 90% were selected. The nucleotide sequences of these genes were aligned and concatenated into a supermatrix. Finally, a tree was inferred on the supermatrix, using the General Time Reversable evolutionary model. The tree was midpoint rooted and visualised with iTol. Additionally, the SNP counts between the genomes of isolates within the same clade were calculated. The whole-genome alignment tool MUMmer was used for these calculations.

3 Results

The data and results obtained in this Master's thesis are presented in this section. In the first part, the dynamics of the VM throughout the three trimesters of pregnancy were investigated for control groups 1 and 2 of the ProPreB trial, followed by analyses of clinical data to unravel possible associations with PTB. In the second part, vertical transmission and persistence of vaginal bacteria were investigated. Hereto, the VM composition and metadata of a subset of Isala mothers and daughters were assessed first. Subsequently, different culturing conditions were screened to tailor the bacterial isolation towards vaginal lactobacilli. The obtained bacterial isolates were identified using 16S rRNA gene sequencing. After identification, phylogenetic analyses were performed based on the 16S rRNA gene sequences and whole genome sequences.

3.1 Examination of vaginal microbiome dynamics and clinical data throughout pregnancy

3.1.1 Global assessment and division of the ProPreB cohort

In total, clinical data of 497 participants were available (Figure 4). For the analyses in this Master's thesis, data from control group 1 (N = 405) and control group 2 (N = 8) were analysed. Within control group 1 (Figure 4, left part), the delivery date of 326 women was recorded, including 302 at term and 24 preterm deliveries. Within control group 2 (Figure 4, middle part), the delivery date of three out of eight women was recorded, including two at term and one preterm delivery. The actual ProPreB study population (Figure 4, right part) includes 84 women up to date. Among them, the delivery date of 60 women was recorded. In addition, microbiome data throughout the three trimesters of pregnancy were available for 92 women of control group 1 and fifteen women of the actual study population. The PTB rate in the population under investigation (i.e., control group 1 and control group 2) was 7.6%. Within the group of participants of whom microbiome data was used (N = 92), five women delivered preterm, resulting in a PTB rate of 5.4%.

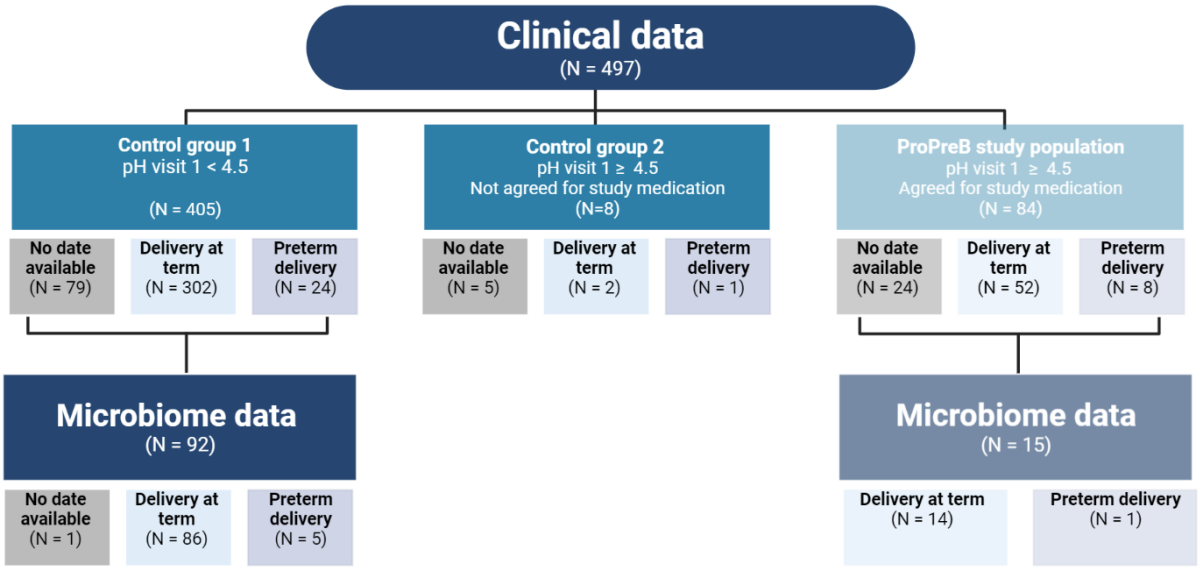

Figure 4: Numbers of ProPreB participants with available clinical and/or microbiome data. *Participants were divided into three groups (i.e., control group 1, control group 2 and the actual study population) based on vaginal pH values and recorded agreement levels at visit 1. Further group divisions were made based on delivery date (including delivery date not available, delivery at term, preterm delivery). In this Master's thesis, data from control group 1 and control group 2 were analysed. Figure made in biorender.com.*

3.1.2 Dynamics of the vaginal microbiome during pregnancy

The VM composition of the ProPreB participants of control group 1 (N = 92) was studied. Microbiome data of the ProPreB study population was not used since an unknown part of this group received probiotic treatment, which is expected to modulate their VM composition. Samples were collected at three time points (each trimester) during pregnancy, which resulted in a total of 276 vaginal swabs. Based on 16S rRNA amplicon sequencing, relative bacterial abundances were determined at genus level during pregnancy (Figure 5A).

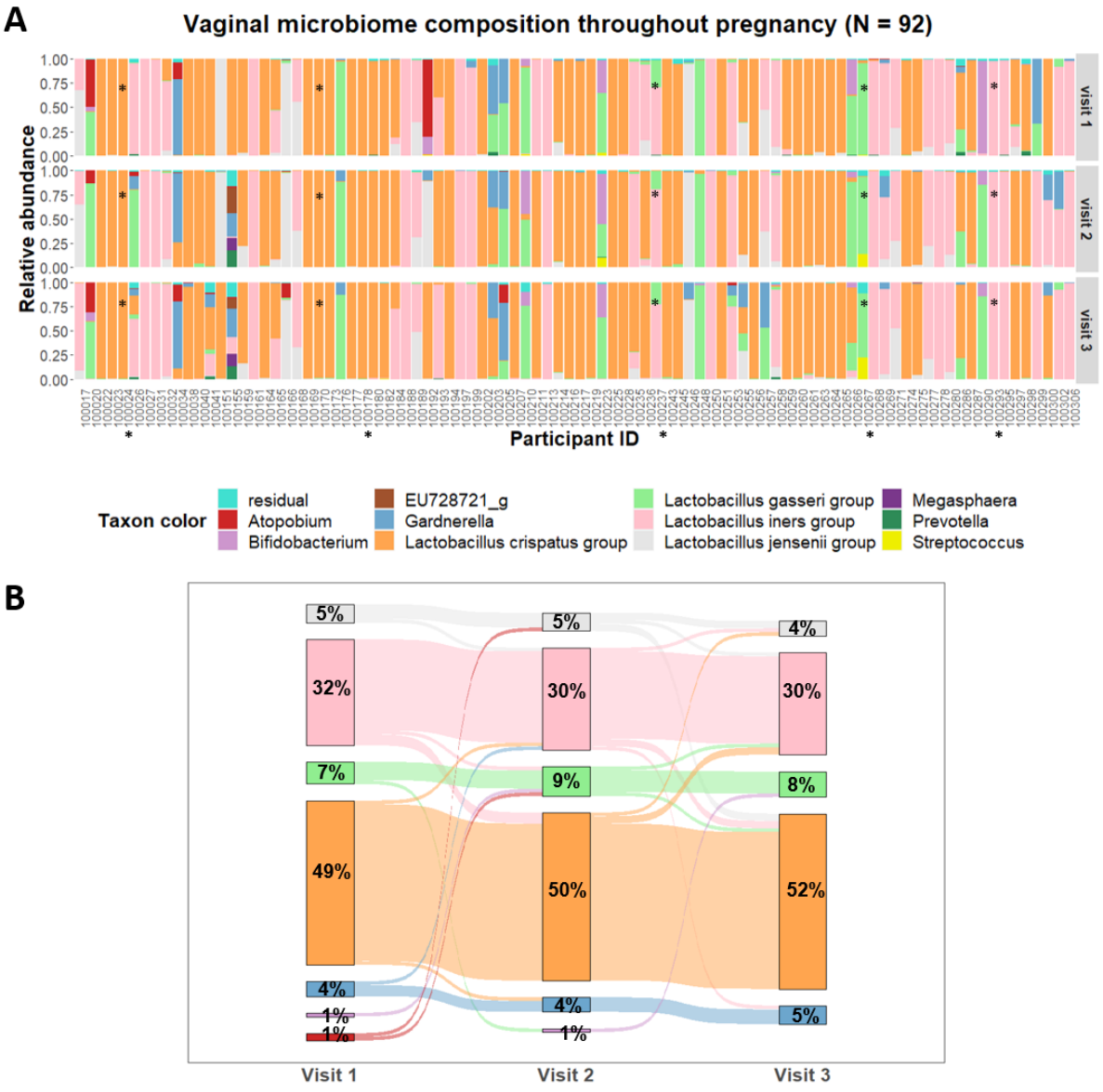

Figure 5: The dynamics of the VM throughout pregnancy. *(A) Relative bacterial abundances in vaginal swabs from ProPreB participants in control group 1 (N = 92), based on 16S rRNA amplicon sequencing. Vaginal swabs were sampled at three different timepoints during pregnancy (i.e., visit 1 (6-13 weeks of GA), visit 2 (18-23 weeks of GA) and visit 3 (35-37 weeks of GA)). Participants that delivered preterm (N = 5) are marked with an asterisk (*). (B) Dynamics of the vaginal microbiome during pregnancy of the ProPreB participants in control group 1 (N = 92). Sankey diagram visualising the dynamics of the VM throughout pregnancy (including three visits), based on the most abundant taxon per participant. The percentages represent the proportion of participants that have a VM dominated by a certain taxon. Legend of panel A is applicable to both panel A and panel B.*

At visit 1 (6-13 weeks of GA), most women had a VM dominated by the *L. crispatus*-group (49%), followed by the *L. iners*-group (32%). Towards visit 2 (18-23 weeks of GA) and visit 3 (35-37 weeks of GA), the dominant taxa were shifted in 17 women. In general, the number of participants with a VM dominated by species of the *L. crispatus*-group increased towards 50% at visit 2 and 52% at visit 3 (Figure 5B). This results in 50% of all collected vaginal swabs being dominated by species of the *L. crispatus*-group. In addition, a VM dominated by the *L. crispatus*-group had the highest stability, as 91% of the women with a VM dominated by the *L. crispatus*-group at visit 1 preserved this dominance at all visits. Among the women who were dominated by the *L. iners-*, *L. gasseri-* and *L. jensenii*-groups at visit 1, respectively 75%, 67% and 40% of them had a VM that was still dominated by the same species group at the following visits. This means that 75% of the included participants had a VM dominated by the same *Lactobacillus* spp.-group at all visits. In general, 89% (82/92) of the participants had a VM dominated by lactobacilli at all visits.

When assessing the total number of identified taxa at each visit as proxy for the overall diversity throughout pregnancy, a decrease was observed from visit 1 to visit 3. More specifically, at visit 1, visit 2 and 3, a total of 141, 86 and 51 genera were identified, respectively. The most frequently observed taxa among all vaginal swabs included (in descending order) species from the *L. crispatus*-group, *L. iners*-group, *L. gasseri*-group, *L. jensenii*-group, *Gardnerella, Prevotella, Peptoniphilus, Anaerococcus, Finegoldia* and *Staphylococcus.* In each collected vaginal swab, vaginal lactobacilli were present. Species from the *L. crispatus*-group were most frequently observed, as they were identified in 93% of samples (258/276) (Table 8).

Table 8: Overview of the ten most frequently detected bacterial taxa in vaginal swabs of control group 1 (N = 92), based on 16S rRNA amplicon sequencing. *The number of vaginal swabs in which these taxa were identified as well as their average relative abundance per visit are shown. In addition, the total number of vaginal swabs (among all visits) in which these taxa were identified as well as their average relative abundance (calculated for this subset of swabs) is shown.*

	Visit 1	**Visit 2**	**Visit 3**	**All visits**
Bacterial Taxa	**Number of swabs (average relative abundance)**	**Number of swabs (average relative abundance)**	**Number of swabs (average relative abundance)**	**Number of swabs (average relative abundance)**
L. crispatus-group	86 (39.6%)	84 (42.2%)	88 (36.5%)	258 (39.4%)
L. iners-group	70 (32.8%)	70 (31.5%)	72 (28.0%)	212 (30.8%)
L. gasseri-group	51 (8.2%)	51 (9.9%)	54 (9.0%)	156 (9.1%)
L. jensenii-group	37 (17.1%)	36 (18.6%)	40 (10.4%)	113 (15.2%)
Gardnerella	32 (6.8%)	27 (8.5%)	32 (8.3%)	91 (7.8%)
Prevotella	37 (0.2%)	19 (0.5%)	19 (0.6%)	75 (0.4%)
Peptoniphilus	31 (0.1%)	21 (0.06%)	11 (0.06%)	63 (0.08%)
Anaerococcus	33 (0.01%)	17 (0.1%)	7 (0.2%)	56 (0.1%)
Finegoldia	33 (0.1%)	15 (0.09%)	4 (0.08%)	52 (0.1%)
Staphylococcus	24 (0.1%)	19 (0.1%)	7 (0.05)	50 (0.1%)

As a measure of bacterial diversity within the VM samples of women in control group 1, alpha diversity was explored by calculating the inverse Simpson's diversity index (D-value) (Figure 7A). Average D-values were not significantly different throughout pregnancy, with values ranging from 1.40, 1.47 and 1.56 at visit 1, 2 and 3, respectively. In addition, per visit, average D-values were calculated for women who delivered at term (N = 86) and preterm (N = 5). Among the women who delivered at term, the average D-values were 1.40, 1.47 and 1.54 at visit 1, 2 and 3, respectively. For the women who delivered preterm, average D values were 1.48, 1.61 and 1.97 for each visit respectively. Here, no significant difference in D-values was observed between women who delivered at term and preterm (Figure 7B). Appendix A Table 1 and 2 provide an overview of the average D-values and p-values, respectively.

3.1.3 Vaginal CSTs throughout pregnancy

The dynamics of the VM throughout pregnancy were also assessed based on the CSTs and sub-CSTs assigned to the vaginal samples according to the VALENCIA algorithm (cfr. Table 1 in section 1.2.2) (60). Over all vaginal swabs (among all visits) , seven CSTs were identified (Figure 6A). Most vaginal swabs (98%) were assigned to a CST that is dominated by lactobacilli (i.e., CST I, II, III and V). Throughout all visits, 52% of vaginal swabs were assigned to CST I (*L. crispatus* dominated), followed by 30% assigned to CST III (*L. iners* dominated), 10% assigned to CST II (*L. gasseri* dominated) and 5.8% to CST V (*L. jensenii* dominated). On the other hand CSTs characterized by a higher abundance of anaerobes, CST IV-A, IV-B and IV-C were assigned to only 1.8% of all vaginal swabs. In addition, nine out of a total of thirteen possible sub-CSTs were identified (Figure 6B), including CST I-A , CST I-B, CST II, CST III-A, CST III-B, CST IV-A, CST IV-B, CST IV-C3, and CST V. At sub-CST level, most vaginal swabs were assigned to CST I-A (46%), followed by CST III-A (28%), at all three visits.

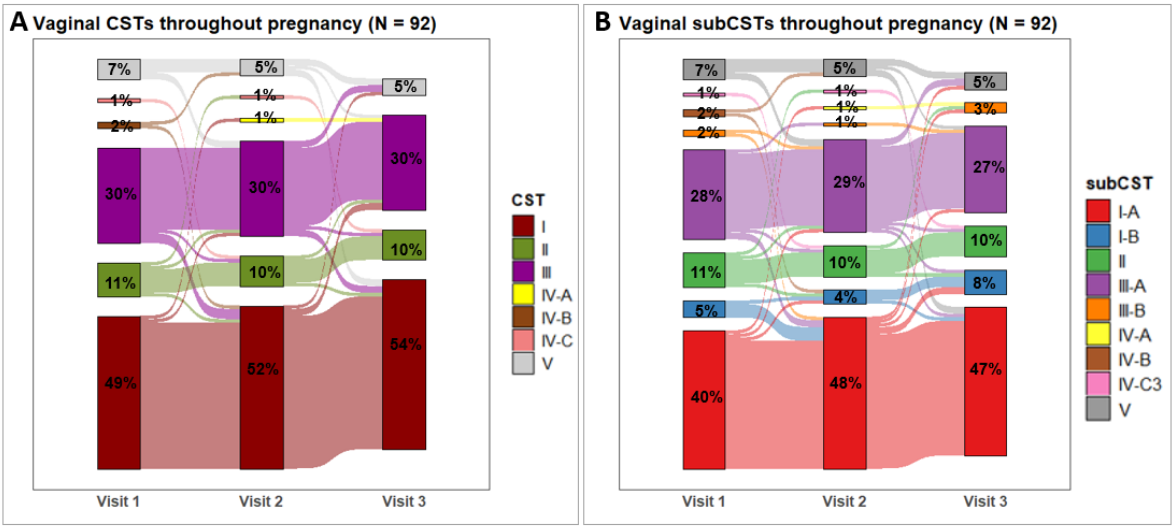

Figure 6: Dynamics of the vaginal microbiome during pregnancy of the ProPreB participants in control group 1 (N = 92) based on the assigned CSTs (A) or sub-CSTs (B). *Sankey diagram visualising the dynamics of the VM throughout pregnancy (including three visits), based on the CSTs (A) or sub-CSTs (B) according to the VALENCIA algorithm (60) The percentages reflect the proportions of participants that have a VM assigned to this particular (sub-)CST. Abbreviations: CST = community state type.*

3.1.4 Vaginal pH throughout pregnancy

To investigate variations in vaginal pH throughout pregnancy and to look for differences between women who delivered at term and preterm, pH measurements were retrieved from the ProPreB participants in the control groups (Figure 7C). These data included pH measurements from 413, 310 and 247 women at visit 1, 2 and 3, respectively. The average pH values measured at visit 1, 2 and 3 were 3.97, 4.03 and 4.10, respectively. pH values significantly differed between visit 1 and visit 2 ($p = 0.0035$), and visit 1 and visit 3 ($p = 6.50E-08$) (Figure 7C). Among the women who delivered at term, the average pH values were 3.97, 4.03 and 4.10 at visit 1, 2 and 3, respectively. For the women who delivered preterm, average pH values were 3.98, 4.05 and 4.04 at each visit, respectively. No significant differences were found when comparing the vaginal pH of women who delivered at term and preterm (Figure 7D). An overview of the average pH values and p-values is shown in Appendix A Table 3 and Table 4, respectively.

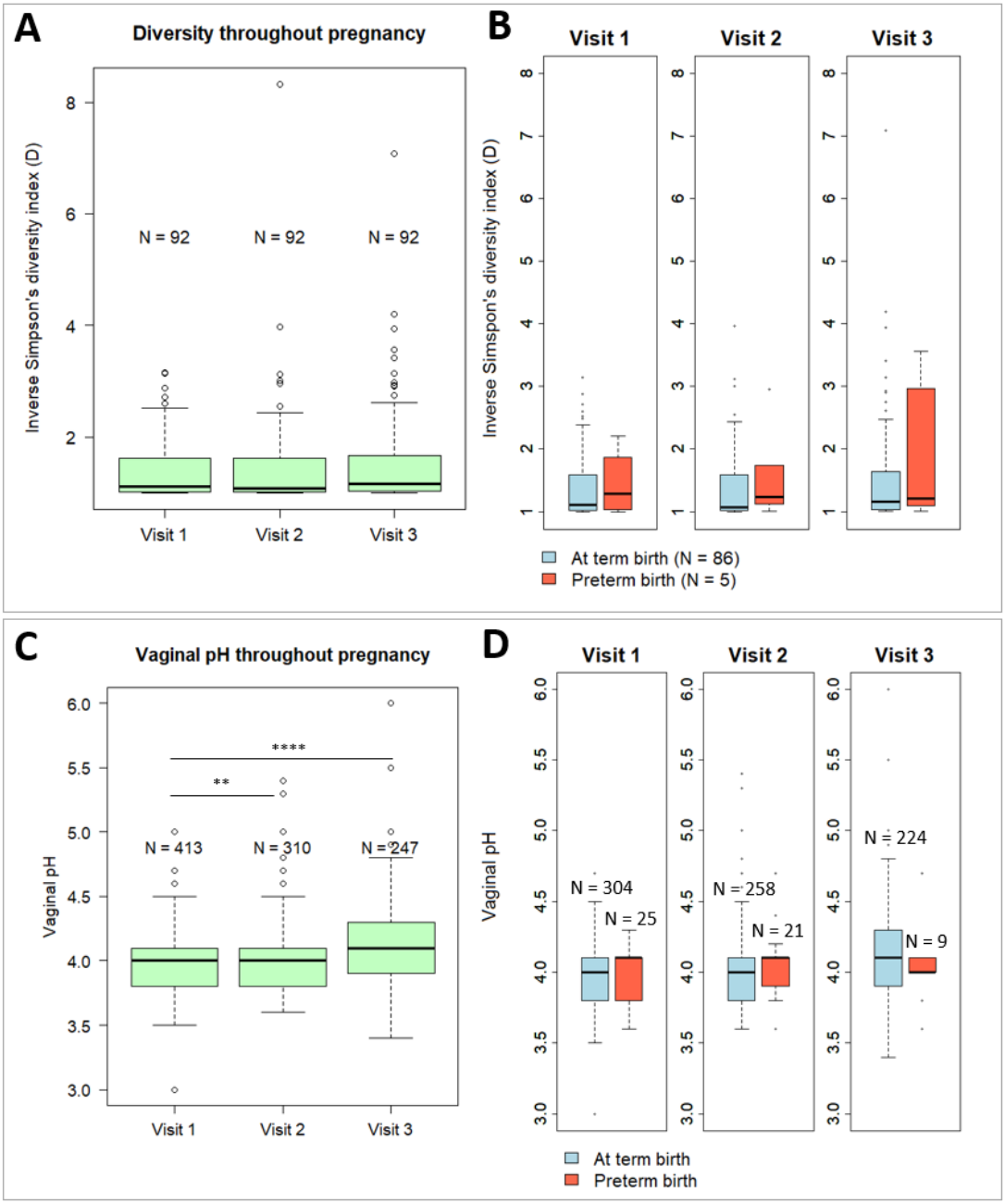

Figure 7: Diversity (A-B) and vaginal pH (C-D) throughout pregnancy. *(A) Inverse Simpson's diversity index (D-values) values at each visit for women in control group 1 (N = 92). Wilcoxon signed-rank test with Bonferroni correction was used to compare all visits. No significant difference in alpha diversity was observed between the different visits. (B) D-values at each visit for women in control group 1 who delivered at term (N = 86) and preterm (N = 5). Wilcoxon rank-sum test was used to compare the groups with at term and preterm deliveries. No significant difference was observed in D-values when comparing women who delivered at term and preterm. (C) Vaginal pH values at each visit for women in control groups 1 and 2. Wilcoxon signed-rank test with Bonferroni correction was used to compare all visits. The vaginal pH was significantly different between visit 1 and visit 2 (p = 0.0035) and between visit 1 and visit 3 (p = 6.50E-08). (D) Vaginal pH values at each visit for women who delivered at term and preterm. Wilcoxon rank-sum test was used to compare the groups with at term and preterm deliveries. No significant difference in vaginal pH values was observed when comparing women who delivered at term and preterm.*

3.1.5 Exploring the associations of several factors with gestational age and preterm birth

In order to explore the potential impact of clinical factors on gestational length, the correlation between GA at delivery and a series of continuous variables was explored. These continuous variables included, the vaginal pH at each visit, cervical length in mm (measured at visit 2), maternal BMI and maternal age (both registered at visit 1). For every parameter, a scatter plot was built and the Spearman correlation coefficients (r_s) were determined (Figure 8 A-F). When examining the r_s -values describing the correlation between GA at delivery and vaginal pH at each visit (Figure 8 A-C), maternal BMI (Figure 8D) and maternal age (Figure 8E), values close to 0 were obtained, suggesting a weak or negligible correlation between GA at delivery and these variables. However, the significant r_s -value describing the correlation between GA at delivery and cervical length indicates a weak positive correlation ($r_s = 0.1315$, $p = 0.0303$) (Figure 8F).

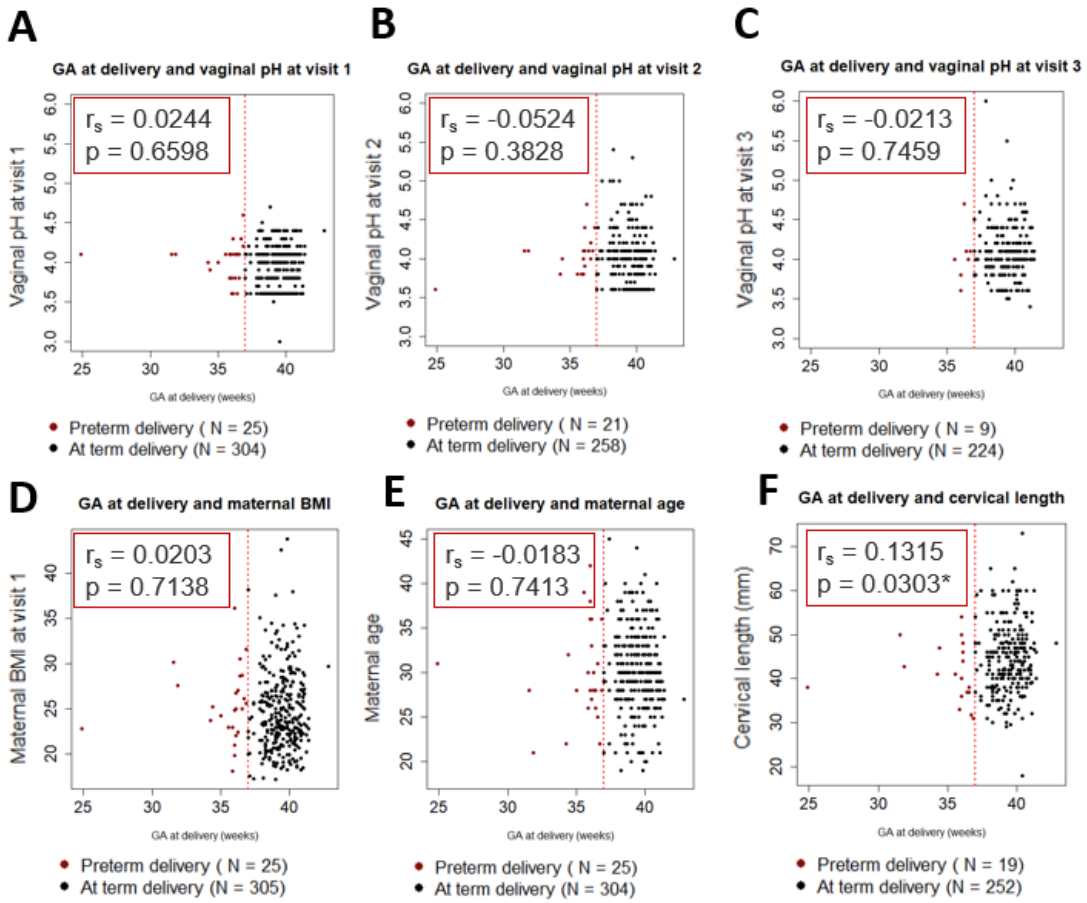

Figure 8: The relationship between GA at delivery and several continuous variables. *Studied variables are the following: vaginal pH at each visit (A-C), maternal BMI at visit 1 (D), maternal age at visit 1 (E) and cervical length measured at visit 2 (F). Spearman correlation coefficients (r_s) and corresponding p-values are shown on the respective graphs. Abbreviations: BMI = body mass index, GA = gestational age.*

Besides this correlation analysis with continuous variables, the association between the occurrence of PTB and a series of categorical variables was examined, including GBS status, 'last time smoked' and the presence of BV at each visit (Figure 9). No significant association was observed between PTB and 'last time smoked' ($p = 1$) (Figure 9A) neither between PTB and the presence of BV at any visit ($p = 1$ for all visits) (Figure 9C-E). However, upon examining the relationship between PTB and GBS status (Figure 9B), a p-value of 0.0060 was obtained, indicating

a significant association between GBS status and PTB. In addition, an odd ratio of 6.8897 was calculated, with a 95% confidence interval (CI) ranging from 1.5024 to 31.6536.

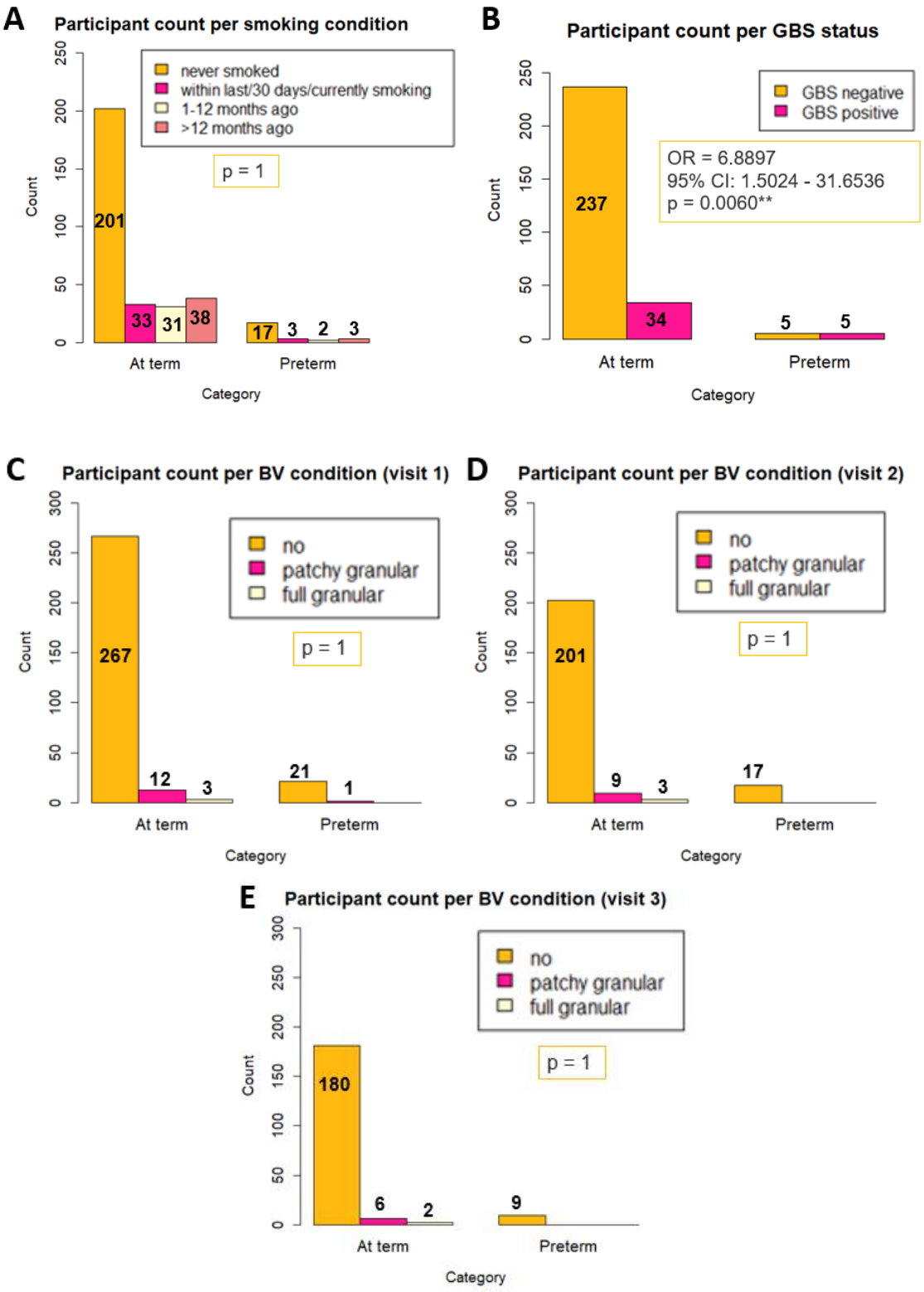

Figure 9: Overview of smoking condition (A), GBS status (B) and BV condition per visit (C-E) along with their participant counts. *These variables were assessed for their association with PTB. The Fisher's exact test was used to examine the significance of association between the aforementioned variables and the occurrence of PTB. The corresponding p-value, odds ratio and 95% confidence interval (if applicable) are shown on the respective graphs. Abbreviations: BV = bacterial vaginosis, CI = confidence interval, GBS = Group B Streptococcus, OR = odds ratio.*

3.1.6 Exploring associations between the vaginal microbiome composition and GBS status

Among participants with available microbiome data and known GBS status in control group 1 (N = 83), 15 women tested positive for GBS at visit 3, resulting in a GBS positive rate of 18% in this group. The VM composition of the GBS-positive women (N = 15) was further investigated (Figure 10A). Among them, two women delivered preterm (13%, indicated by *). At visit 1, 47% of GBS-positive women had a VM assigned to sub-CST III-A (*L. iners* dominated), followed by 33% of women with a VM assigned to sub-CST I-A (*L. crispatus* dominated). At visit two, more GBS positive women had a VM dominated by species of the *L. iners*-group, reflected by 53% of the vaginal swabs being assigned to CST III-A and 7% to CST III-B. The remaining women had a VM dominated assigned to CST I-A (27%) and CST I-B (13%). At visit 3, no changes were observed regarding the most dominant taxon in the vaginal samples compared to visit 2. Overall, none of the vaginal samples was assigned to CST IV at any of the visits (Figure 10B). When determining the prevalence of *Streptococcus* during pregnancy in women with a positive GBS status, *Streptococcus* was only detected in the vaginal swab of one women at visit 3.

Possible associations between GBS status and vaginal sub-CST classification were examined at each visit. The number of GBS-positive and -negative participants per sub-CST per visit is visualised in Figure 11A-C. No significant association was observed between the GBS status and the sub-CST classification at visit 1 ($p = 0.8199$) and visit 3 ($p = 0.0597$). Upon examining the relationship between GBS status and the vaginal sub-CST classification at visit 2, a p-value of 0.0395 was obtained, indicating a significant association between GBS status and the vaginal sub-CST at this visit.

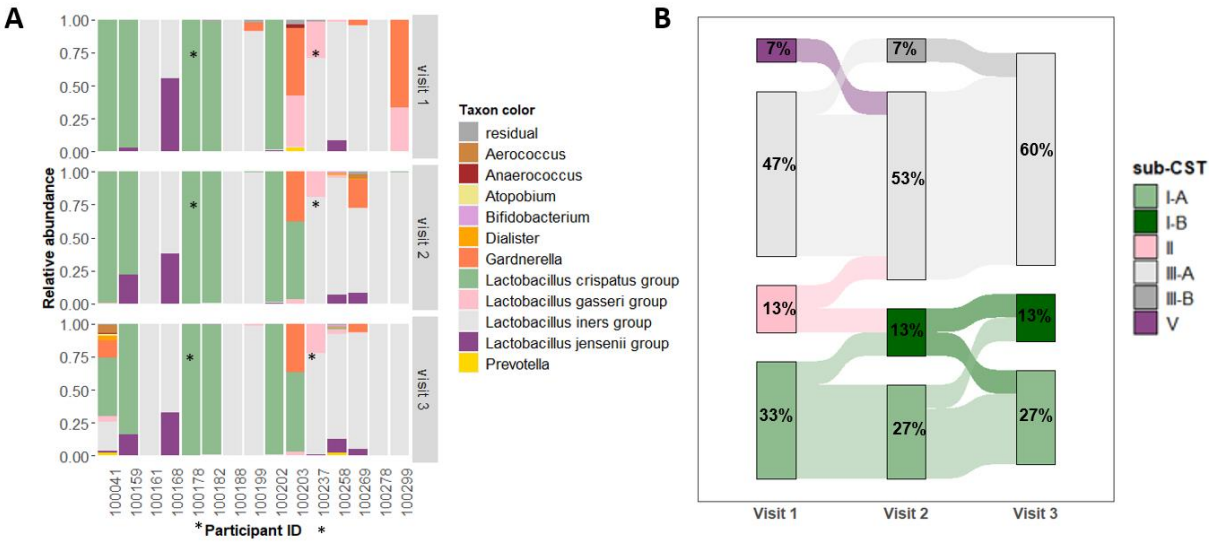

Figure 10: The dynamics of the VM throughout pregnancy in GBS positive women. *(A) Relative bacterial abundances based on 16S rRNA amplicon sequencing of vaginal swabs from ProPreB participants in control group 1, who tested positive for GBS (N = 15). VM composition was assessed at three different timepoints during pregnancy (i.e., visit 1 (6-13 weeks of GA), visit 2 (18-23 weeks of GA) and visit 3 (35-37 weeks of GA)). Participants that delivered preterm (N = 2) are marked with an asterisk (*). (B) Dynamics of the vaginal microbiome during pregnancy of the ProPreB participants in control group 1, who tested positive for GBS (N = 15). Sankey diagram visualising the dynamics of the VM throughout pregnancy (including three visits), based on the sub-CSTs according to the VALENCIA algorithm (60). Percentages reflect the proportion of participants with a VM assigned to a particular sub-CST. Abbreviations: CST = community state type, GBS = Group B Streptococcus.*

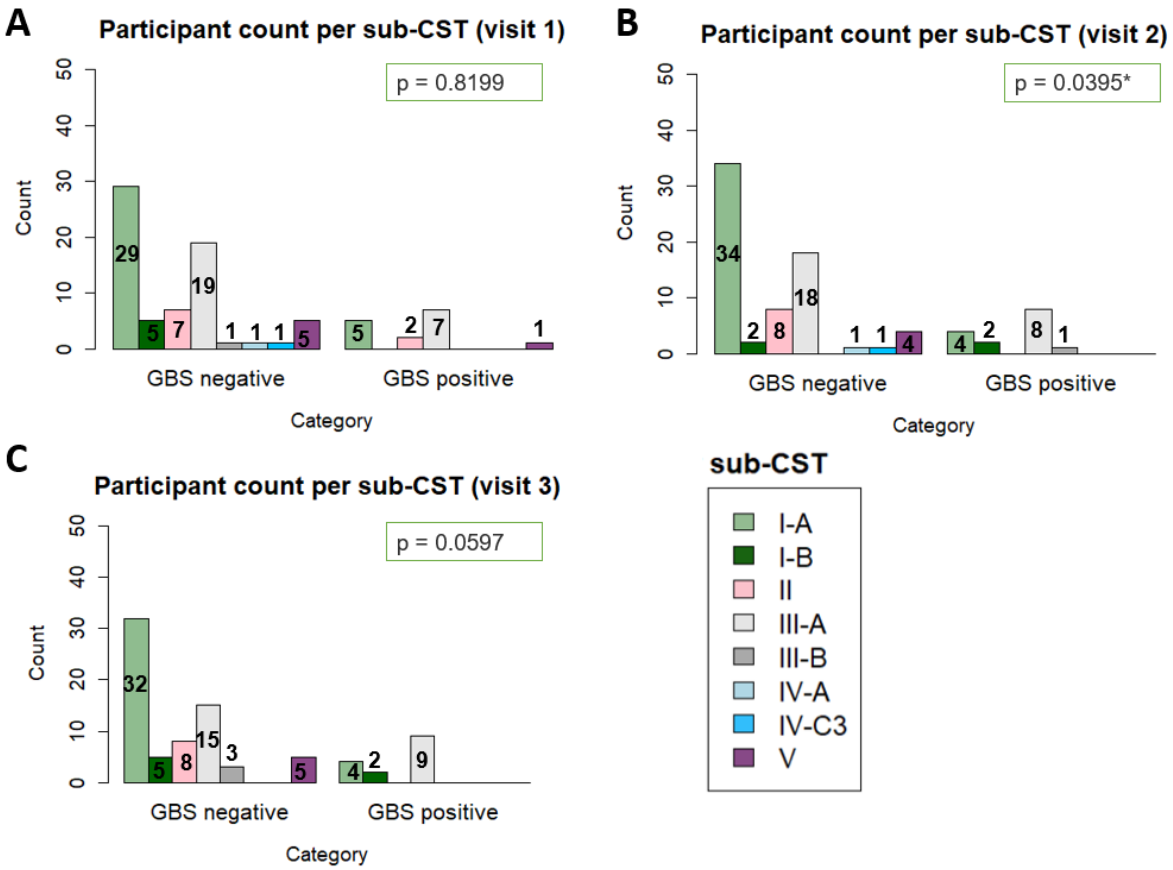

Figure 11: Overview of sub-CST classification along with their participant counts per GBS status, at visit 1 (A), visit 2 (B) and visit 3 (C). *Sub-CSTs were tested for their association with GBS status. This figure includes women from control group 1 who tested positive (N = 15) or negative for GBS (N = 68). The Fisher's exact test was used to examine the significance of association. Corresponding p-values are shown on the graphs. Abbreviations: CST = community state type, GBS = Group B Streptococcus.*

3.2 Examination of vertical transmission and persistence of the vaginal microbiome

3.2.1 Global assessment of the vaginal microbiome composition of the selected Isala mothers and daughters

To analyse vertical transmission of the VM, a selection of seven mother-daughter pairs (P1-P7) and two groups (G8-G9) was made among the Isala participants. The first group (G8) consisted of a grandmother (abbreviated as GM), her daughter (M) and granddaughter (D). The second group (G9) consisted of a mother (M) with four daughters (D1-D4). The taxonomic composition of the VM of these selected Isala mothers and daughters was previously determined based on 16S rRNA amplicon sequencing. The relative bacterial abundances detected within their vaginal swabs are shown in Figure 12. In addition, metadata of the selected participants (N = 22), including their age, age difference with mother, birth mode, pregnancy history, menopausal status and cohabitation status, are summarized in Table 9. The average age of the mothers and daughters was 55.8 years and 27.3 years, respectively. All of the selected daughters, except two, were born vaginally.

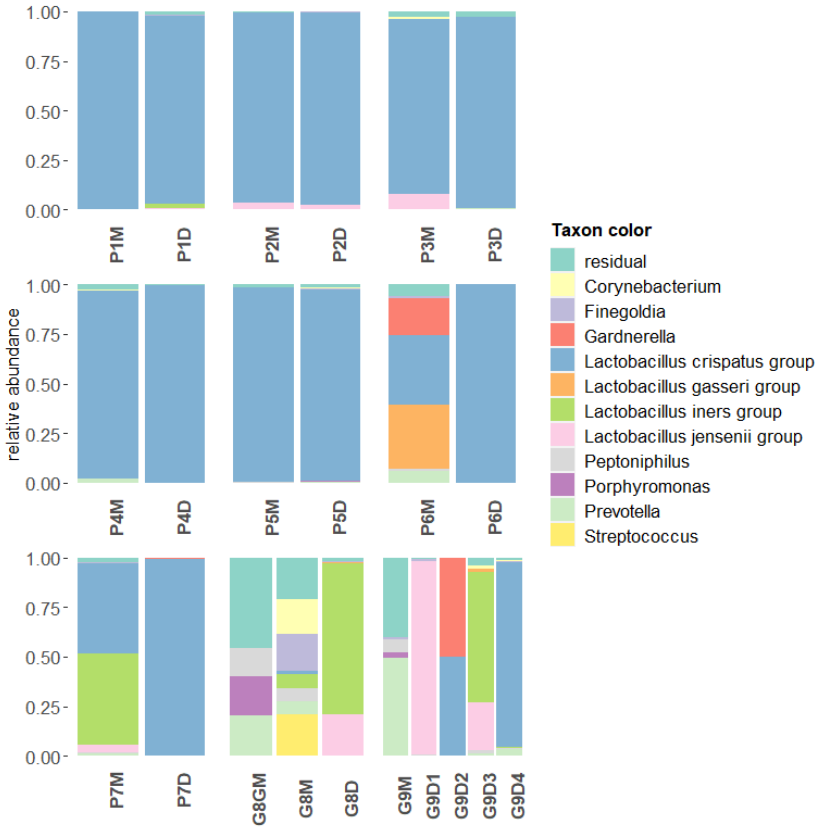

Figure 12: Relative bacterial abundances based on 16S rRNA amplicon sequencing of vaginal swabs from the selected Isala mothers and daughters (N = 22). *All participants were assigned a specific code, starting with the corresponding pair (P1-P7) or group number (G8-G9), followed by the type of relationship (M: mother, D: daughter, GM: grandmother). Abbreviations: P = pair, G = Group, GM = grandmother, M = mother, D = daughter.*

Table 9: Overview of a subset of questionnaire data from the selected Isala mothers and daughters (N = 22). *A total of seven mother-daughter pairs (P1-P7) and two groups (G8-G9) were selected. Abbreviations: C-section = Caesarean section, IVF = in vitro fertilization, PTB = preterm birth, NA: not applicable, P: pair, G: group, GM: grandmother, M: mother, D: daughter.*

Participant code	Age	Age difference with mother	Birth mode	Pregnancy history	Menopause (yes/no)	Cohabitation with mother (yes/no)
P1M	47	24	Vaginal birth	5	No	NA
P1D	23		Vaginal birth	0	No	No
P2M	48	30	Vaginal birth	3 (1 miscarriage)	No	NA
P2D	18		Vaginal birth	0	No	Yes
P3M	57	25	Vaginal birth	4 (2 miscarriages)	Yes	NA
P3D	32		Vaginal birth	1 (IVF trajectory)	No	No
P4M	68	37	Vaginal birth	4 (1 miscarriage, 1 PTB)	Yes	NA
P4D	31		Vaginal birth	0	No	No
P5M	54	32	Vaginal birth	1 (PTB)	No	NA
P5D	22		Vaginal birth	0	No	Yes
P6M	51	29	Vaginal birth	4	Yes	NA
P6D	22		Vaginal birth	0	No	Yes
P7M	49	29	Vaginal birth	2	No	NA
P7D	20		C-section	0	No	Yes

G8GM	96	NA	Vaginal birth	5	Yes	NA
G8M	69	27	Vaginal birth	3	Yes	No
G8D	44	25	C-section	2 (1 abortion)	No	No
G9M	59	NA	C-section	10 (4 miscarriages)	Yes	NA
G9D1	24	35	Vaginal birth	0	No	Yes
G9D2	34	25	Vaginal birth	3 (1 miscarriage)	No	No
G9D3	27	32	Vaginal birth	0	No	Yes
G9D4	31	28	Vaginal birth	0	No	Yes

3.2.2 Optimisation of culturing conditions for vaginal lactobacilli

For the vaginal ESwabs of the selected participants (N = 22), a culturing campaign was set up. Therefore, different laboratory growth media and growth conditions were tested to tailor the bacterial isolation towards vaginal lactobacilli. When directly streaking the diluted vaginal ESwabs of P4M and P4D on CB agar, growth was observed after 24 h on plates with dilution 10^{-2} and dilution 10^{-2} to 10^{-5} from mother and daughter, respectively. A total of 36 colonies were selected from countable plates (10^{-2} for P4M and 10^{-5} for P4D), including twelve from P4M and 24 from P4D. PCR was successful for all selected colonies and Sanger sequencing for 32 colonies. From these colonies, 92% were identified as *Enterococcus faecalis,* 4% as *Streptococcus agalactiae*, and 4% as *Enterococcus dispar* (Figure 14A). In addition, when streaking the vaginal ESwabs on MRS + Cys agar, small colonies were observed on the plates with dilution 10^{-5} after 48 h. A total of sixteen colonies was selected, including six colonies from P4M and ten colonies from P4D. PCR and Sanger sequencing were only successful for the colonies from P4M. Three colonies were identified as *E. faecalis* and three as *S. agalactiae* (Figure 14A). Overall, no vaginal lactobacilli were identified when using this direct streaking technique on CB agar and MRS + Cys agar for P4.

In a next step, adapted culturing conditions were used to tailor the isolation towards vaginal lactobacilli. More specifically, the selective potential of a subculture phase was explored. First, the growth of vaginal isolates retrieved from the host lab biobank was evaluated in MRS pH 4.6 + Cys broth (both in anaerobic and micro-aerophilic conditions), including some of the identified isolates above. The following bacterial strains were selected: *E. faecalis* AMBV2265, *S. agalactiae* AMBV2307, *S. dysgalactiae* AMBV2309, *Shigella flexneri* AMBV2269, *Klebsiella huaxiensis* AMBV2301 and *L. crispatus* AMBV06, AMBV252, AMBV815, and AMBV961. After 24 h, bacterial growth was observed for all four *L. crispatus* strains, both in anaerobic and microaerophilic conditions. In contrast, none of the non-*Lactobacillus* strains did grow, nor after 72 h (Figure 13).

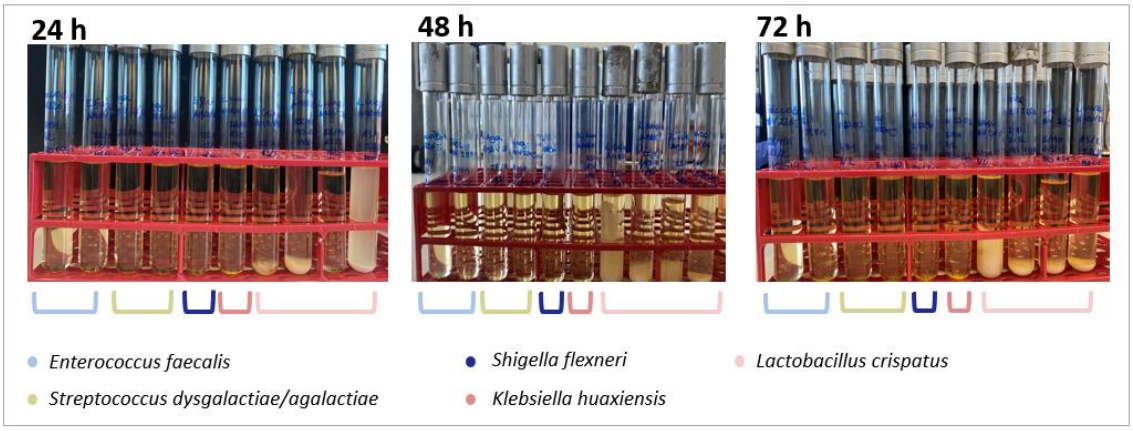

Figure 13: Growth evaluation in MRS pH 4.6 + Cys broth of common vaginal isolates. *Figure shows evaluation of micro-aerophilic growth of ten vaginal isolates. Growth was evaluated after 24, 48 and 72 h, respectively.*

Subsequently, the enrichment of this subculture phase towards vaginal lactobacilli was evaluated on different types of agar (including CB agar, MRS pH 4.6 agar, and MRS agar). Therefore, vaginal ESwabs from P1M and P1D were first inoculated in MRS pH 4.6 + Cys broth under micro-aerophilic conditions. After 48 h, the appropriate dilution factors to obtain countable plates were set to 10^{-4}, 10^{-5} and 10^{-6} based on OD measurements. After 48 h, eight, three and sixteen colonies were selected from CB agar, MRS agar and MRS pH 4.6 agar, respectively. After PCR and Sanger sequencing, five, fifteen and one colonies from CB agar, MRS agar and MRS pH 4.6 agar, respectively, were successfully identified (Figure 14B). For the colonies obtained from CB agar, 20% was identified as *Staphylococcus aureus,* 20% as *Staphylococcus coagulans,* 20% as *Staphylococcus pasteuri,* and 40% as *Staphylococcus epidermidis.* From MRS agar *L. crispatus* (36%), *L. jensenii* (58%) and *L. pasteuri* (6%) were isolated, whereas the only isolate from MRS 4.6 agar was identified as *L. jensenii* (Figure 14B).

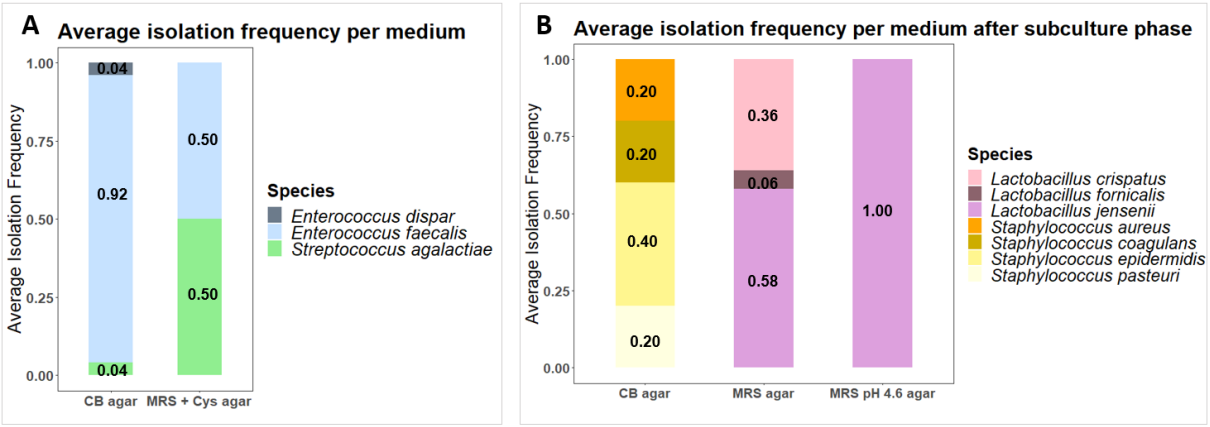

Figure 14: Isolation frequencies per medium without (A) and with (B) a subculture phase. *(A) The average isolation frequency after streaking vaginal ESwabs of P4M and P4D on CB agar and MRS + Cys agar. Average isolation frequencies of different species are calculated for a total of 32 isolates on CB agar and five isolates on MRS + Cys agar. (B) The average isolation frequency per medium after inoculating vaginal ESwabs of P1M and P1D in MRS pH 4.6 + Cys broth. Average isolation frequencies of different species are calculated for five isolates on CB agar, fifteen isolates on MRS agar and one isolate on MRS pH 4.6 agar. Abbreviations: CB = Columbia blood, Cys = cysteine, MRS = De Man – Rogosa – Sharpe, P = pair, M = mother, D = daughter.*

3.2.3 Identification of bacterial isolates from the Isala mothers and daughters

Based on the optimisation steps in the previous section, an optimised experimental pipeline (Figure 15) was established and implemented for all vaginal ESwabs from the selected participants (N = 22). Of note, since no growth was observed when applying this pipeline for the vaginal ESwab of P4M, all incubation steps were performed anaerobically. Table 10 provides an overview of all isolated vaginal lactobacilli per participant per pair or group, including the taxonomic group they belong to. A total of 258 vaginal lactobacilli were isolated and identified by Sanger sequencing of their 16S rRNA gene. Appendix B Table 1 gives an overview of the identified isolates (top-hit), their 'AMBV' strain designation (used by the host lab (LAMB) for bacteria isolated from vaginal (V) ESwabs), their sequence identity and completeness. Isolates were obtained from all participants except P9M, since the vaginal ESwab of this participant showed no growth in the subculture phase (not anaerobically, nor micro-aerophilic). In total 22 different bacterial taxa were isolated. The most frequently isolated species was *L. crispatus* (N = 74), followed by *L. jensenii* (N = 64), *L. gasseri* (N = 32), *L. paragasseri* (N = 29) and *Limosilactobacillus reuteri* (N = 22). Figure 16 gives an overview of the proportions of species that were isolated from each type of agar (i.e., MRS agar or MRS pH 4.6 agar).

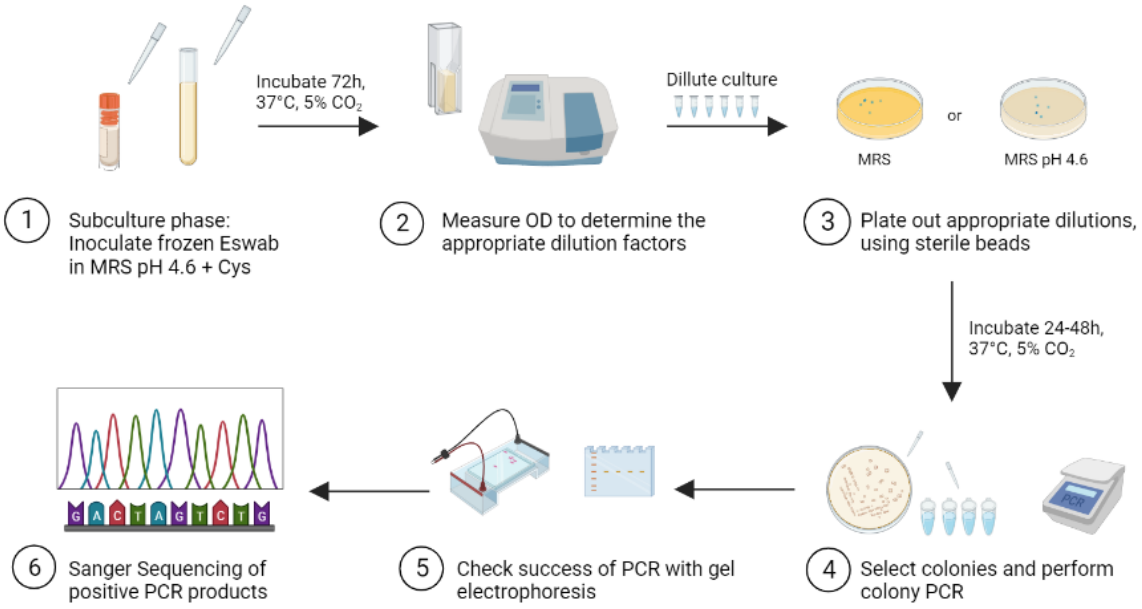

Figure 15: Optimised experimental pipeline. *Figure gives an overview of the optimised experimental pipeline for isolation and identification of vaginal lactobacilli from ESwabs of Isala mothers and daughters. Figure made in biorender.com. Abbreviations: Cys = cysteine, MRS = De Man – Rogosa – Sharpe, OD = optical density, PCR = polymerase chain reaction.*

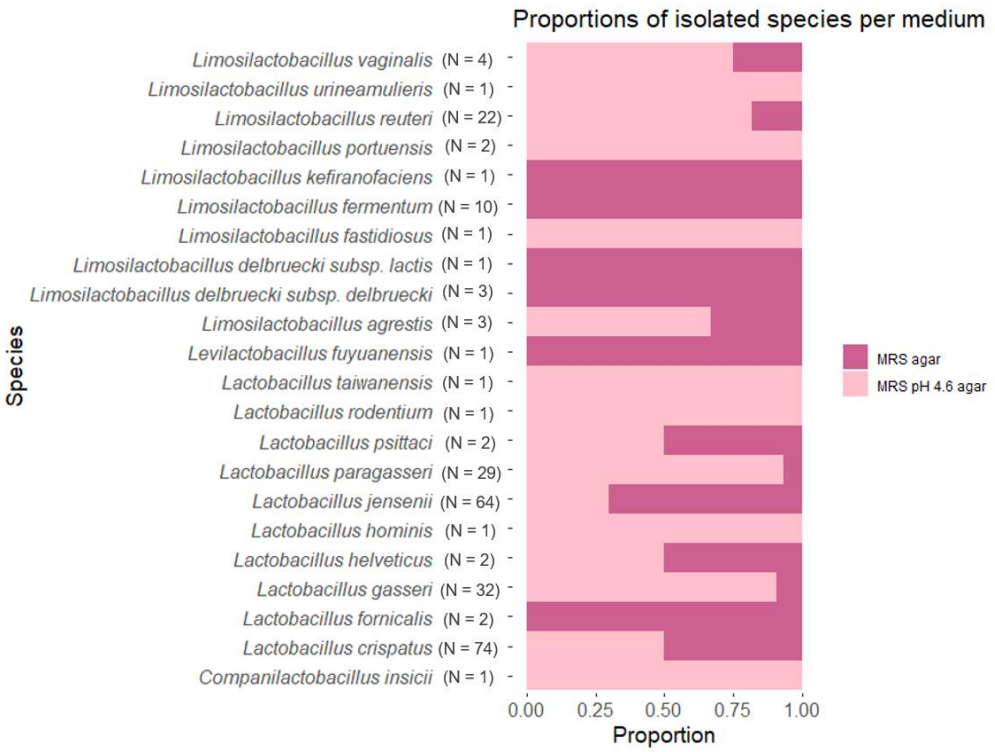

Figure 16: Proportions of vaginal isolates (N = 258) per type of agar after a subculture phase in MRS pH 4.6 + Cys broth. *Proportions are based on the total number of species that were isolated per medium (113 for MRS agar and 145 for MRS pH 4.6 agar). Abbreviations: MRS = De Man – Rogosa – Sharpe.*

Table 10: Isolated vaginal lactobacilli from the selected Isala mothers and daughters (N = 22). *In total, 258 isolates were obtained from all pairs and groups. The species names of the vaginal isolates are shown, identified based on their 16s rRNA sequences (via https://www.ezbiocloud.net/). The number of isolates from a certain species is shown between brackets. Species are coloured according to the taxonomic group to which they belong. The corresponding legend is shown below. Abbreviations: GM = grandmother, M = mother, D = daughter.*

Pair	1	2	3	4	5	6	7
M	*L. crispatus* (7)	*L. jensenii* (9) *L. psittaci* (2)	*L. jensenii* (7)	*L. crispatus* (26)	*L. crispatus* (1) *L. reuteri* (2) *L. agresitis* (2) *L. gasseri* (1)	*L. crispatus* (5) *L. reuteri* (2) *L. vaginalis* (1)	*L. crispatus* (1) *L. jensenii* (6)
D	*L. crispatus* (1) *L. jensenii* (13) *L. fornicalis* (2)	*L. crispatus* (4) *L. jensenii* (5) *L. kefiranofaciens* (1) *L. helveticus* (1) *Companilactobacillus insicii* (1)	*L. crispatus* (1) *L. jensenii* (8)	*L. crispatus* (14)	*L. reuteri* (8) *L. agrestis* (1) *L. fuyuanensis* (1) *L. vaginalis* (1)	*L. crispatus* (7)	*L. crispatus* (6)

Group 8	
GM	*L. gasseri* (12) *L. paragasseri* (7)
M	*L.crispatus* (1) *L. gasseri* (9) *L. paragasseri* (6) *L. rodentium* (1) *L. taiwanensis* (1) *L. hominis* (1)
D	*L. paragasseri* (12) *L. gasseri* (2)

Group 9				
M	/			
D	Daughter 1	Daughter 2	Daughter 3	Daughter 4
	L. jensenii (7) *L. gasseri* (6) *L. paragasseri* (1) *L. helveticus* (1)	*L. delbrueckisubsp. delbrueckii* (3) *L. delbrueckisubsp. lactis* (1) *L. fermentum* (10)	*L. jensenii* (9)	*L. gasseri* (2) *L. paragasseri* (3) *L. reuteri* (10) *L. vaginalis* (2) *L. portuensis* (2) *L. urineamulieris* (1) *L. fastidiosus* (1)

Phylogroups:

- *L. crispatus*-group
- *L. gasseri*-group
- *L. reuteri*-group
- *L. jensenii*-group
- *L. delbrueckii*-group

3.2.4 Phylogenetic analysis of the 16s rRNA gene sequences of isolated vaginal lactobacilli

To determine the degree of similarity between isolates of mothers and daughters, a phylogenetic analysis was performed based on 16S rRNA gene sequencing data. In total, the sequences of 128 isolated vaginal lactobacilli were suitable for phylogenetic analysis. Trees were constructed based on 16S rRNA gene sequences of *L. crispatus, L. jensenii, L. gasseri/L. paragasseri,* and *L. reuteri* isolates. The *L. crispatus* tree included sequences of 53 isolates and the reference strain. The sequences included in the tree were obtained from isolates of P1M (7), P1D (1), P4M (17), P4D (14), P6M (3), P6D (5), P7M (1) and P7D (5) (Figure 17). The *L. jensenii* tree included sequences from eighteen isolates and the reference strain. The sequences included in this tree were obtained from isolates of P2M (3), P2D (3), P3M (6), P3D (1), G9D3 (3) and G9D2 (2) (Appendix C Figure 1). The tree

constructed with sequences of *L. gasseri* and *L. paragasseri* isolates included 49 sequences of isolates and two reference strains (*L. gasseri* and *L. paragasseri*). The sequences included in this tree were obtained from isolates of G8GM (18), G8M (10), G8D (13), G9D1 (4) and G9D4 (4) (Appendix C Figure 2). Finally, the *L. reuteri* tree was constructed using eight sequences and the reference strain sequence. The sequences were obtained from isolates of P5M (2) and P5D (6) (Appendix C Figure 3). In general, among all trees, isolates from the same participant were more often found within the same clade than isolates from different participants. Moreover, in the *L. crispatus* tree, isolates from both mother (P7M AMBV2395) and daughter (P7D AMBV2403) of pair 7 were found within the same clade. In addition, in the *L. gasseri/L. paragasseri* tree, isolates from two sisters (G9D1 and G9D4) of pair 9 were observed within the same clade (Appendix C Figure 2).

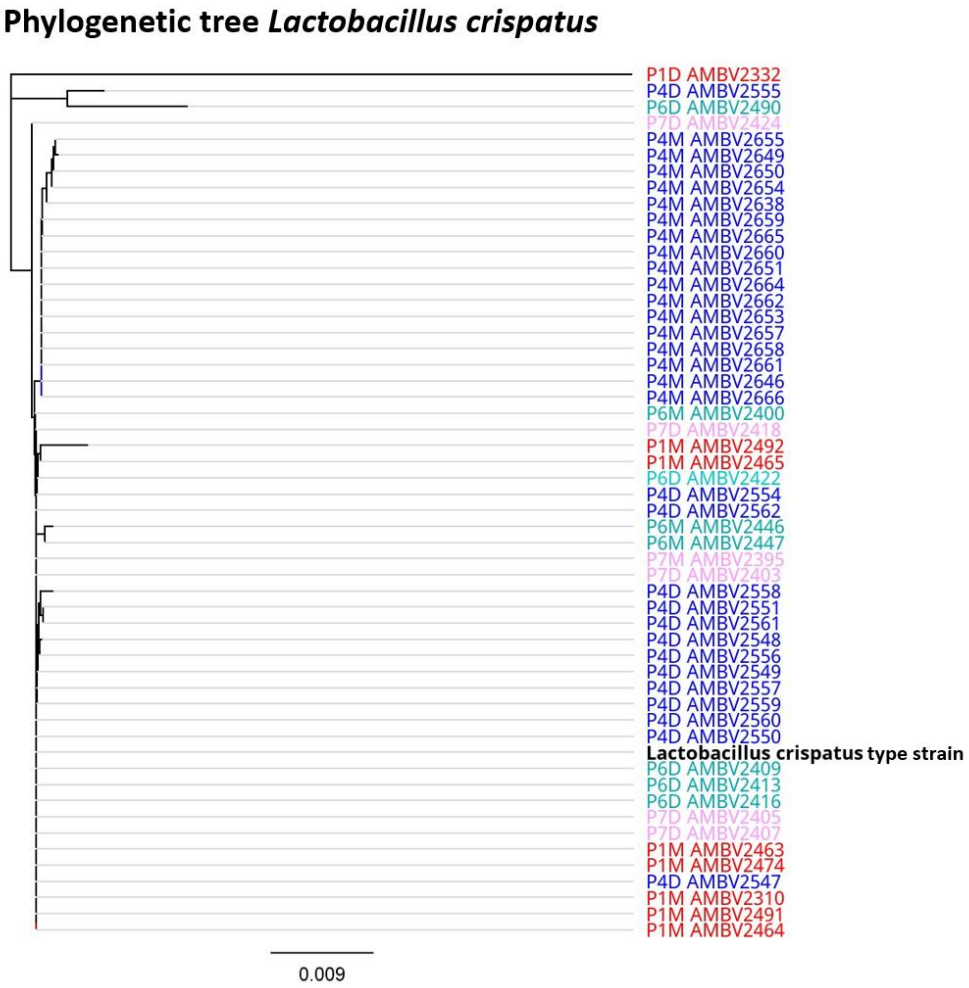

Figure 17: Phylogenetic tree of 16S rRNA gene sequences of L. crispatus isolates and the reference strain. *This tree includes 52 isolates that were identified as L. crispatus. Isolates are coloured according to the pair or group to which they belong. 'AMBV' strain designation is used by the host lab (LAMB) for bacteria isolated from vaginal (V) ESwabs. Tree was built with Geneious Prime (version 2023.0.4). Abbreviations: P = pair, G = group, M = mother, D = daughter.*

3.2.5 Phylogenetic analysis of whole genome sequences of isolated vaginal lactobacilli

In this Master's thesis, a total of 32 vaginal isolates were selected for WGS. After DNA extraction, 24 of these isolates had a DNA concentration above 10 ng/µL and were sent for WGS. Table 11 gives an overview of the isolates sent for WGS, and the assigned quality label based on quality control after arrival at the BGI genomic sequencing facility. Quality level A and B were assigned to 25% and 17% of the isolates, respectively. More than

half of the isolates that were sent for sequencing were assigned to quality level C (58%), thereby excluding them from the sequencing run.

From the 24 vaginal isolates sent for WGS, seven *L. crispatus* genomes were successfully sequenced (Table 11, indicated with asterisk) and could therefore be added to the pool of 32 already available *L. crispatus* genomes vaginal isolates of the host lab. These seven *L. crispatus* isolates were obtained from the mother and daughter of pair 6 and pair 7. All these *L.* crispatus genomes had a completeness higher than 98% and a contamination smaller than 1%, as assessed during quality control using CheckM. The resulting tree including all 39 *L. crispatus* genomes is shown in Figure 18. Sequences from the mother of pair 6 (P6M) were found within the same clade (Figure 18). Their high similarity is reflected by a difference of 2 SNPs only (Figure 19B). In addition, sequences from the daughter of pair 6 (P6D) were found within the same clade (Figure 18). Here, SNP analysis revealed a difference of 13 SNPs (Figure 19B). Interestingly, the sequences of isolates from the mother (P7M) and daughter (P7D) of pair 7 were found within the same clade as well (Figure 18). The isolates from P7D had an average SNP count of 491±2 with the sequence of the maternal isolate, whereas an average SNP count of 13±0 was observed for the isolates of P7D

Table 11: Overview of the isolates sent for WGS. *This table provides an overview of the AMBV numbers and identified species (top-hit) of the 24 isolates selected for WGS, the participant from whom they were isolated, extracted DNA concentration, the sample volume that was sent, and the quality label assigned by the sequencing facility. 'AMBV' strain designation is used by the host lab (LAMB) for bacteria isolated from vaginal (V) ESwabs. Isolates that were used to build a phylogenetic tree are indicated with an asterisk (*). Abbreviations: P = pair, G = group, M = mother, D = daughter.*

AMBV number	Top hit species	Pair code	DNA concentration (ng/µL)	Volume sent (µL)	Quality label
AMBV2332	*L. crispatus*	P1D	40.9	25	C
AMBV2386	*L. reuteri*	P5M	49.1	25	C
AMBV2388	*L. crispatus*	P5M	50	25	C
AMBV2394	*L. reuteri*	P5D	40.3	25	B
AMBV2395*	*L. crispatus*	P7M	56	25	A
AMBV2396	*L. jensenii*	P2M	19.3	50	C
AMBV2400*	*L. crispatus*	P6M	20.9	50	B
AMBV2403*	*L. crispatus*	P7D	42.7	25	A
AMBV2405	*L. crispatus*	P7D	10.7	25	C
AMBV2407*	*L. crispatus*	P7D	30.7	25	A
AMBV2413*	*L. crispatus*	P6D	too high	25	A
AMBV2415*	*L. crispatus*	P7D	54	25	A
AMBV2436	*L. jensenii*	P2M	27.4	25	C
AMBV2439	*L. jensenii*	P2M	14.1	50	C
AMBV2441	*L. jensenii*	P2D	20.4	50	C
AMBV2444	*L. jensenii*	P2D	13	50	C
AMBV2446*	*L. crispatus*	P6M	too high	25	A
AMBV2456	*L. gasseri*	P5M	30	25	B
AMBV2463	*L. crispatus*	P1M	too high	25	C
AMBV2465	*L. crispatus*	P1M	too high	25	B
AMBV2473	*L. jensenii*	P7M	too high	25	C
AMBV2474	*L. crispatus*	P1M	too high	25	C
AMBV2478	*L. jensenii*	P2D	39.2	25	C
AMBV2479	*L. crispatus*	P2D	33.7	25	C

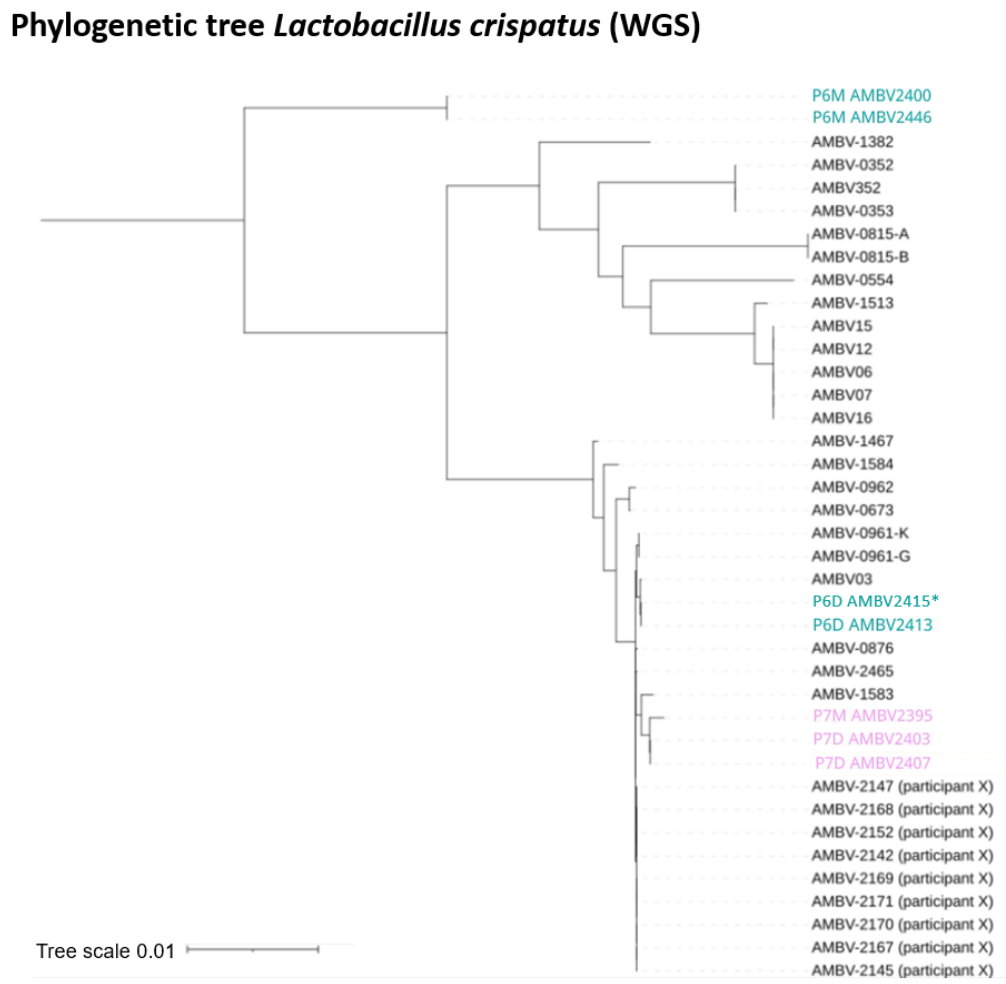

Figure 18: Phylogenetic tree of whole genome sequences of L. crispatus isolates. *This tree includes 39 host lab isolates that were identified as L. crispatus. The coloured isolates are obtained from the selected mothers and daughters. Isolates are coloured according to the pair or group to which they belong. The isolate indicated with an asterisk (*) was not included in the phylogenetic tree based on 16S rRNA gene sequences. 'AMBV' strain designation is used by the host lab (LAMB) for bacteria isolated from vaginal (V) ESwabs. Tree was built by the host lab bio-informaticians. Abbreviations: WGS = whole genome sequencing, P = pair, M = mother, D = daughter.*

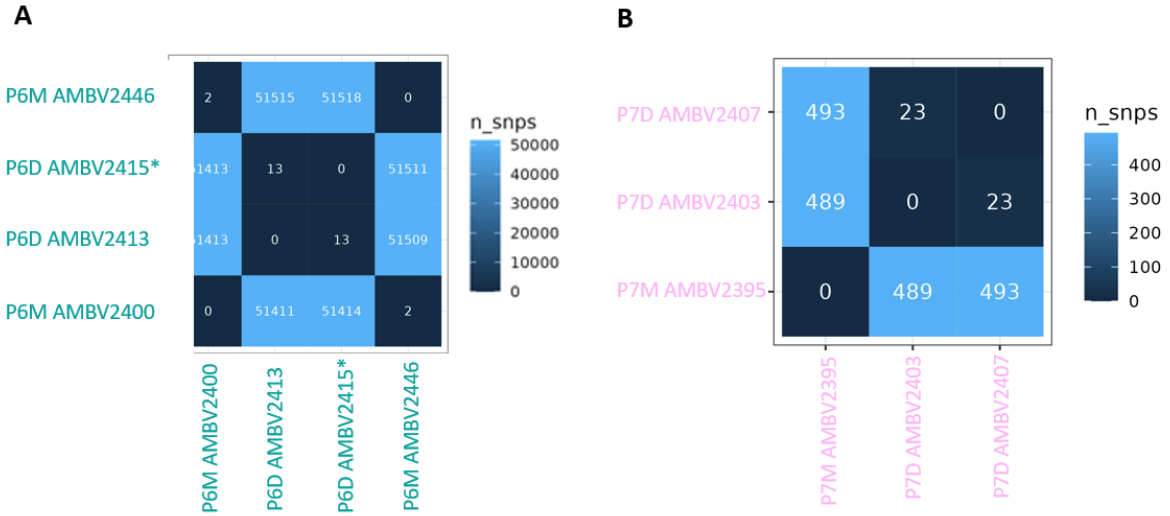

Figure 19: SNP analysis of whole genome sequences obtained from mother and daughter of pair 6 (A) and pair 7 (B). *SNP counts are shown for pairwise comparisons. 'AMBV' strain designation is used by the host lab (LAMB) for bacteria isolated from vaginal (V) ESwabs. Analysis was done by the host lab bio-informaticians. Abbreviations: P = pair, M = mother, D = daughter.*

4 Discussion

In this section, the results and data obtained in this Master's thesis will be discussed in perspective of existing literature, with a focus on the major scientific aims of this Master's thesis. The first major aim was to characterise the VM dynamics throughout the three trimesters of pregnancy. The second one was to explore potential clinical factors that could be associated with PTB. Finally, the third aim was to explore vertical transmission of the VM from mother to daughter as well as persistence towards reproductive-age, with a particular focus on vaginal lactobacilli. Hereto, appropriate culturing conditions towards the isolation of vaginal lactobacilli were optimised.

4.1 Composition and dynamics of the vaginal microbiome throughout pregnancy

In the past decade, different studies have revealed that the VM plays a crucial role in women's health, as well as for their children and partners (66, 104, 111, 149). In the general population, most reproductive-age women have a VM dominated by one or more *Lactobacillus* spp. This vaginal lactobacilli dominance has been associated with vaginal health, due to their protective capacities (46, 54-56, 58, 72). In contrast, a non-lactobacilli dominated VM (also referred to as a dysbiotic VM) could be linked to a number of adverse pregnancy outcomes (including PTB), and urogenital diseases and infections, including BV, STIs, UTIs (46, 64-66, 125, 137). However, women with a VM dominated by anaerobes (including *Prevotella, Atopobium,* and *Dialister*) do not necessarily experience vaginal symptoms or other adverse effects (46, 150). Up until today, it remains to be further elucidated whether these asymptomatic women are at risk for PTB and whether or not they should be (preventively) treated (71, 151). Moreover, a better understanding of the VM composition and dynamics in pregnant women is needed to comprehend how the VM can improve or affect the health of mother, partner and newborn (149).

A large number of studies however still focused on non-pregnant women (46, 48, 59, 78) and thus many knowledge gaps remain considering the dynamics of the VM throughout pregnancy in the general population. Nevertheless, in recent years, the importance of the VM during pregnancy has been stressed by several studies, as PTB remains a large health problem worldwide (89, 91, 92, 94, 121, 152, 153). A clear need exists for more longitudinal studies in order to characterise the dynamics of the VM throughout the different trimesters of pregnancy. In this Master's thesis, the VM dynamics during pregnancy were evaluated for 92 participants, being part of the control group of the ProPreB trial. These women had a vaginal pH below 4.5 at 6-13 weeks of GA and did not receive any treatment. A longitudinal study design was followed including the collection of vaginal swabs at three different time points during pregnancy (each trimester); between 6-13 weeks of GA (visit 1), 18-23 weeks of GA (visit 2) and 35-37 weeks of GA (visit 3), which resulted in a total of 276 collected vaginal swabs.

Among women in this ProPreB control group, the most frequently observed vaginal taxa along all visits belonged to the *L. crispatus*-group, followed by the *L. iners*-group*, L. gasseri*-group*,* and *L. jensenii*-group. Moreover, vaginal lactobacilli were present in all vaginal swabs during pregnancy, irrespective of their relative abundances. This is consistent with the findings in a study of Yeruva *et al.* (2017), who studied the vaginal lactobacilli profile of 27 pregnant Indian women without vaginal dysbiosis (i.e., having a VM dominated by *Lactobacillus* spp.) (154). In that study, *L. crispatus* was observed in 100%*, L. iners* in 77% *, L. jensenii* in 74% and *L. helveticus* in 60% of the samples. More specifically, *L. helveticus* belongs to the *L. crispatus*-group (59). Within another study in 64 pregnant Mexican women without vaginal dysbiosis, lactobacilli were present in 98% of the collected vaginal samples (155). For example, *L. acidophilus*, which also belongs to the *L. crispatus*-group, was observed in the VM of 78% of the analysed samples (59, 155). These reports of high lactobacilli prevalence during pregnancy can be explained by the accompanying rise in circulating oestrogen during pregnancy (156), as this hormone stimulates glycogen accumulation in the vaginal epithelial cells favouring growth of vaginal lactobacilli (40, 44, 45). Other explanations could be an increased endocrine stability and absence of menstrual bleeding (89, 98). In addition, the women in the ProPreB control group all had a low vaginal pH (below 4.5) at visit 1, which also suggests dominance of vaginal lactobacilli. These factors might also explain the high relative abundance of vaginal

lactobacilli during pregnancy within the control group of the ProPreB trial. Species of the *L. crispatus*-group were dominant (i.e., relative abundance above 30%) in 50% of the collected vaginal samples. The other highly abundant taxa included species of the *L. iners-, L. gasseri-* and *L. jensenii*-groups. This was reflected as 52% of all samples being assigned to CST I (*L. crispatus* dominated), 30% to CST III (*L. iners* dominated), 10% to CST II (*L. gasseri* dominated) and 5.8% to CST V (*L. jensenii* dominated). Several other studies have also shown that *L. crispatus* dominates the VM during pregnancy (90, 92, 149). For instance, in a study of MacIntyre *et al.* (2014), samples were collected from 42 pregnant British women at four time points during pregnancy and post-partum (92). Respectively, 40%, 27%, 9% and 13% of the samples were assigned to CST I, CST III, CST II and CST V. In contrast, other studies, including African-American participants report that *L. iners* mostly dominates the VM during pregnancy (76, 91). This could be explained by the difference in ethnicity of the included women in these study cohorts. In general, a VM dominated by *L. crispatus* seems more common in Caucasian women during pregnancy, while dominance by *L. iners* is more frequently observed in pregnant women with African ancestry (74, 76). Within the ProPreB control group, 90% of women self-identified as Caucasian, which aligns with species of the *L. crispatus*-group being dominant in 50% of the samples. In general, the microbiome data collected from this control group confirm that vaginal lactobacilli, and *L. crispatus* particularly, are frequently observed in Caucasian women during pregnancy with high relative abundances. Since samples were exclusively collected during pregnancy, it is however not possible to make comparisons with the VM composition prior to conception. As a consequence, it remains unclear whether these abundances are higher compared to the period preceding pregnancy. Possible ways to investigate this could be on one hand the inclusion of prepartum samples, which however might complicate the ease of recruitment. On the other hand, these abundances could be compared with a subset of Isala participants within a similar age group.

Besides vaginal lactobacilli, anaerobic bacteria, such as *Gardnerella, Prevotella, Peptoniphilus, Anaerococcus,* and *Finegoldia*, were also detected in the collected swabs. On average, the latter four were present in very low relative abundances (0.07-0.4%) calculated over all study visits. Within the Isala cohort, comprising 3345 non-pregnant women, 12% had a VM dominated by *Gardnerella* and 6% by *Prevotella* (59). In contrast, within the ProPreB control group, only 4.7% of all swabs were dominated by *Gardnerella* and none by *Prevotella*, suggesting lower abundances of these anaerobic taxa in pregnant women. Importantly, the size of this study cohort was much smaller compared to the large Isala cohort. Besides, contrary to the Isala cohort for which vaginal pH measurements are missing, the ProPreB control participants were selected based on a low vaginal pH (below 4.5), which can also explain the low abundances of these anaerobic taxa and the aforementioned high abundances of vaginal lactobacilli (40). Another hypothesis could be that anaerobic bacteria decrease in abundance during pregnancy, or that women with high abundances of anaerobes encounter difficulties to get pregnant, thus explaining the low number of ProPreB control participants with a VM dominated by anaerobes. Although, further investigation is needed, such as the inclusion of prepartum samples, to further investigate this hypothesis. When further classifying the vaginal samples according to CST levels, CST IV-A (dominated by *C. Lachanocurva vaginae* and *G. vaginalis*), IV-B (dominated by *G. vaginalis* and *Atopobium* vaginae) and IV-C (low abundance of *G. vaginalis*, high abundance of other anaerobes) were assigned to only 1.8% of samples. Moreover, at visit 3, none of the samples belonged to these CSTs. These data confirm that anaerobic species were rarely dominant during pregnancy in the ProPreB control group. This observation is in line with the study of Romero *et al.* (2014) which also showed significantly lower levels of CST IV-A and IV-B during pregnancy (91).

Upon assessing the VM dynamics during pregnancy in the ProPreB control group, a VM dominated by species of the *L. crispatus*-group showed the greatest stability over time. This was reflected in 45% of women having a VM dominated by species of the *L. crispatus*-group at each visit. A study of Verstraelen *et al.* (2009) supports this observation as they provided evidence that presence of *L. crispatus* ensures ongoing presence of *L. crispatus* (153). Other studies have shown that, in general, the VM during pregnancy is characterised by a greater degree of stability compared to non-pregnant women (91, 94). This increase in stability is suggested to be caused by the increased oestrogen levels during pregnancy, but further research is needed to explore this hypothesis (91). The reported increase in VM stability during pregnancy might play an important role in lowering the susceptibility for vaginal infections, and might therefore be an evolutionary adaptation to increase reproductive success and decreased chances for adverse pregnancy outcomes (91, 157). On the other hand, the study of Verstraelen *et al.*

(2009) showed that dominance by *L. gasseri* and/or *L. iners* was less likely to persist throughout pregnancy, and more prone to switching to a VM dominated by anaerobes (153). In the ProPreB control group, dominance by species of the *L. gasseri*- and *L. iners*-groups was less likely to persist throughout pregnancy compared to *L. crispatus* dominance. However, switches from these dominances to a VM dominated by anaerobes were only observed in one participant. Probably, this might be attributed to the low prevalence of anaerobes in the ProPreB control group, possibly due to the inclusion of women with a low vaginal pH. This observation could also be attributed to the sampling frequency within the ProPreB trial. In this regard, a study of DiGiulio *et al.* (2015) collected vaginal samples weekly during pregnancy and investigated the effects of sampling frequency on the study findings (94). They concluded that sampling less frequently than weekly would have affected the ability to detect changes in CST IV assigned samples, since CST IV has a persistence time of 2.6 weeks on average (94). Thus, as a recommendation for future research, a higher sampling frequency could help in further exploring the dynamics of CST IV assigned vaginal samples.

Throughout all visits, the VM diversity in the studied population remained stable. Moreover, no significant difference in alpha diversity was observed when comparing all visits. Similarly, Nunn *et al.* (2021) examined alpha diversity throughout pregnancy as well as during the postpartum period in 48 American women (99). In that study, no significant difference in alpha diversity was observed during pregnancy, however a major increase in diversity as well as a decrease in vaginal lactobacilli was detected in postpartum samples (99). Currently, longitudinal studies including the postpartum period are rather sparse (92, 99, 149). Nevertheless, the conducted studies suggest that the VM composition is completely altered during the postpartum period, which can play an important role in postpartum pathologies (i.e., endometritis and sepsis) (92, 149). To gain better insights in the effects of this altered VM composition after pregnancy, further research is recommended. A study of Stout *et al.* (2017) (117), examining VM diversity in 77 predominantly African-American pregnant women, showed that the VM diversity remained stable during pregnancy for women who delivered at term (117). In addition, they found a significant difference in alpha diversity in the first trimester for women who delivered at term and those who delivered preterm (117). However, within the ProPreB control group, no significant difference in alpha diversity was observed at any visit between women who delivered at term and preterm. Importantly, the study cohort in the study of Stout *et al.* (2017) consisted mainly of women with African-American ancestry with a much higher rate of PTB (31.5%) compared to the ProPreB control group (PTB rate of 5.4%) (117). Because of a limited study size in this Master's thesis as well as a limited PTB rate, possible associations between VM dynamics during pregnancy and the occurrence of PTB were not further investigated. To this end, a larger study cohort is recommended to obtain significant results.

4.2 Associations between clinical parameters and preterm birth

Currently, several prevention strategies are available for PTB in women who are at risk (158). Therefore, it is essential to detect women at risk for PTB as early as possible. Since PTB is known to be influenced by multiple pathologic processes (126), in this Master's thesis, a series of clinical factors that are possibly associated with PTB were investigated. Clinical data from the participants in both control groups of the ProPreB trial (i.e., vaginal pH below 4.5 and ≥4.5 in the absence of study medication) were used to investigate possible associations with PTB. Data collection started in 2017 and is still ongoing to date. In this Master's thesis, clinical data from 413 women in the ProPreB control groups were analysed. Since data were collected and recorded at various study sites (UZA and RZ T) by multiple clinicians, this could have led to particular discrepancies. For instance, certain measurements (e.g., cervical length) were recorded in different units. Besides, an increase in drop-out rate was observed towards visit 3, thus resulting in an unequal number of data points for all visits. Altogether, these reasons complicated the statistical analyses. Moreover, in this Master's thesis, PTB was defined as delivery earlier than 37 weeks of GA and no further sub-division was made. Several studies however define sub-categories of PTB, ranging from early PTB (before 32 weeks of GA) to late PTB (before 37 weeks of GA), and other subdivisions are also used (159, 160). Since only 25 women delivered preterm in the ProPreB control groups, further sub-divisions would result in small study groups. Comparing findings with other studies was therefore complicated. Moreover, the limited size of the group of women who delivered preterm might explain why some associations

were not found within this study cohort. Another limitation of this Master's thesis includes the lack of differentiation between spontaneous and induced PTBs (125).

The PTB rate in the ProPreB control groups was 7.6%. This corresponds with the Belgian average PTB rate of 7.96% between 2017 and 2020 (161). In Belgium, a decrease in the number of PTBs was observed from 2019 (8.03%) towards 2020 (7.57%) (161). This decline in PTBs was also reported in other developed countries, including the Netherlands and several states in the USA (162-164). Studies suggest that this decline in PTBs could be attributed to the policy measures in response to the COVID-19 pandemic (165-167). Factors that could explain this decline include a reduction in infections due to increased awareness of personal (e.g., social distancing) and environmental hygiene (e.g., clean water, housing/school/public place sanitation), reduced air pollution and a reduction in work-related stress (164, 168). However, other studies observed no difference (169, 170), or even an increase (171) in PTBs in this period. Nevertheless, the impact of the COVID-19 pandemic on the number of PTBs is not yet fully understood and remains to be further explored (164). Therefore, when interpreting the results, it should be taken into consideration that some data of the ProPreB trial were collected during this pandemic.

Many studies have shown that vaginal dysbiosis has been associated with an increased risk for PTB (94, 106, 172, 173). Therefore, it was examined whether there was an association between the presence of BV in the ProPreB control groups at any of the visits and the occurrence of PTB. Surprisingly, no association was found. Currently the gold standard diagnostic tool for BV is the Nugent scoring system, which relies the evaluation of morphotypes on a Gram-stained vaginal smear (174, 175). A main advantage of the Nugent scoring system, is its high reproducibility and sensitivity (176). Another frequently used diagnostic tool are the Amsel's criteria (174). According to the Amsel's criteria, BV is identified by presence of at least three of the following criteria: (i) presence of clue cells on wet mount microscopy,(ii) vaginal pH above 4.5, (iii) presence of vaginal discharge, (iv) positive whiff test (i.e., fishy odour when 10% potassium hydroxide is added to vaginal discharge) (177). However, in the ProPreB trial, only the presence of clue cells was used to characterise BV. The ProPreB control group mainly consisted of women with a pH below 4.5 at visit 1 (N = 405), for 369 of them, the pH remained low at all visits. In addition, the presence of vaginal discharge was not examined in this study. In future research, it would be beneficial to include examination of vaginal discharge to possibly improve the BV classification or the Nugent criteria could be used for diagnosis. Nevertheless, it remains to be further elucidated whether asymptomatic women with BV are at risk for adverse pregnancy outcomes and whether or not this asymptomatic BV should be treated (71, 151). Since vaginal dysbiosis itself is often characterised by an increase in vaginal pH (55, 178), vaginal pH values were compared between women who delivered at term and preterm. Here, no significant differences were found. In addition, no significant relationship was found between vaginal pH at all three visits and GA at delivery. These findings were surprising since previous studies have found significant associations between a high vaginal pH and an increased risk for PTB (179). For example, Hauth *et al.* (2000) studied vaginal markers in early pregnancy that could be predictive for PTB, including vaginal pH (179). Based on the vaginal pH measured in the first or second trimester of pregnancy for 12,041 women, they investigated the differences in PTB rates. In this study, women with a pH ≥ 5 had an increased risk for PTB (179). These findings suggest that an elevated vaginal pH in the first or second trimester of pregnancy is an early pregnancy marker for PTB (179). This finding was also confirmed by another study in 438 pregnant women analysing the vaginal pH in the second trimester of pregnancy (180). Their study indicated that a vaginal pH above 5 was significantly more predictive for PTB than a lower vaginal pH (180). Based on these studies, a negative correlation between vaginal pH and GA at delivery was expected. Since the ProPreB control groups mainly consisted of women with a vaginal pH below 4.5 at the first visit (N = 405), this probably explains the absence of a significant association between pH and GA at delivery in this study cohort. For future research a larger study cohorts of pregnant women, including women with a high vaginal pH and asymptomatic BV is recommended.

Furthermore, a possible correlation between GA at delivery and maternal BMI, maternal age and cervical length was assessed. Previous studies have provided evidence that these factors affect GA at delivery (181). Firstly, being underweight (BMI <18.5kg/m^2), overweight (BMI 25.0-29.9 kg/m^2) or obese (BMI ≥30 kg/m^2) in the pre-pregnancy period has been shown to increase the risk for PTB (181). Importantly, also maternal age and ethnicity

seemed to play a role in this association (181). Within the ProPreB control groups, no relation was found between maternal BMI and GA at delivery. Nevertheless, the association between (pre-)pregnancy BMI and GA remains to be further explored since many studies remain inconclusive (181). Further studies are required to elucidate the roles of ethnicity and maternal age and the type of relationship between maternal BMI and GA at delivery. Secondly, the relationship between advanced maternal age and risk for PTB has been previously addressed by different studies (182-184). In general, these studies have shown an association between advanced maternal age and increased risk for PTB. Contrary, within the ProPreB control groups, no relation was found between maternal age and GA at delivery. It should be noted that it is challenging to study associations between GA at delivery and variables such as BMI and maternal age, since they are often differently categorised. Therefore, within this Master's thesis, these variables were treated as continuous variables, rather than categorical variables. Moreover, a limitation is that only the monotonic relationship between GA at delivery and the aforementioned variables was explored. It is possible that other types of relationships exist, although this has to be further investigated. Thirdly, a short cervical length has already been determined as a risk factor for PTB (185). In line with the expectations, a weak positive correlation was observed between cervical length at visit 2 during the second trimester and GA at delivery within the ProPreB control groups. These findings confirm that measurement of the cervical length is an appropriate screening method to detect women possibly at risk for PTB (158). In addition, for women with a short cervix (i.e., 10-25mm), some studies have shown that administration of vaginal progesterone can significantly reduce the PTB rate (186, 187). Nevertheless, it is still necessary to establish an appropriate cut-off value to determine women at risk and to optimise and streamline measurement methods (188).

Since several studies in the past have examined the association between the occurrence of PTB and smoking behaviour (160, 189), the association between these variables was examined. No significant association was found between the last time a mother had smoked and the occurrence of PTB. Within this ProPreB study cohort, having smoked in the last 30 days or currently smoking did not show an association with the occurrence of PTB. This was unexpected, since several studies in the past have shown an impact of smoking on the risk for PTB (160, 190, 191). Importantly, women self-reported their smoking behaviour, which might be influenced by reporting bias (192, 193) since smoking during pregnancy is socially stigmatised because of reported negative health effects for both mother and unborn child (160, 194, 195). This stigmatisation could have led to incorrect reporting of the actual smoking condition by the mother. In addition, the questionnaire did not include questions on passive smoking, while some studies have suggested that passive smoking could also increase the risk for PTB (189, 196). Nevertheless, smoking during pregnancy remains strongly discouraged as it causes long-lasting epigenetic changes, increased risk for adverse pregnancy outcomes and an increased risk for adverse health outcomes during foetal life and childhood (197). As a recommendation for future research, it could be valuable to include more questions on smoking behaviour of others within the close surroundings of pregnant women, to get more insights on how passive smoking affects the newborn.

4.3 Group B streptococci during pregnancy

Streptococcus agalactiae is an opportunistic pathogen, known as Group B *Streptococcus* (GBS). It colonises the vagina and/or rectum of approximately 25% of pregnant women (198). Within the VM of the control group of the ProPreB trial, 16% of the participants had a positive recto-vaginal GBS swab in the third trimester of pregnancy. This is lower than expected, which might be explained be the high abundances of lactobacilli in the VM of the women in the control group. As shown in a study of Rönnqvist *et al.* (2010), women with high abundances of vaginal lactobacilli are less likely to be a GBS carrier (199). They suggest that lactobacilli might outcompete the colonization by GBS (199). Colonisation by GBS during pregnancy has been linked to neonatal infections, including meningitis (200), sepsis and pneumonia (201), and PTB (202). Although there is a widespread adoption of intrapartum screening and the use of antibiotic prophylaxis for carriers of GBS, there is still need for improvements to reduce adverse pregnancy and neonatal health outcomes (198). Upon examining the association between GBS status and the occurrence of PTB in the ProPreB control group, a significant association ($p = 0.0060$) was found. Although, some studies have found associations between GBS and the risk for PTB, other

studies have reported contradictory findings (203). Therefore further research to understand the mechanisms by which GBS could attribute to a higher risk for PTB is recommended.

In addition, the VM composition of fifteen women who had a positive GBS recto-vaginal swab was examined more in detail. In the past, only a limited number studies have looked into the link between the VM composition and GBS colonisation (204-206). In general, these studies indicated that a high abundance of vaginal lactobacilli lowers vaginal colonisation with GBS (205). In particular, *L. crispatus* dominance seemed to be the most protective against GBS colonisation (205). However, a knowledge gap remains on which VM composition might increase the risk for GBS colonisation. For the women in the ProPreB control group, an association was found between the VM composition in the second trimester (at visit 2) at sub-CST level and a GBS status. However, a larger data set is recommended to apply a chi-square test, allowing to see which CST shows an association with GBS status. Given the impact of GBS colonisation during pregnancy on mother and newborn, and considering that the VM might be an important determinant for the susceptibility for GBS colonisation, further studies are strongly advised. Another remarkable finding emerged upon examining the VM composition of GBS positive women. When looking at the prevalence of streptococci in the VM of these women, species of the *Streptococcus* genus were only detected in one women at visit 3. This could mean that streptococci were not present in the vagina of these women, but originated from the rectum. This could be a possibility, since the rectum is known to be a major reservoir for GBS (198). Nevertheless, it is more likely that they were present in the VM, but that they did not get detected within the vaginal swabs. This stresses the importance of collecting rectal samples for GBS screening.

4.4 Optimised culturing conditions for isolation of lactobacilli from vaginal swabs

Since vaginal lactobacilli are essential for maintaining a healthy vaginal environment (40) and for the initial colonization of a newborn (103), these bacteria were of main interest in the culturing campaign set up in this Master's thesis to study vertical transmission of vaginal microbiota members from mother to daughter. Difficulties with isolating oral and faecal lactobacilli were previously reported in literature since these bacteria only represent a small proportion of the total microbial composition in these niches (207). In contrast to their low abundances in the gastro-intestinal tract, lactobacilli often comprise more than 70% of the human vaginal microbiota (51). In this Master's thesis, isolating lactobacilli from vaginal swabs of pair 4 (both dominated by *Lactobacillus* spp.) was however not successful when directly streaking the swabs on CB agar and MRS + Cys agar. More specifically, this direct streaking technique resulted in the isolation of enterococci and *Streptococcus agalactiae,* despite the frequent use of these agar types for the cultivation of lactobacilli (208, 209). Furthermore, MRS is often supplemented with cysteine since this amino acid enhances the growth of lactobacilli by removing oxygen present in the agar, thereby supporting a microaerophilic environment which is in favour of these bacteria (210). In 2020, after sample collection at home, the vaginal swabs were transferred to the host lab by postal service with varying shipping times. This transport process at room temperature might have affected the bacterial composition within the swabs. As a result, a culturing bias towards vaginal pathobionts such as enterococci and streptococci might be introduced, at the expense of lactobacilli, since these are more adapted to cope with different environments and replicate more rapidly at room temperature (211). Therefore, it was required to investigate more suitable *in vitro* culturing conditions to specifically enhance the growth of vaginal lactobacilli. To this end, the use of a subculture phase in acidified MRS broth supplemented with cysteine (MRS pH 4.6 + Cys) was implemented. Importantly, MRS + Cys broth was acidified to pH 4.6 to mimic the acidity of the vaginal environment and incubation at 37°C with 5% CO_2 was performed to recreate the microaerophilic conditions present in the human vagina (212). Implementing this subculture phase was successful to tailor the bacterial isolation towards vaginal lactobacilli, instead of vaginal pathobionts of genera such as *Enterococcus, Shigella, Klebsiella* and *Streptococcus* (16). It should however be noted that staphylococci were still isolated from CB agar after subculturing the vaginal swabs of pair 1 at pH 4.6. This is not surprising since staphylococci are able to grow in a wide pH range, with values ranging from 4 to 10 (213). Besides, this could be explained by the presence of iron in CB agar, which is known to enhance the growth of staphylococci (214, 215). Surprisingly,

enterococci and streptococci were not able to grow in this subculture phase, while being known to produce lactic acid themselves and to adapt to wide pH ranges as well (202, 216, 217). Some studies have been investigating the response to acid stress of these LAB but mechanistic research is rather limited to date (202). As a result, some knowledge gaps remain regarding the acidic stress response of these bacteria. Thus, further investigation is recommended since the mechanisms by which growth of these bacteria in MRS pH 4.6 + Cys broth is inhibited are unknown. In general, after subculturing the vaginal swabs, it was shown that streaking on both MRS agar and MRS pH 4.6 agar was suitable for the isolation of vaginal lactobacilli. In contrast to streaking on CB agar, no staphylococci were observed on these MRS agar types.

By using the optimised culturing protocol including subculture phase, a total of 258 vaginal lactobacilli were isolated from vaginal swabs of one grandmother, nine mothers and twelve daughters. In descending order, the most frequently isolated species were *L. crispatus, L. jensenii, L. gasseri, L. paragasseri* and *L. reuteri.* Remarkably, besides the species with the highest relative abundance in the VM of these participants, also isolates from less abundant species were retrieved. For instance, *L. jensenii* and *L. reuteri* were isolated from vaginal swabs, while *L. crispatus* had a relative abundance of more than 90% for these swabs. Secondly, *L. gasseri* and *L. paragasseri* were isolated from the grandmother and granddaughter in group 8. However, these taxa were not observed in the VM profile of the grandmother, and only with a relative abundance of 0.5% in the VM of the granddaughter. Importantly, based on 16S rRNA gene sequencing, relative bacterial abundances can be determined for a vaginal sample, which does not provide any information on absolute bacterial cell counts (218). For instance, in case the *L. crispatus*-group dominates a particular VM profile, this does not provide information on the absolute abundance of *L. crispatus* nor other bacteria within this particular swab (219, 220). If the total bacterial biomass in a sample is lower compared to other samples, a high relative abundance of a particular taxon in that sample can still correspond to a lower absolute cell count of this taxon compared to other samples (221). This might explain the frequent isolation of bacteria other than the dominant one, since these non-dominant bacteria are probably present in sufficient numbers in the original vaginal swab as well. Here, quantitative PCR (qPCR) can be used to determine absolute bacterial counts in a sample, however this technique is time intensive and expensive. Moreover, a separate assay has to be developed for every bacterium present in a diverse sample or species of interest should be selected, which can result in investigator bias (218). In this respect, species-specific primers are a useful tool to tailor the isolations towards a particular species. Currently the host lab is working on the development of such primers by creating a pangenome of sequences of strains of the family *Lactobacillaceae*. Based on this pangenome, specific core genes could be selected as species-specific targets to design primers. This approach is a promising future step to optimise sequencing, identification and quantification of vaginal lactobacilli. In general, relative abundances obtained by 16S rRNA gene sequencing are certainly valuable to study the interactions and associations with microbiome associated conditions. Nevertheless, absolute bacterial counts might be a better predictor for disease risk (218). Therefore, for future experiments, a combination of qPCR and 16S rRNA gene sequencing might be recommended. Another possible explanation for isolating other taxa than the dominant could be the presence of picking bias, since not all colonies were picked up from the agar plates for PCR and sanger sequencing.

All identified isolates, except for *Levilactobacillus fuyuanensis*, were host-adapted strains (i.e., associated with invertebrate or vertebrate hosts) (222). *L. fuyuanensis* was isolated from the daughter of pair 5 with a species identity of 77.11% and a sequence completeness of 67.7%. A species identity below 97%, suggests that this isolate likely represents another (possibly novel) species or genus (223). Although, it can also be caused by a low sequencing quality. It is recommended to perform WGS for this isolate to be able to more accurately identify and classify it compared to 16S rRNA gene sequencing (224). In general, host-adapted vaginal lactobacilli (e.g., *L. crispatus* and *L. gasseri*,) are sensitive to the antibiotic vancomycin. In that regard, MRS supplemented with vancomycin can be used to tailor the isolation of vaginal lactobacilli towards nomadic strains (i.e., strains that can be encountered in a variety of different environments), such as *Lactiplantibacillus plantarum* and *Lacticaseibacillus casei.* These species have a higher capacity to adapt themselves to various environments, contrary to host-adapted strains (225-227). Since MRS supplemented with vancomycin was not used to isolate vaginal lactobacilli in this Master's thesis, mainly host-adapted strains were isolated. In general, the optimised culturing protocol in this Master's thesis seems to be optimal for isolating host-adapted lactobacilli from vaginal

swabs. In follow-up research, as an alternative for MRS medium, simulated vaginal fluid can be used to isolate bacteria from vaginal swabs, as this medium is known to more closely mimic the vaginal microenvironment and therefore might increase the isolation rate of vaginal lactobacilli compared to MRS (228, 229).

Remarkably, two vaginal swabs (of P4M and P9M) did not show growth upon applying the optimised culturing protocol. For the mother of pair 4 (P4M), the swab showed growth in the subculture phase in MRS pH 4.6 + Cys, but not when subsequently streaking it on MRS agar or MRS pH 4.6 agar. This was unexpected since the VM profile was almost completely dominated by *L. crispatus* (relative abundance of 95%). However, upon growing the swab under anaerobic conditions, *L. crispatus* could be isolated from both MRS agar and MRS pH 4.6 agar. These observations suggest that *L. crispatus* can grow both under micro-aerophilic and anaerobic conditions (212, 227). Secondly, from the mother of group 9, no isolates were obtained since no growth was observed after subculturing and incubating under micro-aerophilic or anaerobic conditions. It could be that lactobacilli were hardly present in this vaginal swab. Based on 16S rRNA gene sequencing, a relative abundance of 0.06% and 0.08% was determined for species of the *L. crispatus*- and *L. iners*-group, respectively. It was reported that this mother was in her menopause at the time of sampling, which goes accompanied by a reduction in vaginal lactobacilli and an increase in anaerobic taxa (157). Her VM profile was dominated by *Prevotella*. In addition, *Porphyromonas* (relative abundance of 2.5%), *Peptoniphillus* (relative abundance of 6.4%) and other taxa known to be involved in BV were present. These taxa typically grow under strict anaerobic conditions (230-232). Moreover, since MRS is a selective medium for lactobacilli but not for anaerobes such as *Prevotella*, this choice of medium might also explain the lack of growth. Overall, no *L. iners* isolates were obtained in this Master's thesis, despite *L. iners* being present in the VM profiles of twelve selected participants. For the mother of pair 7, the granddaughter of group 8 and the daughter of pair 9, the *L. iners*-group was even dominant in their VM profile with relative abundances of 46%, 76% and 66%, respectively. However, it is not surprising that this species was not isolated, since it is a rather unusual *Lactobacillus* spp., with a relatively small genome size and variable Gram morphology, that does not easily show growth on MRS agar (233, 234). Instead, for future experiments, this species can be isolated from blood agar after incubation under anaerobic conditions, typically with small, smooth, non-pigmented colonies (234, 235). In addition, New York City broth (NYCIII) supplemented with horse serum seems to be suitable for cultivating *L. iners* (232).

4.5 Vertical transmission of the vaginal microbiome from mother to daughter

At birth, a newborn is exposed to a variety of microorganisms for the first time (113). Despite the ongoing debate on *in utero* seeding (103, 142, 236), it is known that the maternal microbiome is of major importance for initial colonisation of a newborn upon delivery and subsequently for a newborn's health (113). Several studies have shown that vaginally delivered newborns harbour bacterial communities similar to the microbiota of their mother (97, 103, 113). These studies however have mainly focussed on the impact of the maternal microbiota on the development of the infant gut microbiome (104, 144, 237, 238). In more detail, directly following delivery, maternal skin, vaginal and gut strains were already found in the infant gut (104). The abundance of vaginal and skin species decreased over time, suggesting that they were only transient inhabitants (104). The maternal gut strains, however, seemed to persist the longest in the infant, as these bacteria were still highly abundant four months after delivery (104). Increasing evidence suggests that this vertical transmission of maternal gut microbiota members is essential for the development of the infant gut microbiome, while the role of the maternal VM remains unclear (239-241). Up until today, the origin of the VM and the roles of the maternal VM in its initial colonisation are not yet fully understood. One hypothesis is that vaginal microbiota members, for example vaginal lactobacilli, are transferred from a mother to a newborn upon delivery. If this hypothesis holds true, it still remains unclear how these lactobacilli can persist throughout early childhood and pre-menarche until reproductive-age (44). Therefore this Master's thesis aimed to further explore this hypothesis based on a selection of seven pairs and two groups of mothers and daughters from the Isala project. Vaginal isolates from this subset of mothers and their adult daughters were cultured and sequenced (16S rRNA gene sequencing and/or WGS) whereafter phylogenetic analysis was performed.

Upon examining the phylogenetic trees based on 16S rRNA gene sequences, the sequences of one mother's *L. crispatus* isolate and one daughter's isolate of pair 7 were found within the same clade. For the phylogenetic tree based on whole genome sequences, the same sequences as well as another daughter isolate of this pair were found within the same clade. To reveal how closely genetically related these isolates are, a genome-wide SNP analysis was performed. This analysis revealed the presence of 489 and 493 SNPs between the two daughter isolates and the mother's isolate. SNPs are defined as single base differences in DNA between individual organisms, and are often used to examine genetic diversity and to perform phylogenetic analysis (242). As a future step in the host lab, the exact genomic location of these SNPs will be determined, using a precise position tracking pipeline, to possibly support the hypothesis of vertical transmission at birth. In more detail, random accumulation of SNPs along the genomes allows these SNPs to be used to calculate the *in vivo* doubling time. On the other hand, if SNPs are found close together, this could indicate that there have been horizontal gene transfers (243), which complicates the estimation of the *in vivo* doubling time. To calculate the *in vivo* doubling time based on recombination-filtered SNPs, the same procedure as the study of Bassis *et al*. (2023) can be used (147). As such, the *in vivo* doubling time for *L. crispatus* can be estimated using the following formula: (mutation rate)(daughter's age)(genome length)/(#mutations). However, the mutation rate of *L. crispatus* in the human vagina is currently unknown. Although as an alternative, the *in vitro* mutation rate of *L. casei* in the absence of antibiotics ($1.0x10^{-9}$ bp/generation) can be used (147, 226). The study of Bassis *et al.* (2023) has been assuming that the isolates arose from a common ancestor and that all mutations are non-convergent (147). Therefore, they assume that the number of mutations acquired by each isolate equals the number of SNPs between the mother's isolate and the daughter's isolate divided by two. If doubling time would be in a reasonable range compared to other bacteria in their natural environment (i.e., *Escherichia coli* (15 hours) and *Salmonella enterica* (25 hours)) the observed number of SNPs can be explained by accumulation of independent mutations during the years of persistence in both mother and daughter since birth (147). Overall, these analyses should elucidate whether the observed SNPs are accumulated over the daughters life-time (20 years) or that they have occurred at a later stage in her live, indicating another source.

Notably, the daughter of pair 7 was delivered by C-section, which raises the question whether vertical transmission of vaginal microbiota at birth did occur. However, currently it is not known whether this was an elective or an emergency C-section or whether vaginal seeding occurred after delivery. Since an emergency C-section is known to affect the gut microbiome in a different way compared to an elective one, as there has been some contact with the vaginal microbiota, this might also be of importance for the VM (241, 244). Moreover, the practice of vaginal seeding (i.e., wiping the newborn's mouth, face and skin with the mother's vaginal secretions after C-section) has increased in recent years (245). This procedure is also known to affect the composition of the newborn's microbiome, and can still support the hypothesis of vertical transmission (246, 247). As a future step, additional questions on the delivery mode will be asked to the involved participants, to further explore all possible hypotheses. Overall, within the subset of selected participants, only two daughters were delivered by C-section. Therefore, the differences in VM composition with vaginally delivered daughters cannot profoundly be studied in this study cohort. Previous studies have however shown that birth mode can affect the microbial composition (113, 248). For instance, a study of Dominquez-Bello *et al.* (2010) showed that upon vaginal delivery, the first microbial communities in a newborn resemble the mother's vaginal microbiota (113). On the other hand, newborns delivered by C-section are more likely to have communities resembling the maternal skin microbiota (113). These differences can affect further development of the infant's microbiome as has been shown for the gut microbiome of newborns delivered by C-section (249). Another recommendation for future research is to include a higher number of mother-daughter pairs with daughters delivered by C-section.

Although the importance of delivery mode has been stressed by several studies (113, 145), it is also known that the microbial differences caused by varying birth modes decrease with increasing age of the newborns (103). This suggests that exposure to external sources (in later life) could be of importance for the composition of the microbiome (i.e., diet, environment, horizontal transmission) (250). Therefore, several studies also focus on transmission routes other than vertically (250, 251). For instance within households or social networks, transmission of microbiota members could also occur. It has been shown that social-interactions (such as cohabitation) shape the gut microbiome (250, 252). However, up until today, a knowledge gap remains on how

social contacts shape the VM. For the selected mothers and daughters in this Master's thesis, seven daughters still lived together with their mother at the moment of sampling. Moreover, within group 9, three out of four sisters lived together with their mother at that time. Vaginal bacterial isolates from two of these sisters were observed in the same clade of the *L. gasseri/L. paragasseri* tree based on their 16S rRNA gene sequences, suggesting that these isolates are very similar. This could indicate that both sisters acquired the same strains from their environment (e.g., using the same washing machine or eating the same food). However, they could also have acquired this microbiota member from their mother at birth. Unfortunately, it was not possible to further explore this latter hypothesis, since no vaginal isolates from the mother were obtained. In addition, contrary to the available 16S rRNA gene sequences, whole genome sequences of these isolates were not yet available to perform a more in-depth genome analysis. Although 16S rRNA gene sequences are often used in bacterial phylogenetics, some limitations are encountered as mentioned by Hassler *et al.* (2022) (253). Since the 16S rRNA gene has a low rate of nucleotide substitution in general, these sequences do not capture all evolutionary changes (253). Therefore, future steps include obtaining whole genome sequences from all isolates of the mothers and daughters to better analyse their genetic relatedness and evolutionary history. In addition, more research is recommended to explore other routes of transmission of vaginal microbiota members, such as via the environment, diet, or direct contact (e.g., during intercourse). Investigating these other transmission routes can provide new insights into the origin of the VM. Since the Isala study cohort also included other pairs of related participants, such as partners, sisters and housemates, the influence of these relationships on the VM composition can also be assessed by the host lab in the future. Investigating the vaginal swabs from these participants is promising to reveal more and novel insights in the effects of cohabitation on the VM composition.

Another noteworthy finding obtained from the phylogenetic analyses was that 16S rRNA gene sequences from the same participant were often observed within the same clade, which means that they are closely genetically related. As mentioned before, the use of 16S rRNA gene sequences might be less reliable when comparing sequences of the same species since this gene generally undergoes limited nucleotide substitutions (253). However, within the phylogenetic analysis using whole genome sequences, two isolates of the mother of pair 6 clustered together in a clade, similarly for two isolates of her daughter in another clade and for two isolates of the daughter of pair 7 as well. Here, the number of SNPs was determined to be 2, 13 and 24, respectively. These observations suggest that these isolates originated from a common ancestor within the lifespan of the respective participant, suggesting the occurrence of sub-lineage diversification for each of them (254). However, this observation could also be due to culturing bias, since it might be that some variants are better culturable than others. Nevertheless, further investigation is required to confirm these hypotheses.

5 Conclusions

A better understanding of the VM during pregnancy might be a large help in unravelling the pathophysiology of several adverse pregnancy outcomes, including PTB (94). A considerable amount of studies however still focuses on characterising the VM in non-pregnant women, which limits the knowledge on the VM dynamics during pregnancy in the general population (46, 48, 59, 78). Therefore, a first aim of this Master's thesis was to characterise the dynamics of the VM throughout pregnancy. Hereto, the VM composition during pregnancy was examined at tree timepoints (in each trimester) in a cohort of 92 women from the ProPreB control group. It was shown that most women (89%) had a VM dominated by vaginal lactobacilli at all visits. Especially, a VM dominated by species from the *L. crispatus*-group was frequently observed as it accounted for 50% of all collected vaginal samples. Moreover, a VM dominated by species from the *L. crispatus*-group showed the highest stability throughout all visits. On the other hand, a VM dominated by anaerobes (e.g., *Gardnerella* and *Prevotella*) was rarely observed during pregnancy in this cohort. Finally, it was also shown that the overall diversity of the VM remained stable throughout pregnancy. Overall, the findings in this Master's thesis contribute to the pool of available knowledge on the VM dynamics during pregnancy, by providing support for previous findings. Nevertheless, further research on the VM throughout pregnancy remains of crucial importance, as well as an exploration of the (possibly) influencing factors such as vaginal pH and ethnicity. In addition, in future studies, it is highly recommended to focus on both the pre- and postpartum period to get better insights in how the VM changes at the onset, during and after pregnancy and how this affects both mother and child (102).

Currently, several strategies are available for prevention of PTB in women who are at risk (158). In general, to guarantee success of these prevention measurements, it is essential to detect women at risk for PTB as early as possible. Therefore, this Master's thesis aimed to find associations between several clinical parameters at multiple time points during pregnancy and the risk for PTB. Hereto, clinical data from 413 ProPreB control participants was used to perform statistical analyses. Firstly, the performed analyses revealed a significant correlation between cervical length and GA at delivery, thereby confirming the efficacy of cervical length measurement as a screening method to detect women at risk for PTB. Secondly, a significant association was found between GBS status in the third trimester and the occurrence of PTB. In addition, a significant association was observed between the vaginal composition at sub-CST level in the second trimester and GBS status. This observation certainly warrants further investigation, as a knowledge gap remains on how the VM composition influences the risk for GBS colonisation. Although no associations were found between PTB and BV status, vaginal pH, maternal BMI, maternal age and maternal smoking behaviour, further investigations are definitely recommended given the limited participant count with preterm delivery. In general, the findings in this Master's thesis contribute to the identification of factors to detect women at risk for PTB.

Lastly, vertical transmission of vaginal microbiota members from mother to daughter at birth was explored. For that purpose, a culturing campaign was set up to isolate vaginal lactobacilli from vaginal swabs of mothers and daughters from the Isala project. This resulted in an optimised pipeline for isolating vaginal lactobacilli from vaginal swabs, which is highly relevant for future isolation campaigns. By using this optimised protocol, a total of 258 vaginal lactobacilli were isolated. 16S rRNA gene and whole genome sequences were subsequently used for phylogenetic analyses to assess the genetic relationship between mother and daughter isolates. In general, based on this phylogenetic analyses, several interesting findings were obtained. Firstly, *L. crispatus* isolates from one mother-daughter pair were observed within the same clade. This daughter was delivered by C-section, thereby raising questions on the possibility of vertical transfer and stressing the importance of further research. Secondly, bacterial isolates of two sisters, living together at the moment of sampling, were found within the same clade. Lastly, it was shown that isolates from the same participant often cluster together within the same clade, suggesting that they originate from a common bacterial ancestor. All together, these findings provide dedicated research questions and stress the importance of further research on the origin of the VM and the effects of different transmission routes.

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Supplementary Material

Appendix A: Analysis of alpha-diversity and vaginal pH throughout pregnancy

Table 1: Mean inverse Simpson diversity indices (D-values). *Table shows the mean D-values per visit for participants who delivered at term (N= 86), preterm (N = 5) and the total population (N = 92).*

Visit	Mean value at term	Mean value preterm	Mean value (total population)
1	1.40	1.48	1.40
2	1.47	1.61	1.47
3	1.54	1.97	1.56

Table 2: Overview of the performed statistical tests and p-values to compare the inverse Simpson diversity indices (D-values). *Table shows the performed statistical tests and p-values to compare D-values between participants who delivered at term (N = 86) and participants who delivered preterm (N = 5) for each visit, and to compare the inverse Simpson diversity indices between all visits in the total population (N = 92).*

Compared	Statistical test	p-value
Visit 1 preterm – Visit 1 at term	Wilcoxon rank sum test	0.7872
Visit 2 preterm – Visit 2 at term	Wilcoxon rank sum test	0.3886
Visit 3 preterm – Visit 3 at term	Wilcoxon rank sum test	0.5137
Visit 1-Visit 2 (whole population)	Wilcoxon signed rank test + Bonferroni correction	>1.0
Visit 2-Visit 3 (whole population)	Wilcoxon signed rank test + Bonferroni correction	0.3764
Visit 1 – Visit 3 (whole population)	Wilcoxon signed rank test + Bonferroni correction	0.3457

Table 3: Mean vaginal pH values per visit. *Table shows the mean pH values for participants who delivered at term and preterm, and the whole population. The number of participants (N) differs per group as specified between brackets.*

Visit	Mean value at term	Mean value preterm	Mean value (whole population)
1	3.97 (N = 304)	3.98 (N = 25)	3.973 (N = 413)
2	4.03 (N = 258)	4.05 (N = 21)	4.026 (N = 310)
3	4.10 (N = 224)	4.04 (N = 9)	4.099 (N = 247)

Table 4: Overview of the performed statistical tests and p-values to compare the vaginal pH. *Table shows the performed statistical tests and p-values to compare vaginal pH between participants who delivered at term and participants who delivered preterm for each visit, and to compare the vaginal pH between all visits in the total population.*

Compared	Statistical test	p-value
Visit 1 preterm – visit 1 at term	Wilcoxon rank sum test	0.859
Visit 2 preterm – visit 2 at term	Wilcoxon rank sum test	0.590
Visit 3 preterm – visit 3 at term	Wilcoxon rank sum test	0.5137
Visit 1-Visit 2	Wilcoxon signed rank test + Bonferroni correction	3.4891E-3
Visit 1-Visit 3	Wilcoxon signed rank test + Bonferroni correction	6.5080E-8****
Visit 2– Visit 3	Wilcoxon signed rank test + Bonferroni correction	00.0531*

Appendix B: Overview of the obtained lactobacilli

***Table 1: Overview of all vaginal lactobacilli (N = 258**). Table gives an overview of all vaginal lactobacillus isolates obtained from selected mothers and daughters, their pair code, medium from which they were isolated and their species identity and completeness. In total, 258 isolates were obtained from all pairs and groups. The species names of the vaginal isolates are shown, identified based on their 16s rRNA gene sequences (via https://www.ezbiocloud.net/) unless mentioned otherwise in comments column. Abbreviations: P = pair, G = group, GM = grandmother, M = mother, D= daughter.*

AMBV nummer	Pair code	Medium	Top hit species	Species identity (%)	Completeness (%)	Comments
AMBV2310	P1M	MRS	*Lactobacillus crispatus*	100	31.3	
AMBV2311	P1D	MRS pH 4.6	*Lactobacillus jensenii*	93.08	37	
AMBV2317	P1D	MRS	*Lactobacillus jensenii*	100	36.9	
AMBV2318	P1D	MRS	*Lactobacillus jensenii*	100	34.7	
AMBV2325	P1D	MRS	*Lactobacillus jensenii*	100	33.5	
AMBV2326	P1D	MRS	*Lactobacillus jensenii*	100	36.4	
AMBV2327	P1D	MRS	*Lactobacillus jensenii*	100	37.2	
AMBV2328	P1D	MRS	*Lactobacillus fornicalis*	83.97	70.9	
AMBV2329	P1D	MRS	*Lactobacillus jensenii*	100	16	
AMBV2330	P1D	MRS	*Lactobacillus jensenii*	100	33.9	
AMBV2331	P1D	MRS	*Lactobacillus jensenii*	78.12	68.1	
AMBV2332	P1D	MRS	*Lactobacillus crispatus*	75.96	48.4	
AMBV2334	P1D	MRS	*Lactobacillus jensenii*	100	36.3	
AMBV2335	P1D	MRS	*Lactobacillus jensenii*	78.51	69.4	
AMBV2336	P1D	MRS	*Lactobacillus jensenii*	100	37.6	
AMBV2337	P1D	MRS	*Lactobacillus fornicalis*	77.33	61	
AMBV2383	P3M	MRS	*Lactobacillus jensenii*	100	18.9	
AMBV2384	P3M	MRS	*Lactobacillus jensenii*	100	41.7	
AMBV2386	P5M	MRS	*Limosilactobacillus reuteri*	99.63	36.3	
AMBV2387	P5M	MRS	*Limosilactobacillus agrestis*	98.92	39.6	
AMBV2388	P5M	MRS	*Lactobacillus crispatus*	100	28.8	
AMBV2389	P5M	MRS	*Limosilactobacillus reuteri subsp. reuteri*	78.37	55.2	
AMBV2390	P5D	MRS	*Levilactobacilus fuyuanensis*	77.11	67.7	
AMBV2391	P5D	MRS	*Limosilactobacillus vaginalis*	100	100	Identified with NCBI nucleotide BLAST
AMBV2392	P5D	MRS	*Limosilactobacillus reuteri*	100	100	Identified with NCBI nucleotide BLAST
AMBV2394	P5D	MRS	*Lactobacillus reuteri*	100	29.8	
AMBV2395	P7M	MRS	*Lactobacillus crispatus*	100	42.2	
AMBV2396	P2M	MRS	*Lactobacillus jensenii*	77.7	34	
AMBV2397	P2M	MRS	*Lactobacillus jensenii*	100	7.5	
AMBV2398	P2M	MRS	*Lactobacillus jensenii*	100	38.9	
AMBV2399	P2M	MRS	*Lactobacillus psittaci*	76.44	55	
AMBV2400	P6M	MRS	*Lactobacillus crispatus*	99.82	29.3	
AMBV2401	P6M	MRS	*Lactobacillus crispatus*	99.48	39.8	
AMBV2402	P6D	MRS	*Lactobacillus crispatus*	100	38.1	Duplicate of AMBV2415
AMBV2403	P7D	MRS	*Lactobacillus crispatus*	100	18.3	
AMBV2404	P7D	MRS	*Lactobacillus crispatus*	100	18.3	Duplicate of AMBV2403
AMBV2405	P7D	MRS	*Lactobacillus crispatus*	100	39.9	
AMBV2406	P7D	MRS	*Lactobacillus crispatus*	100	39.9	Duplicate of AMBV2405
AMBV2407	P7D	MRS	*Lactobacillus crispatus*	100	20.8	
AMBV2408	P7D	MRS	*Lactobacillus crispatus*	100	20.8	Duplicate of AMBV2407
AMBV2409	P6D	MRS	*Lactobacillus crispatus*	100	34.9	
AMBV2410	P6D	MRS	*Lactobacillus crispatus*	100	34.9	Duplicate of AMBV2409
AMBV2411	P2D	MRS	*Lactobacillus crispatus*	100	18.9	
AMBV2412	P2D	MRS	*Lactobacillus crispatus*	100	18.9	Duplicate of AMBV2411
AMBV2413	P6D	MRS	*Lactobacillus crispatus*	100	43.5	
AMBV2414	P6D	MRS	*Lactobacillus crispatus*	100	43.5	Duplicate of AMBV2413
AMBV2415	P6D	MRS	*Lactobacillus crispatus*	100	38.1	
AMBV2416	P6D	MRS	*Lactobacillus crispatus*	100	38.1	Duplicate of AMBV2415
AMBV2417	P2D	MRS	*Lactobacillus kefiranofaciens subsp. kefirgranum*	80.35	71.1	
AMBV2418	P7D	MRS	*Lactobacillus crispatus*	100	39.1	
AMBV2419	P2M	MRS	*Lactobacillus psittaci*	76.44	55	
AMBV2420	P2M	MRS	*Lactobacillus jensenii*	98.18	42	
AMBV2421	P6D	MRS	*Lactobacillus crispatus*	100	28.8	
AMBV2422	P6D	MRS	*Lactobacillus crispatus*	100	20.9	
AMBV2423	P7D	MRS	*Lactobacillus crispatus*	100	42.6	
AMBV2424	P7D	MRS	*Lactobacillus crispatus*	100	21.7	

AMBV2429	P1D	MRS pH 4.6	*Lactobacillus jensenii*	99.01	43.9	
AMBV2430	P3M	MRS pH 4.6	*Lactobacillus jensenii*	99.77	29.3	
AMBV2431	P3M	MRS pH 4.6	*Lactobacillus jensenii*	76.6	59.5	
AMBV2432	P3M	MRS pH 4.6	*Lactobacillus jensenii*	100	15.4	
AMBV2433	P3M	MRS pH 4.6	*Lactobacillus jensenii*	100	100	Identified with NCBI nucleotide BLAST
AMBV2434	P3M	MRS pH 4.6	*Lactobacillus jensenii*	100	32.7	
AMBV2436	P2M	MRS pH 4.6	*Lactobacillus jensenii*	99.84	42.4	
AMBV2437	P2M	MRS pH 4.6	*Lactobacillus jensenii*	100	49.6	
AMBV2439	P2M	MRS pH 4.6	*Lactobacillus jensenii*	100	26.9	
AMBV2440	P2M	MRS pH 4.6	*Lactobacillus psittaci*	76.6	59.5	
AMBV2441	P2M	MRS pH 4.6	*Lactobacillus jensenii*	100	25.5	
AMBV2442	P2M	MRS pH 4.6	*Lactobacillus jensenii*	100	33	
AMBV2443	P2D	MRS pH 4.6	*Lactobacillus jensenii*	100	22	
AMBV2444	P2D	MRS pH 4.6	*Lactobacillus jensenii*	100	24.6	
AMBV2445	P6M	MRS pH 4.6	*Limosilactobacillus reuteri*	99.78	30.6	
AMBV2446	P6M	MRS pH 4.6	*Lactobacillus crispatus*	100	20.4	
AMBV2447	P6M	MRS pH 4.6	*Lactobacillus crispatus*	100	31.1	
AMBV2448	P6M	MRS pH 4.6	*Lactobacillus crispatus*	99.45	26.5	
AMBV2449	P5D	MRS pH 4.6	*Limosilactobacillus reuteri subsp. reuteri*	80.66	64.1	
AMBV2450	P5D	MRS pH 4.6	*Limosilactobacillus reuteri*	100	26.2	
AMBV2451	P5D	MRS pH 4.6	*Limosilactobacillus agrestis*	100	38.4	
AMBV2452	P5D	MRS pH 4.6	*Limosilactobacillus reuteri*	100	53	
AMBV2453	P5D	MRS pH 4.6	*Limosilactobacillus reuteri*	100	35.9	
AMBV2454	P5D	MRS pH 4.6	*Limosilactobacillus reuteri*	100	19.3	
AMBV2455	P5M	MRS pH 4.6	*Limosilactobacillus agrestis*	97.52	42.6	
AMBV2456	P5M	MRS pH 4.6	*Lactobacillus gasseri*	100	37.3	
AMBV2459	P2D	MRS pH 4.6	*Lactobacillus jensenii*	99.29	39.6	
AMBV2460	P6M	MRS pH 4.6	*Limosilactobacillus vaginalis*	100	17.1	
AMBV2461	P6M	MRS pH 4.6	*Lactobacillus reuteri*	99.81	34.4	
AMBV2462	P5D	MRS pH 4.6	*Limosilactobacillus reuteri*	94.85	96	
AMBV2463	P1M	MRS pH 4.6	*Lactobacillus crispatus*	97.82	28.7	
AMBV2464	P1M	MRS pH 4.6	*Lactobacillus crispatus*	100	30.6	
AMBV2465	P1M	MRS pH 4.6	*Lactobacillus crispatus*	100	32.8	
AMBV2466	P3D	MRS pH 4.6	*Lactobacillus crispatus*	100	29.5	
AMBV2468	P7M	MRS pH 4.6	*Lactobacillus jensenii*	100	21.5	
AMBV2469	P7M	MRS pH 4.6	*Lactobacillus jensenii*	100	20.6	
AMBV2470	P7M	MRS pH 4.6	*Lactobacillus jensenii*	100	22.7	
AMBV2471	P7M	MRS pH 4.6	*Lactobacillus jensenii*	100	18.5	
AMBV2472	P7M	MRS pH 4.6	*Lactobacillus jensenii*	100	25.6	
AMBV2473	P7M	MRS pH 4.6	*Lactobacillus jensenii*	100	14.1	
AMBV2474	P1M	MRS pH 4.6	*Lactobacillus crispatus*	100	35.9	
AMBV2475	P1M	MRS pH 4.6	*Lactobacillus crispatus*	100	30.6	Duplicate of AMBV2464
AMBV2476	P2D	MRS pH 4.6	*Lactobacillus jensenii*	100	35.3	
AMBV2477	P2D	MRS pH 4.6	*Companilactobacillus insicii*	99.17	8.5	
AMBV2478	P2D	MRS pH 4.6	*Lactobacillus jensenii*	100	15.9	
AMBV2479	P2D	MRS pH 4.6	*Lactobacillus crispatus*	100	29.9	
AMBV2480	P2D	MRS	*Lactobacillus crispatus*	100	25.1	
AMBV2481	P2D	MRS	*Lactobacillus crispatus*	100	30.9	
AMBV2482	P3D	MRS pH 4.6	*Lactobacillus jensenii*	100	32.4	
AMBV2483	P3D	MRS pH 4.6	*Lactobacillus jensenii*	100	30.8	
AMBV2484	P3D	MRS	*Lactobacillus jensenii*	100	25.6	
AMBV2485	P3D	MRS	*Lactobacillus jensenii*	100	14.9	
AMBV2486	P3D	MRS	*Lactobacillus jensenii*	100	36.8	
AMBV2487	P3D	MRS	*Lactobacillus jensenii*	99.37	33.5	
AMBV2488	P3D	MRS	*Lactobacillus jensenii*	92.01	45.9	
AMBV2489	P3D	MRS	*Lactobacillus jensenii*	100	36.4	
AMBV2490	P6D	MRS	*Lactobacillus crispatus*	95.6	21.4	
AMBV2491	P1M	MRS pH 4.6	*Lactobacillus crispatus*	100	27	
AMBV2492	P1M	MRS pH 4.6	*Lactobacillus crispatus*	100	22.3	
AMBV2493	P2D	MRS	*Lactobacillus crispatus*	100	30.9	
AMBV2494	P2D	MRS	*Lactobacillus helveticus*	100	8.7	
AMBV2495	P8M	MRS pH 4.6	*Lactobacillus gasseri*	99.73	27.5	
AMBV2496	P8M	MRS pH 4.6	*Lactobacillus rodentium*	85.49	70.4	
AMBV2497	P8M	MRS pH 4.6	*Lactobacillus taiwanensis*	90.12	83.6	
AMBV2498	P8M	MRS pH 4.6	*Lactobacillus gasseri*	96.24	98	Identified with NCBI nucleotide BLAST
AMBV2499	P8M	MRS pH 4.6	*Lactobacillus paragasseri*	99.1	69.4	
AMBV2500	P8M	MRS pH 4.6	*Lactobacillus paragasseri*	96.26	67	
AMBV2501	P8M	MRS pH 4.6	*Lactobacillus hominis*	99.7	66.6	
AMBV2502	P8M	MRS pH 4.6	*Lactobacillus paragasseri*	96.31	68.8	
AMBV2503	P8M	MRS pH 4.6	*Lactobacillus paragasseri*	97.1	68.6	
AMBV2504	P8M	MRS pH 4.6	*Lactobacillus paragasseri*	94.29	68.3	
AMBV2505	P8GM	MRS pH 4.6	*Lactobacillus paragasseri*	99.9	64.7	

AMBV2507	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	98.88	37.1	
AMBV2508	P8GM	MRS pH 4.6	*Lactobacillus paragasseri*	100	65.9	
AMBV2509	P8GM	MRS pH 4.6	*Lactobacillus paragasseri*	99.29	68.2	
AMBV2510	P8GM	MRS pH 4.6	*Lactobacillus paragasseri*	98.58	67.8	
AMBV2511	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	96.89	70.8	
AMBV2512	P8GM	MRS pH 4.6	*Lactobacillus paragasseri*	100	68.6	
AMBV2513	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	33.4	
AMBV2514	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	52.6	
AMBV2515	P8M	MRS pH 4.6	*Lactobacillus paragasseri*	100	37.8	
AMBV2516	P8M	MRS pH 4.6	*Lactobacillus gasseri*	99.93	43.5	
AMBV2517	P8M	MRS pH 4.6	*Lactobacillus gasseri*	100	32.6	
AMBV2518	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	32.5	
AMBV2519	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	34.8	
AMBV2521	P8M	MRS pH 4.6	*Lactobacillus crispatus*	100	10.2	
AMBV2522	P8M	MRS pH 4.6	*Lactobacillus gasseri*	100	23.2	
AMBV2523	P8M	MRS pH 4.6	*Lactobacillus gasseri*	100	42.1	
AMBV2524	P8M	MRS pH 4.6	*Lactobacillus gasseri*	100	29.4	
AMBV2525	P8M	MRS pH 4.6	*Lactobacillus gasseri*	99.13	42.5	
AMBV2526	P8M	MRS pH 4.6	*Lactobacillus gasseri*	100	37.2	
AMBV2527	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	31.8	
AMBV2528	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	31.9	
AMBV2529	P8GM	MRS pH 4.6	*Lactobacillus paragasseri*	100	37.1	
AMBV2530	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	32	
AMBV2531	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	33.3	
AMBV2532	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	34.3	
AMBV2533	P8GM	MRS pH 4.6	*Lactobacillus paragasseri*	100	39.3	
AMBV2534	P8GM	MRS pH 4.6	*Lactobacillus gasseri*	100	36.3	
AMBV2535	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	96.2	46.1	
AMBV2536	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	99.81	69.6	
AMBV2537	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	95.29	51.4	
AMBV2538	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	99.8	68.1	
AMBV2539	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	99.9	66.4	
AMBV2540	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	100	67.3	
AMBV2541	P8D	MRS pH 4.6	*Lactobacillus gasseri*	100	28.3	
AMBV2542	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	99.9	69.0	
AMBV2543	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	99.9	65.8	
AMBV2544	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	100	65.7	
AMBV2545	P8D	MRS pH 4.6	*Lactobacillus gasseri*	98.75	29.8	
AMBV2546	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	99.9	64.3	
AMBV2547	P4D	MRS	*Lactobacillus crispatus*	100	67.4	
AMBV2548	P4D	MRS	*Lactobacillus crispatus*	99.9	69.7	
AMBV2549	P4D	MRS	*Lactobacillus crispatus*	100	65.1	
AMBV2550	P4D	MRS	*Lactobacillus crispatus*	100	62.2	
AMBV2551	P4D	MRS	*Lactobacillus crispatus*	99.9	66.6	
AMBV2552	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	99.7	69.4	
AMBV2553	P8D	MRS pH 4.6	*Lactobacillus paragasseri*	99.9	65.8	
AMBV2554	P4D	MRS	*Lactobacillus crispatus*	100	29.9	
AMBV2555	P4D	MRS	*Lactobacillus crispatus*	99.47	65	
AMBV2556	P4D	MRS	*Lactobacillus crispatus*	100	70.3	
AMBV2557	P4D	MRS	*Lactobacillus crispatus*	100	67.3	
AMBV2558	P4D	MRS	*Lactobacillus crispatus*	99.81	70.4	
AMBV2559	P4D	MRS	*Lactobacillus crispatus*	100	68	
AMBV2560	P4D	MRS	*Lactobacillus crispatus*	100	68.2	
AMBV2561	P4D	MRS	*Lactobacillus crispatus*	99.9	67.8	
AMBV2562	P4D	MRS	*Lactobacillus crispatus*	100	70.1	
AMBV2563	P9D2	MRS	*Lactobacillus delbruecki subsp. delbrueckii*	100	33.8	
AMBV2564	P9D2	MRS	*Lactobacillus delbruecki subsp. delbrueckii*	100	31.6	
AMBV2565	P9D2	MRS	*Lactobacillus delbruecki subsp. Lactis*	100	37.4	
AMBV2567	P9D2	MRS	*Limosilactobacillus fermentum*	100	30.7	
AMBV2568	P9D2	MRS	*Limosilactobacillus fermentum*	100	35.1	
AMBV2569	P9D2	MRS	*Limosilactobacillus fermentum*	100	32.8	
AMBV2570	P9D2	MRS	*Lactobacillus delbrueckii subsp. delbrueckii*	100	32.7	
AMBV2571	P9D2	MRS	*Limosilactobacillus fermentum*	99.85	44.6	
AMBV2572	P9D2	MRS	*Limosilactobacillus fermentum*	100	55.6	
AMBV2573	P9D2	MRS	*Limosilactobacillus fermentum*	100	32.6	
AMBV2574	P9D2	MRS	*Limosilactobacillus fermentum*	100	32.5	

AMBV2575	P9D2	MRS	*Limosilactobacillus fermentum*	100	32.6	
AMBV2576	P9D2	MRS	*Limosilactobacillus fermentum*	100	30	
AMBV2577	P9D2	MRS	*Limosilactobacillus fermentum*	100	33.3	
AMBV2578	P9D3	MRS	*Lactobacillus jensenii*	99.3	41	
AMBV2580	P9D3	MRS	*Lactobacillus jensenii*	100	33.2	
AMBV2581	P9D3	MRS	*Lactobacillus jensenii*	100	32.2	
AMBV2583	P9D3	MRS	*Lactobacillus jensenii*	100	39.7	
AMBV2584	P9D3	MRS	*Lactobacillus jensenii*	100	25.1	
AMBV2586	P9D3	MRS	*Lactobacillus jensenii*	100	25.6	
AMBV2587	P9D3	MRS	*Lactobacillus jensenii*	98.81	47.9	
AMBV2588	P9D3	MRS	*Lactobacillus jensenii*	95.87	47.1	
AMBV2589	P9D3	MRS	*Lactobacillus jensenii*	100	39	
AMBV2591	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	99.87	30.7	
AMBV2592	P9D4	MRS pH 4.6	*Limosilactobacillus vaginalis*	99.58	16.5	
AMBV2593	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	99.78	30.1	
AMBV2594	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	99.8	32.6	
AMBV2595	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	100	22.9	
AMBV2596	P9D4	MRS pH 4.6	*Limosilactobacillus vaginalis*	99.66	40.3	
AMBV2597	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	99.73	24.6	
AMBV2598	P9D4	MRS pH 4.6	*Limosilactobacillus portuensis*	99.81	34.8	
AMBV2599	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	99.79	32	
AMBV2600	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	99.79	32.4	
AMBV2601	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	99.8	33.3	
AMBV2603	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	99.8	33.7	
AMBV2606	P9D1	MRS	*Lactobacillus jensenii*	98.87	31.2	
AMBV2607	P9D1	MRS	*Lactobacillus gasseri*	100	33.1	
AMBV2608	P9D1	MRS	*Lactobacillus jensenii*	99.77	29.2	
AMBV2610	P9D1	MRS	*Lactobacillus jensenii*	100	36.2	
AMBV2611	P9D1	MRS	*Lactobacillus jensenii*	99.8	33.8	
AMBV2612	P9D1	MRS	*Lactobacillus jensenii*	99.77	30.8	
AMBV2613	P9D1	MRS	*Lactobacillus jensenii*	97.11	41.1	
AMBV2615	P9D1	MRS	*Lactobacillus jensenii*	100	29	
AMBV2621	P9D4	MRS pH 4.6	*Limosilactobacillus urineamulieris*	99.73	48.8	
AMBV2622	P9D4	MRS pH 4.6	*Limosilactobacillus fastidiosus*	99.21	26.4	
AMBV2623	P9D4	MRS pH 4.6	*Limosilactobacillus reuteri*	100	20.3	
AMBV2624	P9D4	MRS pH 4.6	*Limosilactobacillus portuensis*	99.83	38.4	
AMBV2625	P9D4	MRS pH 4.6	*Lactobacillus paragasseri*	99.86	46.8	
AMBV2626	P9D4	MRS	*Lactobacillus paragasseri*	100	39.7	
AMBV2627	P9D4	MRS	*Lactobacillus gasseri*	100	38.4	
AMBV2628	P9D4	MRS	*Lactobacillus paragasseri*	100	38.4	
AMBV2629	P9D4	MRS	*Lactobacillus gasseri*	100	35.3	
AMBV2630	P9D1	MRS pH 4.6	*Lactobacillus helveticus*	100	11.5	
AMBV2631	P9D1	MRS pH 4.6	*Lactobacillus gasseri*	100	36.9	
AMBV2632	P9D1	MRS pH 4.6	*Lactobacillus gasseri*	99.2	37.2	
AMBV2633	P9D1	MRS pH 4.6	*Lactobacillus gasseri*	100	33.9	
AMBV2634	P9D1	MRS pH 4.6	*Lactobacillus gasseri*	100	37.7	
AMBV2635	P9D1	MRS pH 4.6	*Lactobacillus gasseri*	100	35.2	
AMBV2636	P9D1	MRS pH 4.6	*Lactobacillus paragasseri*	95.8	51	
AMBV2638	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	30.6	Grown under anaerobic conditions
AMBV2640	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.81	35.3	Grown under anaerobic conditions
AMBV2641	P4M	MRS pH 4.6	*Lactobacillus crispatus*	98.29	34.7	Grown under anaerobic conditions
AMBV2642	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.76	30.6	Grown under anaerobic conditions
AMBV2643	P4M	MRS pH 4.6	*Lactobacillus crispatus*	95.57	26.6	Grown under anaerobic conditions
AMBV2644	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	32.3	Grown under anaerobic conditions
AMBV2645	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	36.7	Grown under anaerobic conditions
AMBV2646	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	32	Grown under anaerobic conditions
AMBV2648	P4M	MRS	*Lactobacillus crispatus*	99.64	37.3	Grown under anaerobic conditions
AMBV2649	P4M	MRS	*Lactobacillus crispatus*	100	32.4	Grown under anaerobic conditions
AMBV2650	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	30.4	Grown under anaerobic conditions
AMBV2651	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	29.7	Grown under anaerobic conditions
AMBV2652	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.42	35.1	Grown under anaerobic conditions
AMBV2653	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	33.2	Grown under anaerobic conditions
AMBV2654	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.85	44.4	Grown under anaerobic conditions
AMBV2655	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.84	44.5	Grown under anaerobic conditions
AMBV2656	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.36	34.9	Grown under anaerobic conditions
AMBV2657	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.81	35.1	Grown under anaerobic conditions

AMBV2658	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.82	38.6	Grown under anaerobic conditions
AMBV2659	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	36.3	Grown under anaerobic conditions
AMBV2660	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	29.7	Grown under anaerobic conditions
AMBV2661	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	30.8	Grown under anaerobic conditions
AMBV2662	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	35.6	Grown under anaerobic conditions
AMBV2664	P4M	MRS pH 4.6	*Lactobacillus crispatus*	100	32.4	Grown under anaerobic conditions
AMBV2665	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.84	42.1	Grown under anaerobic conditions
AMBV2666	P4M	MRS pH 4.6	*Lactobacillus crispatus*	99.82	37.6	Grown under anaerobic conditions

Appendix C: Phylogenetic trees based on 16S rRNA gene sequences

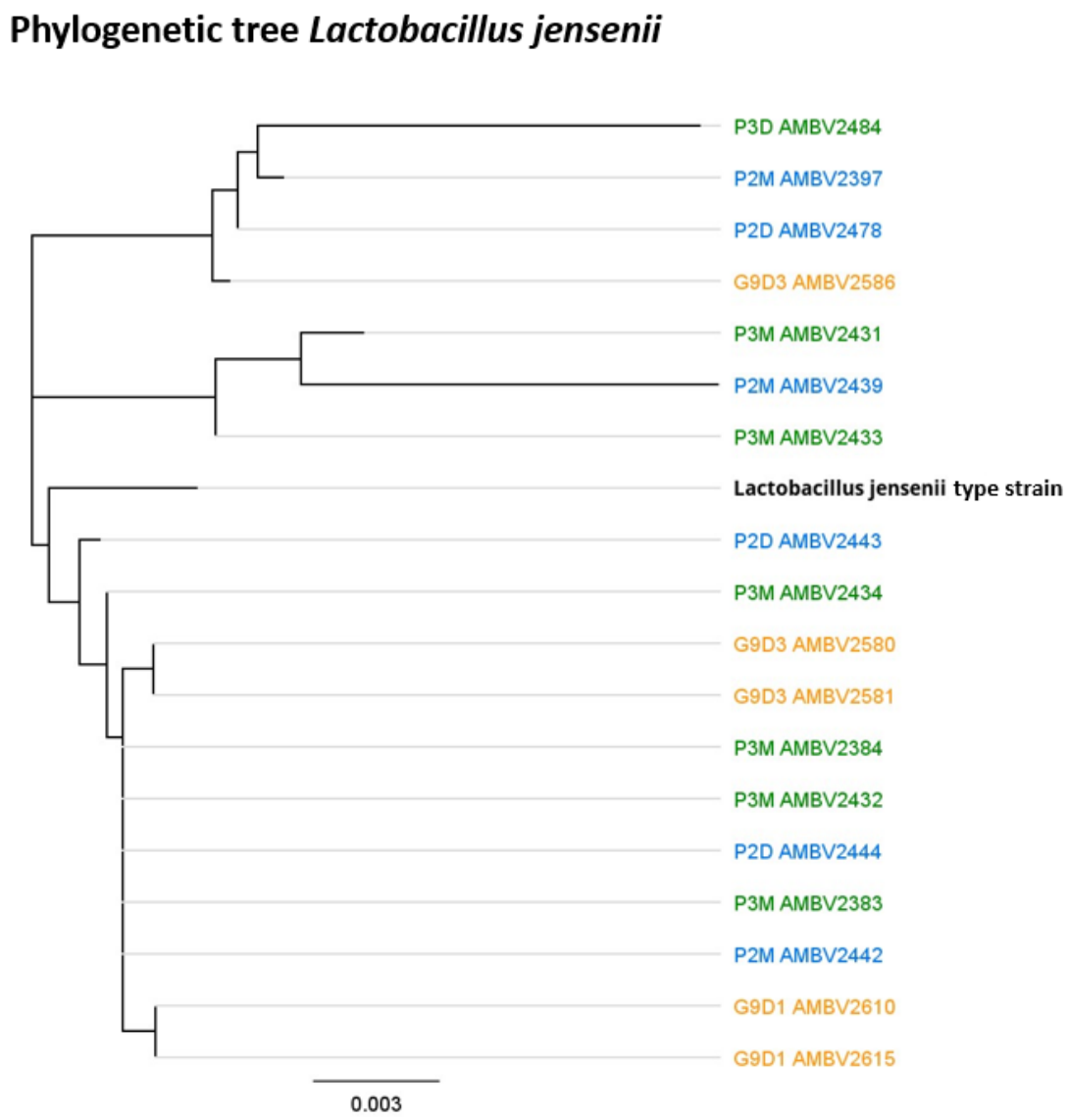

Figure 1: Phylogenetic tree of 16S rRNA gene sequences of L. jensenii isolates and the reference strain. *This tree includes eighteen isolates that were identified as L. jensenii. Isolates are coloured according to the pair or group to which they belong. 'AMBV' strain designation is used by the host lab (LAMB) for bacteria isolated from vaginal (V) ESwabs. Tree was built with Geneious Prime (version 2023.0.4). Abbreviations: P = pair, G = group, M = mother, D = daughter.*

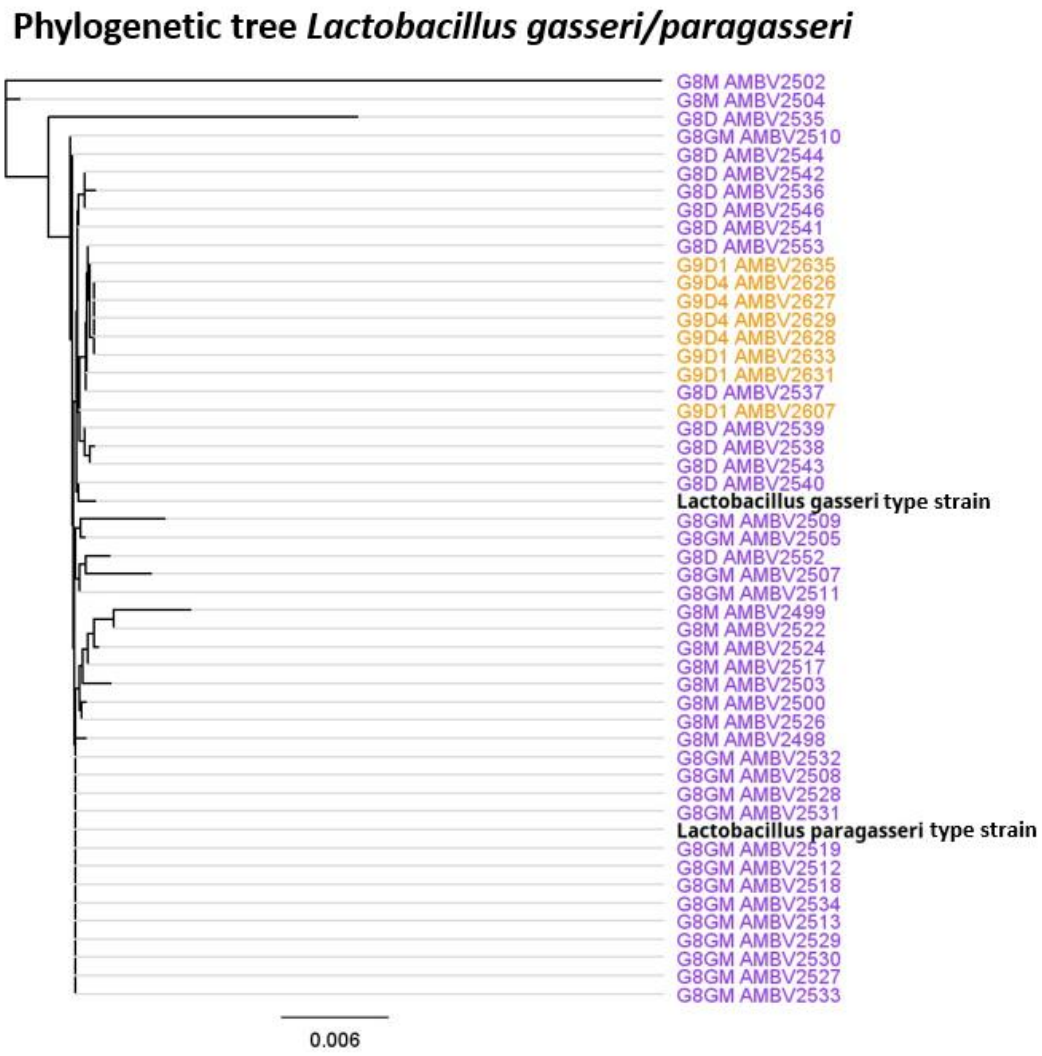

Figure 2: Phylogenetic tree of 16S rRNA gene sequences of L. gasseri and L. paragasseri isolates and their reference strains. *This tree includes 49 isolates that were identified as L. gasseri or L. paragasseri. Isolates are coloured according to the pair or group to which they belong. 'AMBV' strain designation is used by the host lab (LAMB) for bacteria isolated from vaginal (V) ESwabs. Tree was built with Geneious Prime (version 2023.0.4). Abbreviations: P = pair, G = group, GM = grandmother, M = mother, D = daughter.*

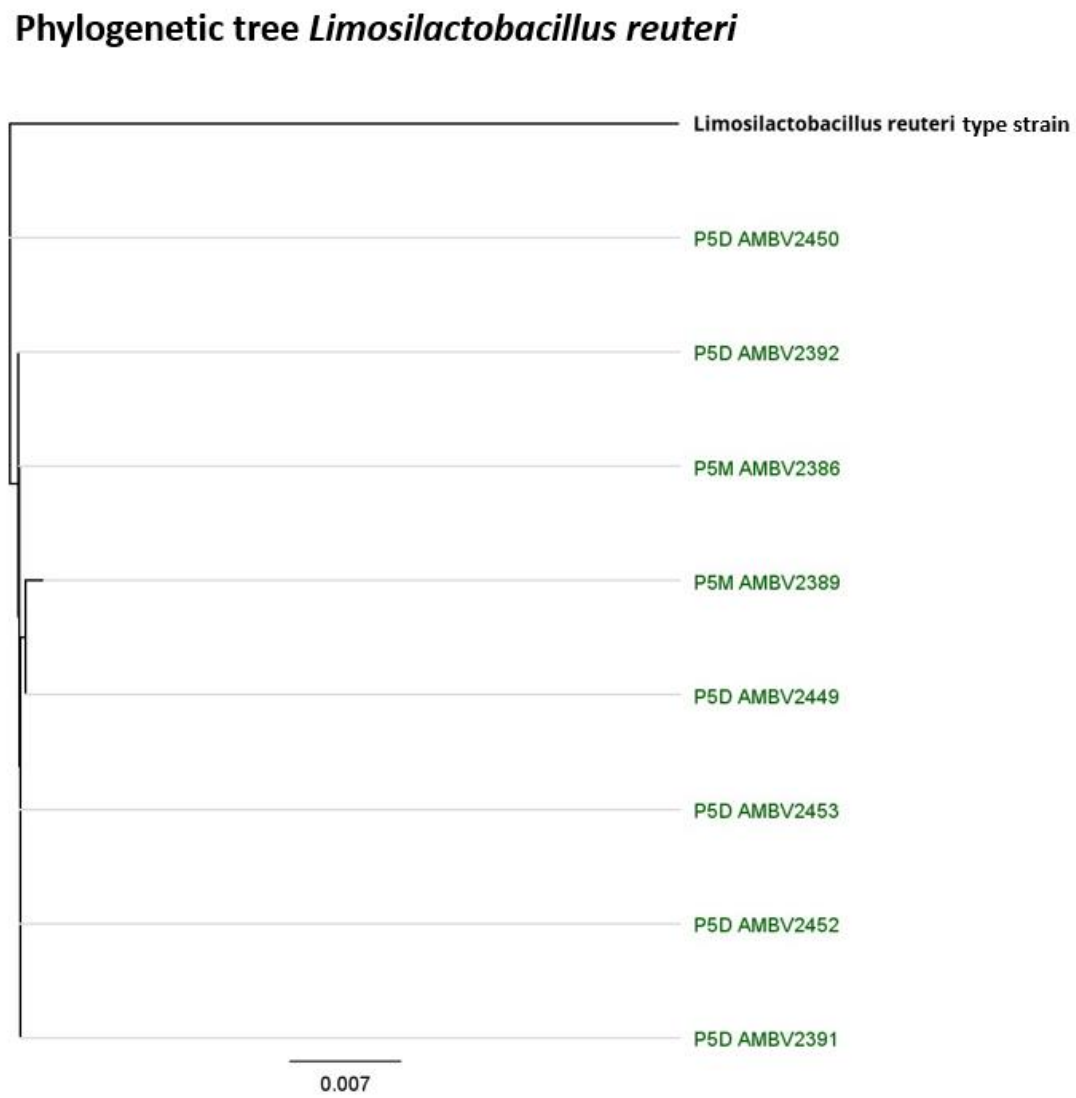

Figure 3: Phylogenetic tree of 16S rRNA gene sequences of L. reuteri isolates and the reference strain. *This tree includes eight isolates that were identified as L. reuteri. Isolates are coloured according to the pair or group to which they belong. 'AMBV' strain designation is used by the host lab (LAMB) for bacteria isolated from vaginal (V) ESwabs. Tree was built with Geneious Prime (version 2023.0.4). Abbreviations: P = pair, M = mother, D = daughter.*