Pediatric RESEARCH

CLINICAL RESEARCH ARTICLE

Lactobacilli-dominated cervical microbiota in women with preterm prelabor rupture of membranes

Marian Kacerovsky[1,2], Lenka Pliskova[3], Radka Bolehovska[3], Romana Gerychova[4], Petr Janku[4], Petr Matlak[5], Ondrej Simetka[5], Tomas Faist[1], Jan Mls[1], Petr Vescicik[1], Helena Zemlickova[6], Bo Jacobsson[7,8,9] and Ivana Musilova[1]

BACKGROUND: To determine the association between microbial invasion of the amniotic cavity (MIAC) and the presence of *Lactobacillus crispatus*- or *Lactobacillus iners*-dominated cervical microbiota in pregnancies with preterm prelabor rupture of membrane. Next, to assess the relationship between the presence of *L. crispatus*- or *L. iners*-dominated cervical microbiota and short-term neonatal morbidity.

METHOD: A total of 311 women were included. Cervical samples were obtained using a Dacron polyester swab and amniotic fluid samples were obtained by transabdominal amniocentesis. Bacterial DNA, *L. crispatus*, and *L. iners* in the cervical samples were assessed by PCR. Cervical microbiota was assigned as *L. crispatus*- or *L. iners*-dominated when the relative abundance of *L. crispatus* or *L. iners* was ≥50% of the whole cervical microbiota, respectively.

RESULTS: Women with MIAC showed a lower rate of *L. crispatus*-dominated cervical microbiota (21% vs. 39%; $p = 0.003$) than those without MIAC. *Lactobacillus crispatus*-dominated cervical microbiota was associated with a lower rate of early-onset sepsis (0% vs. 5%; $p = 0.02$).

CONCLUSIONS: The presence of *L. crispatus*-dominated cervical microbiota in women with preterm prelabor rupture of membrane was associated with a lower risk of intra-amniotic complications and subsequent development of early-onset sepsis of newborns.

Pediatric Research (2020) 87:952–960; https://doi.org/10.1038/s41390-019-0692-1

INTRODUCTION

Preterm prelabor rupture of membrane (PPROM) is defined as the rupture of fetal membranes with amniotic fluid leakage before the onset of regular uterine activity prior to 37 weeks' gestation.[1,2] Although the underlying pathophysiology leading to preterm fetal membrane thinning, weakening, and rupture is well understood, PPROM remains an unpredictable and unpreventable condition.[3]

Approximately between one-third and one-half of all PPROM cases, depending on the assessment techniques, are complicated by the presence of bacteria in the amniotic fluid.[4–6] This condition, known as microbial invasion of the amniotic cavity (MIAC), can be further categorized as intra-amniotic infection or colonization when intra-amniotic inflammation (IAI) is present or absent.[4,7] In most cases of MIAC, bacteria reach the amniotic fluid by ascension from the vagina, cervix, or choriodecidual space. Given that the thinning and rupture of membranes in PPROM predisposes women to MIAC,[8] studies are needed to determine why only a subset of women with PPROM develops MIAC. Understanding the biological mechanisms of MIAC development is critical for devising optimal management strategies for PPROM.

It was previously demonstrated that alterations in the cervicovaginal microbiota may affect the integrity of the cervical epithelial barrier.[9,10] The maintenance of this integrity is crucial for preventing microbial ascension from the vagina or cervix to the choriodecidual space or amniotic cavity.[9] Therefore, the composition of cervicovaginal microbiota may contribute to the development of MIAC and/or IAI in PPROM.

Cervicovaginal microbiota in pregnancy is typically characterized by the dominance of a limited number of *Lactobacillus* species.[11–13] In pregnancies complicated by PPROM, cervicovaginal microbiota dominated by *Lactobacillus* have been observed in at least half of the women studied.[14,15] Among them, the most common species are *Lactobacillus crispatus* and *Lactobacillus iners*.[14,15]

The presence of *L. crispatus*-dominated cervicovaginal microbiota is considered to represent a healthy cervicovaginal state and lower risk of bacterial vaginosis.[16] In PPROM pregnancies, *L. crispatus*-dominated cervicovaginal microbiota has been shown to be associated with a low risk of intra-amniotic infection and early-onset sepsis in newborns from PPROM pregnancies.[14,15] In contrast, *L. iners*-dominated cervicovaginal microbiota has been shown to be associated with both normal and abnormal (bacterial vaginosis) cervicovaginal flora.[17] Additionally, the presence of *L. iners*-dominated cervicovaginal microbiota at week 16 of

[1]Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Charles University Faculty of Medicine in Hradec Kralove, Hradec Kralove, Czech Republic; [2]Biomedical Research Center, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic; [3]Institute of Clinical Biochemistry and Diagnostics, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic; [4]Department of Obstetrics and Gynecology, University Hospital Brno, Faculty of Medicine Masaryk University, Brno, Czech Republic; [5]Department of Obstetrics and Gynecology, University Hospital Ostrava, Ostrava, Czech Republic; [6]Institute of Clinical Microbiology, University Hospital in Hradec Kralove, Faculty of Medicine Hradec Kralove, Charles University, Hradec Kralove, Czech Republic; [7]Department of Obstetrics and Gynecology, Institute of Clinical Science, Sahlgrenska Academy, Gothenburg University, Gothenburg, Sweden; [8]Department of Obstetrics and Gynecology, Region Västra Götaland, Sahlgrenska University Hospital, Gothenburg, Sweden and [9]Department of Genetics and Bioinformatics, Domain of Health Data and Digitalisation, Institute of Public Health, Oslo, Norway
Correspondence: Marian Kacerovsky (kacermar@fnhk.cz)

Received: 19 June 2019 Revised: 28 October 2019 Accepted: 31 October 2019
Published online: 2 December 2019

gestation has been shown to be associated with an increased risk of a short cervix or preterm birth.[18]

It is well known that *L. crispatus* exerts several antibacterial properties by producing lactic acid and hydrogen peroxide, and can up-regulate the production of antimicrobial peptides and bacteriocins.[9,19] *Lactobacillus crispatus* can inhibit pathogens associated with bacterial vaginosis, as well as *Escherichia coli*.[20] Recently, *L. crispatus* has been shown to protect the epithelial barrier in the presence of inflammatory stimuli.[9] Taking together, the protective effect of *L. crispatus* may play an important role in preventing MIAC.

Although *L. crispatus*- or *L. iners*-dominated cervicovaginal microbiota is detected in most PPROM pregnancies,[14,15] its association with MIAC and/or IAI, as well as with the potential maternal and neonatal consequences in PPROM, remains unclear.

Therefore, this study was conducted to determine the association between the presence of *L. crispatus*- or *L. iners*-dominated cervical microbiota and MIAC and/or IAI in PPROM pregnancies. We also assessed the association between the presence of *L. crispatus*- or *L. iners*-dominated cervical microbiota and development of postpartum endometritis and short-term neonatal morbidity.

MATERIALS AND METHODS

This retrospective cohort study of pregnant women between 24^{+0} and 36^{+6} weeks' gestation who were admitted to the Department of Obstetrics and Gynecology of the University Hospital Hradec Kralove in the Czech Republic was conducted between August 2015 and December 2018. Women with singleton pregnancies complicated by the presence of PPROM and aged ≥18 years were recruited. Women with pregnancy-related and other medical complications, such as fetal growth restriction, congenital or chromosomal fetal abnormalities, gestational or pregestational diabetes, gestational hypertension, preeclampsia, signs of fetal hypoxia, or significant vaginal bleeding, were excluded from the study.

Gestational age was established by first-trimester fetal biometry. Women with PPROM between 24^{+0} and 34^{+6} weeks' gestation were treated with antibiotics and corticosteroids to accelerate lung maturation and reduce neonatal mortality and morbidity, whereas antibiotic treatment alone was administered to those beyond 34^{+6} weeks' gestation. Women with proven microbial-associated IAI (amniotic fluid interleukin-6 [IL-6] ≥745 pg/mL and the presence of MIAC) beyond 28 weeks' gestation were actively managed. In actively managed women, labor was induced, or an elective cesarean section was performed within 72 h of membrane rupture. The remaining women with PPROM were subjected to expectative management.

PPROM was diagnosed by examining the women using a sterile speculum to verify the pooling of amniotic fluid in the vagina. If clinical PPROM was suspected, amniotic fluid leakage was confirmed by the presence of insulin-like growth factor-binding proteins (Actim PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

This study was approved by the Institutional Review Board Committee (July 2014; No. 201408 S07P), and informed consent was obtained from all participants, who were all Caucasian.

Cervical sampling

Cervical samples were obtained prior to administration of corticosteroids, antibiotics, or tocolytics using a Dacron polyester swab placed in the cervical canal for 20 s to achieve saturation. Upon collection, the Dacron polyester swab was inserted into a polypropylene tube containing 1.5 mL of phosphate-buffered saline. Each tube was shaken for 20 min, followed by centrifugation at $300 \times g$ for 15 min at room temperature. The supernatant was aliquoted and stored at −70 °C until further analyses. The pellet was sent to the molecular biology laboratory to assess the presence of bacterial DNA, *L. crispatus*, and *L. iners*.

Amniotic fluid sampling

Ultrasonography-guided transabdominal amniocentesis was performed prior to administering corticosteroids, antibiotics, or tocolytics. Approximately 5 mL of amniotic fluid was aspirated, and a tube containing uncentrifuged amniotic fluid was transported to the laboratory for DNA isolation and PCR detection of *Ureaplasma* spp., *Mycoplasma hominis*, *Chlamydia trachomatis*, and 16S ribosomal RNA (rRNA) gene sequencing, as well as for aerobic and anaerobic cultivation.

Detection of cervical L. crispatus and *L. iners*

Nucleic acid was isolated from the pellets using the tissue protocol with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Detection of *L. crispatus* and *L. iners* was performed by in-house reverse transcription-PCR (RT-PCR). Primers and hydrolysis probes for *L. crispatus* were designed from the 16S rRNA region to amplify a 150-base pair (bp) amplicon (forward primer LC F35 GCG AGC GGA ACT AAC AGA TT, reverse primer LC R184 TGA TCA TGC GAT CTG CTT TC and probe-labeled FAM-BHQ1 CTG CCC CAT AGT CTG GGA TA). For *L. iners* PCR detection, we used a set of previously published primers and probes (forward primer LI F AGT CTG CCT TGA AGA TCG G, reverse primer LI R CTT TTA AAC AGT TGA TAG GCA TCA TC and probe FAM-BHQ1 CCA AGA GAT CGG GAT AAC ACC T).[21] RT-PCR was performed on a Rotor-Gene Q instrument (Qiagen) in 25-μL reactions containing universal 2× gb IPC PCR Master Mix (Generi Biotech, Hradec Kralove, Czech Republic) with an internal positive control, primers at concentrations of 400 nM each, and dual-labeled hydrolysis probes (FAM-BHQ1) at a concentration of 200 nM. Primers and probes were synthesized by Generi Biotech. Amplification parameters were as follows: 95 °C for 5 min, followed by 45 cycles of 95 °C 15 s and 60 °C for 30 s. PCR detection was performed by absolute quantification and a standard curve was generated from serial 10-fold dilutions of linearized and normalized plasmids containing the cloned target sequences, first for *L. crispatus* and then for *L. iners* both at concentrations of 10^7 copies/μL (Generi Biotech, Hradec Kralove, Czech Republic). We also tested our quantitative PCR method using a collection of strains for each of the bacterial targets as well as using related bacterial species, and only the intended targets showed positive results (data not shown).

To identify the dominant bacteria (*L. crispatus* or *L. iners*), total bacterial DNA detection was performed by quantitative RT-PCR—BactQuant.[22] To quantify the bacterial load, we used the forward primer CCT ACG GGD GGC WGC A, reverse primer GGA CTA CHV GGG TMT CTA ATC, and hydrolysis probe FAM-BHQ1 CAG CCG CGG TA, and a calibration curve was generated using 10-fold dilutions of linearized and normalized plasmid containing the cloned target sequence of the 466-bp region in the V3–V4 domain of 16S rRNA at a concentration of 10^7 copies/μL (Generi Biotech). Lactobacilli dominance was expressed by relative quantification as the ratio of the DNA quantity of *L. crispatus* or *L. iners* to the total bacterial DNA load. The PCR conditions used in the BactQuant assay were the same as those used for lactobacilli.

Detection of *Ureaplasma* species, *M. hominis*, and *C. trachomatis*

DNA was isolated from the amniotic fluid using a QIAamp DNA Mini Kit according to the manufacturer's instructions (using the protocol for isolating bacterial DNA from biological fluids). RT-PCR was performed using a Rotor-Gene 6000 instrument using the commercial AmpliSens® *C. trachomatis*/*Ureaplasma*/*M. hominis*-FRT Kit (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect the DNA from *Ureaplasma* species, *M. hominis*, and *C. trachomatis* in the same PCR tube. As a control, we amplified β-actin, a housekeeping gene, to exclude the presence of PCR inhibitors. The concentration of *Ureaplasma* spp. DNA (copies/mL) was determined using an absolute quantification technique using an external calibration curve. Plasmid DNA (pCR4; Invitrogen, Carlsbad, CA) was used to construct the calibration curve.

Detection of other bacteria in amniotic fluid

Bacterial DNA was identified by PCR targeting *16S rRNA* using the following primers: 5′-CCAGACTCCTACGGGAGGCAG-3′ (V3 region) and 5′-ACATTTCACAACACGAGCTGACGA-3′ (V6 region).[23] Each reaction contained 3 µL target DNA, 500 nM forward and reverse primers, and Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) in a total volume of 25 µL. Amplification was performed using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The products were visualized on an agarose gel. Positive reactions yielded 950-bp products that were subsequently analyzed by sequencing. The 16S PCR products were purified and sequenced by PCR using the above primers and BigDye Terminator Kit v3.1 (Thermo Fisher Scientific, Waltham, MA). The bacteria were then typed using the sequences obtained from BLAST® and SepsiTest™ BLAST.

Aerobic and anaerobic cultures of amniotic fluid

The amniotic fluid samples were cultured in Columbia agar with sheep's blood, *Gardnerella vaginalis* selective medium, MacConkey agar, *Neisseria*-selective medium (modified Thayer–Martin medium), Sabouraud agar, and Schaedler anaerobe agar. The plates were cultured for 6 days and checked daily. The species were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using MALDI Biotyper software (Bruker Daltonics, Billerica, MA).

Amniotic fluid IL-6 concentrations

IL-6 concentrations were assessed using a Milenia QuickLine IL-6 lateral flow immunoassay using a Milenia POCScan reader (Milenia Biotec, GmbH, Giessen, Germany). The measurement range was 50–10,000 pg/mL. The intra- and inter-assay variations were 12.1% and 15.5%, respectively.[24]

Definition of *L. crispatus*- and *L. iners*-dominated cervical microbiota

Cervical microbiota was assigned as *L. crispatus*- or *L. iners*-dominated when the relative abundance of *L. crispatus* or *L. iners* was ≥50% of the total cervical microbiota, respectively (cervical microbial loads of either *L. crispatus* or *L. iners* ≥50% of amount of the cervical bacterial DNA).[25]

Diagnosis of MIAC

MIAC was determined based on a positive PCR analysis of *Ureaplasma* species, *M. hominis*, *C. trachomatis*, or a combination of these species or positivity for 16S rRNA or aerobic/anaerobic cultivation of the amniotic fluid or a combination of these parameters.

Diagnosis of IAI

IAI in PPROM pregnancies was defined as amniotic fluid bedside IL-6 concentrations ≥745 pg/mL.[26,27]

Based on the presence of MIAC and/or IAI, the women were categorized into four groups: with intra-amniotic infection (both MIAC and IAI), with sterile IAI (IAI without MIAC), with colonization (MIAC without IAI), and without MIAC or IAI.

Definition of postpartum endometritis

Postpartum endometritis was defined as the presence of fever (temperature ≥38 °C) and/or elevated (>10 mg/L) maternal serum C-reactive protein in combination with low abdominal pain, an enlarged, tender uterus, or foul-smelling discharge during the first 10 days after delivery, with no other obvious source of infection.[28]

Definitions of selected aspects of short-term neonatal morbidity

Maternal and perinatal medical records were reviewed by four investigators (M.K., T.F., J.M., P.V.). Data regarding short-term neonatal morbidity were reviewed for all newborns. "Compound neonatal morbidity" was defined in this study as follows: the need for intubation, and/or need for nasal continuous positive airway pressure, and/or respiratory distress syndrome (defined by the presence of two or more of the following criteria: evidence of respiratory compromise, persistent oxygen requirement for more than 24 h, administration of exogenous surfactant, radiographic evidence of hyaline membrane disease); and/or transient tachypnea of newborns (defined as any oxygen supplement requirement during the first 6 h that did not increase during the subsequent 18 h as clinical conditions improved within 3–6 h and chest radiographs either normal or indicating reduced translucency, infiltrates, and hyperinsufflation of the lungs); and/or bronchopulmonary dysplasia (defined as infant oxygen requirement at 28 days of age); and/or pneumonia (diagnosed by abnormal findings on chest X rays); and/or retinopathy of prematurity (identified using retinoscopy); and/or intraventricular hemorrhage (diagnosed by cranial ultrasound examinations according as described by Papile et al.[29]); and/or necrotizing enterocolitis (defined as radiologic findings of either intramural gas or free intra-abdominal gas); and/or early-onset (during the first 72 h of life) or late-onset (between the ages of 4 and 120 days) sepsis (either proven by bacterial culture or clinically highly suspected sepsis); and/or neonatal death before hospital discharge.

Statistical analyses

The normality of the data was tested using the D'Agostino–Pearson omnibus normality test and Shapiro–Wilk test. Continuous variables were compared by nonparametric Mann–Whitney *U* test and presented as the median value (interquartile range [IQR]). Categorical variables were compared using Fisher's exact test or Cochran–Armitage test for trends, as appropriate, and presented as a number (%). A partial correlation was used to adjust the results for gestational age at delivery. Differences were considered significant at $p < 0.05$. All p values were obtained from two-tailed tests, and all statistical analyses were performed using GraphPad Prism version 6.0 h software for Mac OS X (GraphPad Inc., San Diego, CA, USA) and SPSS version 19.0 for Mac OS X (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 356 women with singleton pregnancies at 24^{+0} to 36^{+6} weeks' gestation were admitted during the study period. Nineteen women were excluded because cervical fluid was not obtained ($n = 12$), amniocentesis failure ($n = 2$), unconfirmed PPROM ($n = 2$), and delivery prior to amniocentesis ($n = 3$). Twenty-six women were subsequently excluded for the following medical reasons: gestational diabetes mellitus ($n = 9$), fetal growth restriction ($n = 6$), chronic hypertension ($n = 3$), pre-gestation diabetes mellitus ($n = 3$), preeclampsia ($n = 3$), and pregnancy induced hypertension ($n = 2$). A total of 311 women were included in the study.

MIAC was observed in 23% (73/311) of the women. The women's demographic and clinical data based on the presence and absence of MIAC are shown in Table 1. IAI was observed in 17% (53/311) of the women.

MIAC and amount of cervical bacterial DNA, microbial loads of cervical *L. crispatus* and *L. iners*

No differences were found in the amounts of cervical bacterial DNA between women with and without MIAC (with MIAC: median 2.76×10^6 copies DNA/mL, IQR 3.00×10^5–1.82×10^7 vs. without MIAC: median 3.37×10^6 copies DNA/mL, IQR 7.38×10^5–2.14×10^7; $p = 0.22$).

The cervical presence of *L. crispatus* DNA was identified in 77% (56/73) and 74% (177/238) of women with and without MIAC, respectively. Women with MIAC had a lower cervical microbial load of *L. crispatus* than women without MIAC (with MIAC: median 1.63×10^4 copies DNA/mL, IQR 2.94×10^2–1.34×10^6 vs. without

Table 1. Maternal and clinical characteristics of pregnancies complicated by preterm prelabor rupture of membranes with respect to the presence and the absence of microbial invasion of the amniotic cavity.

Characteristic	With microbial invasion of the amniotic cavity ($n = 73$)	Without microbial invasion of the amniotic cavity ($n = 238$)	p Value
Maternal age [years, median (IQR)]	30 (24–34)	31 (27-34)	**0.04**
Primiparous [number (%)]	36 (49%)	152 (64%)	**0.03**
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	22.1 (19.6–25.2)	23.6 (21.0–27.6)	**0.009**
Smoking [number (%)]	16 (22%)	37 (16%)	0.22
Interval between PPROM and amniocentesis [hours, median (IQR)]	4 (3–8)	4 (3-7)	1.00
Gestational age at admission [weeks, median (IQR)]	33 + 3 (29 + 6–35 + 3)	34 + 3 (32 + 4–35 + 3)	**0.009**
Gestational age at delivery [weeks, median (IQR)]	33 + 4 (30 + 2–35 + 4)	34 + 4 (33 + 0–35 + 6)	**0.002**
Latency between PPROM and delivery [hours, median (IQR)]	60 (22–111)	38 (15–110)	0.19
Intra-amniotic inflammation [number (%)]	34 (47%)	19 (8%)	**<0.0001**
CRP levels at admission [mg/L, median (IQR)]	5.8 (2.5–13.3)	4.6 (2.4–8.3)	0.10
WBC count at admission [×10^9 L, median (IQR)]	12.6 (10.4–15.5)	12.2 (10.1–14.6)	0.44
Administration of antibiotics [number (%)]	66 (90%)	203 (85%)	0.33
Administration of corticosteroids [number (%)]	51 (70%)	148 (62%)	0.27
Spontaneous vaginal delivery [number (%)]	54 (74%)	160 (67%)	0.31
Forceps delivery [number (%)]	18 (25%)	73 (31%)	0.38
Cesarean delivery [number (%)]	1 (1%)	5 (2%)	1.00
Birth weight [g, median (IQR)]	2020 (1530–2495)	2275 (1890–2615)	**0.01**
Apgar score <7; 5 min [number (%)]	5 (7%)	7 (3%)	0.16
Apgar score <7; 10 min [number (%)]	2 (3%)	3 (1%)	0.33
Postpartum endometritis [number (%)]	3 (4%)	3 (1%)	0.14

CRP C-reactive protein, *IQR* interquartile range, *PPROM* preterm prelabor rupture of membranes, *WBC* white blood cells.
Continuous variables were compared using a nonparametric Mann–Whitney *U* test. Categorical variables were compared using the Fisher's exact test.
Continuous variables are presented as median (IQR) and categorical as number (%).
Statistically significant results are marked in bold.

MIAC: median 2.64×10^5 copies DNA/mL, IQR 1.18×10^3–3.87×10^6; $p = 0.04$; Fig. 1a)

The presence of *L. iners* DNA in the cervical fluid was observed in 88% (64/73) and 85% (203/238) of women with and without MIAC, respectively. Cervical microbial loads of *L. iners* between women with and without MIAC did not significantly differ (with MIAC: median 4.83×10^5 copies DNA/mL, IQR 3.77×10^3–5.55×10^6 vs. without MIAC: median 3.81×10^5 copies DNA/mL, IQR 1.74×10^3–3.37×10^6; $p = 0.41$; Fig. 1b).

MIAC and *L. crispatus*- and *L. iners*-dominated cervical microbiota

Lactobacillus crispatus- and *L. iners*-dominated cervical microbiota were found in 35% (109/311) and 41% (126/311) of women, respectively. Women with MIAC had a lower rate of *L. crispatus*-dominated cervical microbiota [with MIAC: 21% (15/73) vs. without MIAC: 39% (94/238); $p = 0.003$; Fig. 2a] than women without MIAC. No difference in the rate of *L. iners*-dominated cervical microbiota [with MIAC: 48% (35/73) vs. without MIAC: 38% (91/238); $p = 0.17$; Fig. 2a] was observed between women with and without MIAC.

When women with MIAC were split into three subgroups based on the presence of *Ureaplasma* spp. and/or other bacteria in the amniotic fluid, the subgroup of women with only *Ureaplasma* spp. in the amniotic fluid had a higher rate of *L. crispatus*-dominated cervical microbiota than the other subgroups, [*Ureaplasma* spp.: 30% (12/40) vs. *Ureaplasma* spp. + other bacteria: 13% (1/8) vs. other bacteria: 8% (2/25); $p = 0.03$; Fig. 2b]. No difference in the rates of *L. iners*-dominated cervical microbiota among these subgroups was observed [*Ureaplasma* spp.: 45% (18/40) vs. *Ureaplasma* spp. + others: 50% (4/8) vs. others: 52% (9/25); $p = 0.50$; Fig. 2b].

MIAC and/or IAI and amount of cervical bacterial DNA, microbial loads of cervical *L. crispatus* and *L. iners*

No differences were found in the amounts of cervical bacterial DNA between women with intra-amniotic infection, sterile IAI, colonization, and without MIAC and IAI (with infection: median 4.24×10^6 copies DNA/mL, IQR 3.00×10^5–3.10×10^7, sterile IAI: median 5.62×10^6 copies DNA/mL, IQR 6.57×10^5–7.37×10^7, colonization: median 2.41×10^6, IQR 2.96×10^5 –1.07×10^7, without: median 3.34×10^6, IQR 7.38×10^5–2.26×10^7; $p = 0.22$).

No differences were found in the amounts of *L. crispatus* DNA between women with intra-amniotic infection, sterile IAI, colonization, and without MIAC and IAI (with infection: median 6.81×10^2 copies DNA/mL, IQR 0–2.63×10^5, sterile IAI: median 3.61×10^2 copies DNA/mL, IQR 0–1.29×10^6, colonization: median 1.90×10^3, IQR 3.57×10^1–3.24×10^5, without: median 6.94×10^3, IQR 2.10×10^0–2.20×10^6; $p = 0.49$; Fig. 3a)

No differences were found in the amounts of *Lactobacillus iners* DNA between women with intra-amniotic infection, sterile IAI,

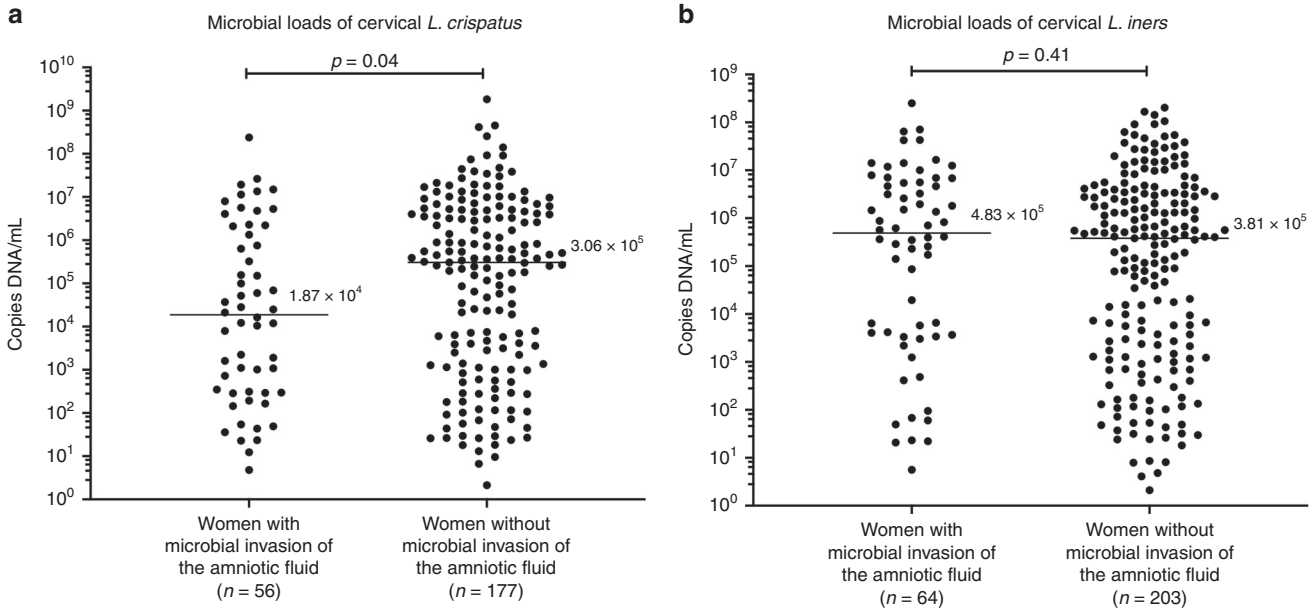

Fig. 1 Microbial loads (copies DNA/mL) of cervical *L. crispatus* (**a**) and *L. iners* (**b**) based on the presence or absence of microbial invasion of the amniotic cavity.

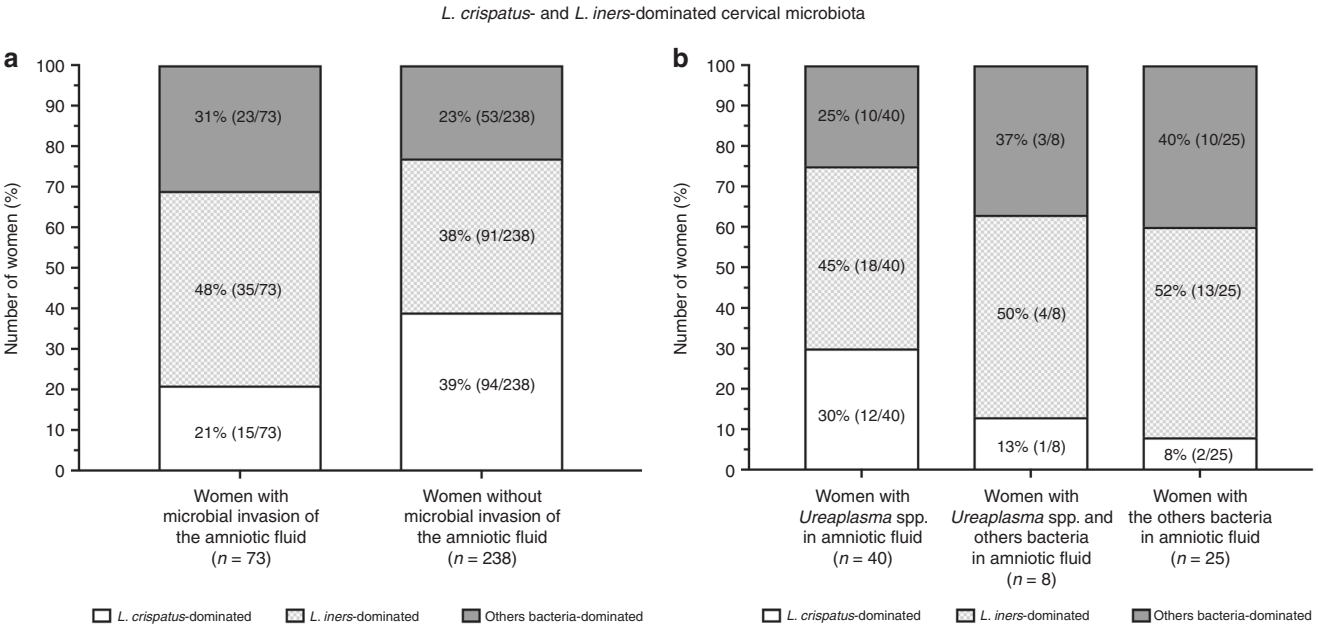

Fig. 2 Rates of *L. crispatus*- and *L. iners*-dominated cervical microbiota based on the presence or absence of microbial invasion of the amniotic cavity (**a**) and the presence of *Ureaplasma* spp. and/or other bacteria in the amniotic fluid (**b**).

colonization, and without MIAC and IAI (with infection: median 5.35×10^5 copies DNA/mL, IQR 1.0×10^3–6.7×10^6, sterile IAI: median 6.42×10^5 copies DNA/mL, IQR 1.80×10^2–3.61×10^6, colonization: median 1.41×10^5, IQR 6.79×10^3 – 2.59×10^6, without: median 7.89×10^4, IQR 1.18×10^2 - 2.41×10^6; $p = 0.36$; Fig. 3a)

MIAC and/or IAI and *L. crispatus*- and *L. iners*-dominated cervical microbiota

Differences in the rates of *L. crispatus*- and *L. iners*-dominated cervical microbiota were found among women with intra-amniotic infection, sterile IAI, and colonization, and among women without MIAC and IAI [*L. crispatus* infection: 15% (5/34), sterile IAI: 42% (8/19), colonization: 26% (10/39), without: 39% (86/219); $p = 0.01$; *L. iners* infection: 56% (19/34), sterile IAI: 53% (10/19), colonization: 41% (16/39), without: 37% (81/219); $p = 0.02$; Fig. 4].

Postpartum endometritis and *L. crispatus*- and *L. iners*-dominated cervical microbiota

Six women with PPROM developed postpartum endometritis. A higher rate of postpartum endometritis was found in women with *L. iners*-dominated than in women without *L. iners*-dominated cervical microbiota [with 4% (5/126) vs. without 0.5% (1/185); $p = 0.04$]. Women *L. crispatus*-dominated cervical microbiota showed a lower rate of postpartum endometritis than those without, but the difference was not significant [with 0% (0/109) vs. with 3% (6/202); $p = 0.09$].

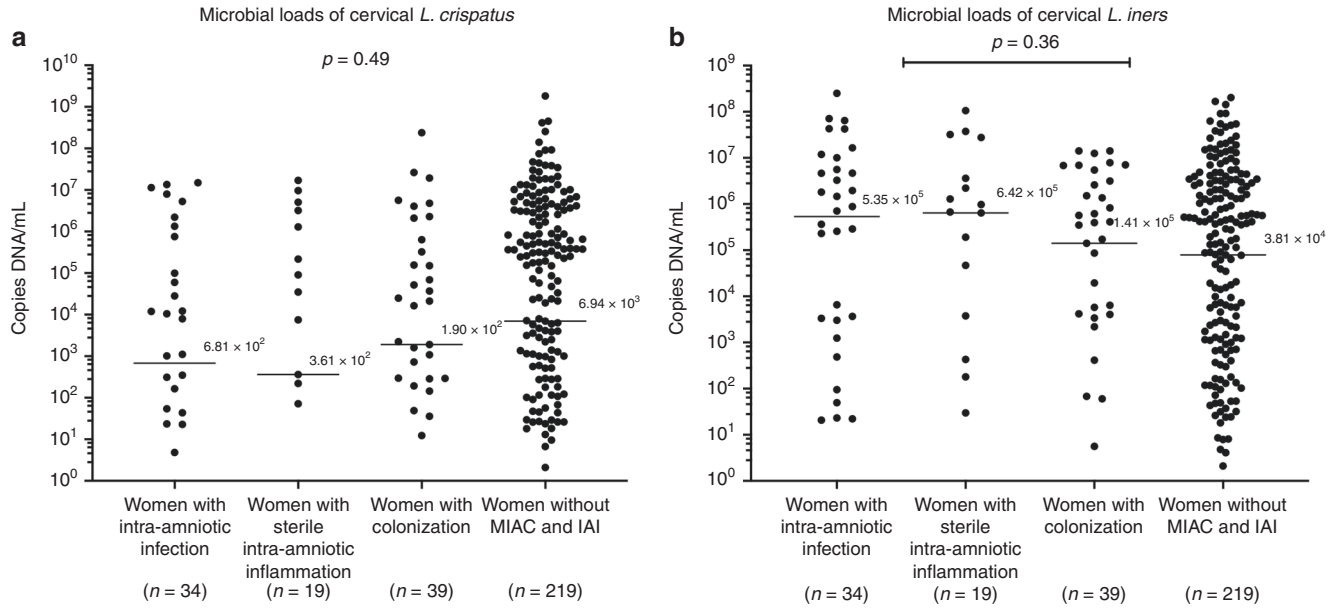

Fig. 3 Microbial loads (copies DNA/mL) of cervical *L. crispatus* (**a**) and *L. iners* (**b**) based on the presence or absence of microbial invasion of the amniotic cavity and/or intra-amniotic inflammation.

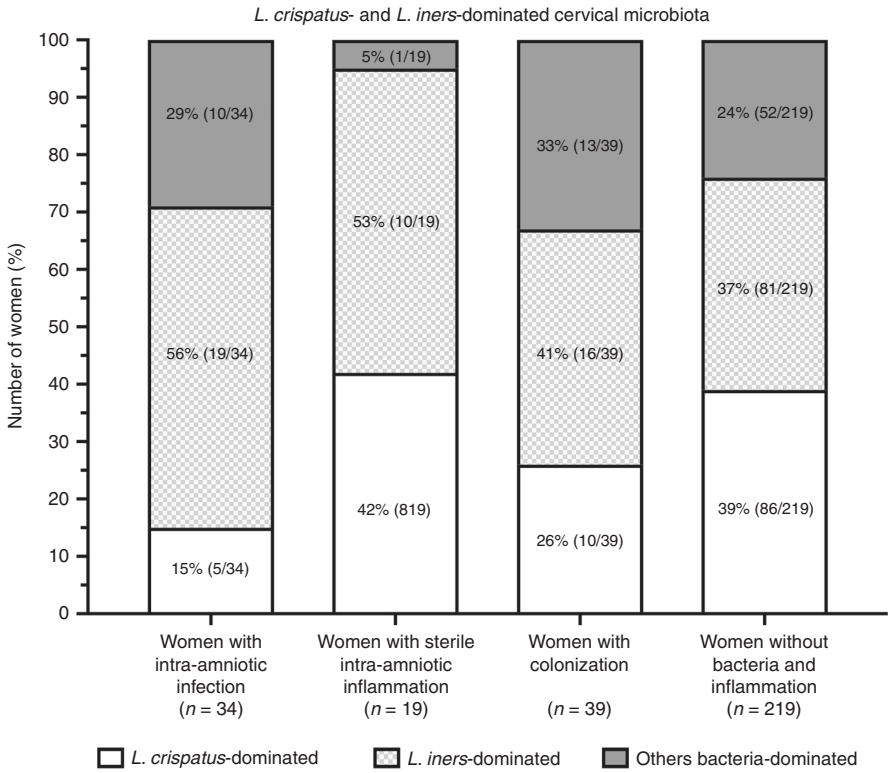

Fig. 4 Rates of *L. crispatus*- and *L. iners*-dominated cervical microbiota based on the presence or absence of microbial invasion of the amniotic cavity and/or intra-amniotic inflammation.

Short-term neonatal morbidity and *L. crispatus*- and *L. iners*-dominated cervical microbiota

The associations between *L. crispatus*- and *L. iners*-dominated cervical microbiota and selected aspects of short-term neonatal morbidity are listed in Table 2. The presence of *L. crispatus*-dominated cervical microbiota was related to a lower rate of early-onset sepsis compared to the absence of *L. crispatus*-dominated cervical microbiota [0% (0/109) vs. 5% (10/202); $p = 0.02$], which was maintained after adjusting for gestational age at delivery ($p = 0.02$).

DISCUSSION

Revealing the association between cervicovaginal microbiota and infection-related and inflammatory intra-amniotic complications is essential for understanding the underlying pathophysiology of these specific complications of PPROM, as well as for developing effective PPROM management strategies to improve neonatal outcomes.

The principal findings of this study are as follows: (i) women with MIAC had lower microbial loads of cervical *L. crispatus* and a

Table 2. Selected aspects of short-term neonatal morbidity with respect to the presence or absence *L. crispatus*- and *L. iners*-dominated cervical microbiota.

	L. crispatus-dominated cervical microbiota		*p* Value[a]	*L. iners*-dominated cervical microbiota		*p* Value[b]
	With ($n = 109$)	Without ($n = 202$)		With ($n = 126$)	Without ($n = 185$)	
Transient tachypnea of newborns	5 (5%)	8 (4%)	0.77	5 (4%)	8 (4%)	1.00
Respiratory distress syndrome	19 (17%)	50 (25%)	0.15	28 (22%)	41 (22%)	1.00
Respiratory disorders	24 (22%)	58 (29%)	0.23	33 (26%)	49 (26%)	1.00
Bronchopulmonary dysplasia	6 (6%)	16 (8%)	0.50	8 (6%)	14 (8%)	0.82
Need for intubation	4 (4%)	9 (5%)	1.00	4 (3%)	9 (5%)	0.57
Intraventricular hemorrhage (I–II)	13 (12%)	26 (13%)	0.86	15 (12%)	24 (13%)	0.86
Retinopathy of prematurity	4 (4%)	2 (1%)	0.19	0 (0%)	6 (3%)	0.09
Necrotizing enterocolitis	0 (0%)	3 (1%)	0.55	1 (1%)	2 (1%)	1.00
Early-onset sepsis	0 (0%)	10 (5%)	**0.02**	7 (6%)	3 (2%)	0.10
Late-onset sepsis	2 (2%)	4 (2%)	1.00	3 (2%)	3 (2%)	0.69
Compound neonatal morbidity	34 (31%)	72 (36%)	0.45	41 (33%)	65 (35%)	0.72
Neonatal death	2 (2%)	3 (2%)	1.00	3 (2%)	2 (1%)	0.40

Respiratory disorders were defined as a respiratory distress syndrome or transient tachypnea of newborns.
Compound neonatal morbidity was defined as a need for intubation and/or respiratory distress syndrome and/or transient tachypnea of newborns and/or pneumonia and/or bronchopulmonary dysplasia and/or retinopathy of prematurity and/or intraventricular hemorrhage and/or necrotizing enterocolitis and/or early-onset sepsis and/or late-onset sepsis and/or neonatal death before hospital discharge.
Intraventricular hemorrhage grades III–IV was not considered in the analysis because of no occurrence in the cohort.
Categorical variables were compared using the Fisher's exact test.
[a]*p* Value—the comparison between women with and without *L. crispatus*-dominated cervical microbiota.
[b]*p* Value—the comparison between women with and without *L. iners*-dominated cervical microbiota.
Statically significant results are marked in bold.

lower rate of *L. crispatus*-dominated cervical microbiota; (ii) the presence of only *Ureaplasma* spp. in the amniotic fluid was associated with the highest rate of *L. crispatus*-dominated cervical microbiota among women with MIAC; (iii) women with intra-amniotic infection showed a lower rate of *L. crispatus*-dominated cervical microbiota and a higher rate of *L. iners*-dominated cervical microbiota than women with sterile IAI, colonization, and without MIAC and IAI; (iv) *L. iners*-dominated cervical microbiota was related to a higher rate of puerperal endometritis; and (v) *L.* crispatus-dominated cervical microbiota was associated with a lower rate of early-onset sepsis.

The presence *L. crispatus*- or *L. iners*-dominated cervicovaginal microbiota represents the most common cervicovaginal microbiota during pregnancy, even when PPROM is present.[12–15] In this study, 75% of women with PPROM had either *L. crispatus*- or *L. iners*-dominated cervicovaginal microbiota.

In this study, the presence of MIAC was related to a lower cervical microbial load of *L. crispatus* and lower rate of *L. crispatus*-dominated cervical microbiota. These findings support the observations described above. Notably, leaking amniotic fluid may have contributed to depletion of the cervical microbial load of *L. crispatus* in the subgroup of women with MIAC; however, no differences in the cervical loads of microbial DNA and *L. iners* between women with and without MIAC were observed.

Various bacteria can cause MIAC; however, *Ureaplasma* spp. represent the most commonly identified bacteria in the amniotic fluid in PPROM.[4,6,30,31] *Ureaplasma* spp. may colonize uterine cavity (choriodecidual space), even in uncomplicated pregnancies.[32,33] Therefore, the choriodecidual space may serve as a source of amniotic fluid *Ureaplasma* spp. in PPROM pregnancies characterized with thin fetal membranes. Because the presence of *Ureaplasma* spp. alone in amniotic fluid may represent a unique subtype of MIAC in which *Ureaplasma* spp. did not need to breach the cervical epithelial bacteria and only passed through the fetal membranes, we hypothesized that this subgroup of PPROM pregnancies is related to a higher rate of cervical *L. crispatus* than the subgroups of PPROM containing other bacteria in the amniotic fluid. In this study, we confirmed that the subgroup of PPROM with *Ureaplasma* spp. alone had a higher rate of *L. crispatus*-dominated cervical microbiota compared to PPROM with other amniotic fluid bacteria and that accompanied by *Ureaplasma* spp.

In our previous study on the cervical microbiota in women with PPROM, the subset of women with community state types I (*L. crispatus*-dominated cervical microbiota) was completely without the presence of intra-amniotic infection.[14] This clinically interesting finding was partly confirmed in this study since *L. crispatus*-dominated cervical microbiota was identified in the subgroup of PPROM with intra-amniotic infection; however, with a lower rate (15%) than in the subgroups of the women with sterile IAI, colonization, and without MIAC and IAI. On the other hand, the subset of the women with intra-amniotic infection was associated with the highest rate of *L. iners*-dominated cervical microbiota.

Endometritis, defined as infection of the upper genital tract, commonly occurs postpartum, when bacteria from cervicovaginal microbiota can access the upper genital tract.[34] The rate of postpartum endometritis is significantly higher in women with PPROM compared to in women who deliver at term (2.8% vs. 1.4%).[35] However, the association between cervicovaginal microbiota and risk of the development of endometritis in PPROM is not well understood. In this study, we found that the presence of L. *iners*-dominated cervical microbiota was related to a higher risk of developing postpartum endometritis. This is expected because *L. iners*-dominated cervicovaginal microbiota has been shown to be less stable or more in transition than *L. crispatus*-dominated microbiota.[17] However, these results should be considered with caution because of the small number of women in this study who developed endometritis (5/311, 1.6%).

Finally, we assessed the association between selected aspects of short-term neonatal morbidity and the presence of *L. crispatus*- and *L. iners*-dominated microbiota. As expected, neither *L. crispatus*- nor

L. iners-dominated cervical microbiota affected short-term neonatal outcomes, except for early-onset sepsis of newborns. All women who developed early-onset sepsis showed absence of *L. crispatus*-dominated vaginal microbiota (7× *L. iners*-dominated vaginal microbiota and 3× other bacteria-dominated vaginal microbiota). This finding agrees with the results of a study by Brown et al.[15] in which vaginal microbiota in PPROM cases who developed early-onset sepsis was enriched for *Catonella* spp. and *Sneathia* spp., while *L. crispatus* was overexpressed in cases that did not develop this complication. Our results and those of Brown et al.[15] are clinically very relevant, as they may help clinicians to distinguish PPROM pregnancies at a low risk of subsequent development of early-onset sepsis of the newborn from those at high risk.

One strength of this study was the large, very well-defined (information about MIAC and IAI, and short-term neonatal outcomes) cohort of women with PPROM in whom the cervical presence of *L. crispatus* and *L. iners* were evaluated before administration of antibiotics and corticosteroids. Another strength is that we assessed *L. crispatus* and *L. iners* by specific RT-PCR with a detection limit of 1–10 copies DNA/mL. This study also had some limitations. First, amniotic fluid leakage through the cervix may have affected the composition of the cervical microbiota, as amniotic fluid has been shown to have antimicrobial properties and can inhibit the growth of bacteria such as *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus faecalis*.[36,37] Second, amniotic fluid leakage may have diluted the microbial loads of cervical DNA and *L. crispatus* and *L. iners*, as well as contributed to the rapid transition to an abnormal microbial profile during the interval between membrane rupture and sampling. However, amniotic fluid leaking is an unavoidable confounder when studying cervical microbiota in PPROM. Additionally, because of changes in the amniotic fluid proteome during pregnancy and variable duration of exposure to amniotic fluid, the effects of amniotic fluid leakage on the cervical microbiota remain unclear.[38] Last, the population of this study was very homogeneous (all women in this study were Caucasian). This finding should be considered as a weakness since it prevents from translation of the finding on populations that are racially/ethnically more diverse.[39] In addition, it is well known that African-American population has a higher prevalence of *L. iners* in vaginal microbiota and a higher rate of preterm delivery.[40]

In conclusion, the presence of *L. crispatus*-dominated cervical microbiota in women with PPROM was associated with a lower risk of intra-amniotic infection-related and inflammatory complications and subsequent development of early-onset sepsis in newborns. The presence of *L. iners*-dominated cervical microbiota was related to a higher risk of developing postpartum endometritis.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Ministry of Health of the Czech Republic (16-28587A).

AUTHOR CONTRIBUTIONS

M.K., L.P., R.B., and I.M. were responsible for concept and design. M.K., L.P., R.B., R.G., P.J., P.M., O.S., T.F., J.M., P.V., H.Z., B.J., and I.M. contributed to data acquisition, analysis, and interpretation. M.K., B.J., and I.M. drafted and revised the manuscript. All authors approved the final manuscript

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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