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FULL PAPER

HPV/*Chlamydia trachomatis* co-infection: metagenomic analysis of cervical microbiota in asymptomatic women

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SUMMARY

HPV and *Chlamydia trachomatis* are the most common causes of sexually transmitted diseases worldwide. Most infections are asymptomatic and left untreated lead to severe reproductive tract sequelae such as cervical cancer and infertility. Interestingly, *C. trachomatis* may also increase the susceptibility to HPV infection as well as contribute to viral persistence.

Recently, a growing body of evidence has suggested that the composition of the cervico-vaginal microbiota plays a key role in the susceptibility and outcome of genital infections caused by several pathogens, including HPV and *C. trachomatis*.

The aim of our study was to undertake a metagenomic analysis of sequenced 16s rRNA gene amplicons to characterize the cervical microbiota from asymptomatic women with HPV/*C. trachomatis* co-infection. The composition of the cervical microbiota from HPV-positive or *C. trachomatis*-positive women was also analysed.

The main finding of our study showed that the cervical microbiota in HPV/*C. trachomatis* co-infected women had a higher microbial diversity than the cervical microbiota in healthy controls ($p<0.05$). In addition, *Aerococcus christensenii* was associated with C. *trachomatis* infection.

In conclusion, the increased cervical microbial diversity observed in HPV/*C. trachomatis* co-infected women and the detection of potential microbiological biomarkers of *C. trachomatis* infection will open the way to innovative approaches that may be helpful to identify women at risk of co-infection.

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INTRODUCTION

Human Papillomavirus (HPV) and *Chlamydia trachomatis* are the most common causes of sexually transmitted diseases worldwide. According to World Health Organization (WHO) estimates, approximately 290 and 131 million new cases of HPV and *C. trachomatis* respectively occur each year (Newman *et al.,* 2015; de Sanjosé *et al.,* 2007).

HPV and *C. trachomatis* share similar risk factors**,** like younger age and a higher number of sexual partners, as well as common clinical features, such as the lack of symptoms in the majority of infected women. In fact, both HPV and *C. trachomatis* are responsible for asymptomatic infections in approximately 90% and 80% of women respectively and, hence, remain unrecognized and untreated, leading to persistent infection and to onward transmission in the population (Shaw *et al.,* 2011; Mitra *et al.,* 2016**)**.

Key words:
HPV, *Chlamydia trachomatis*, Coinfection, Cervical microbiota, Metagenomic analysis.

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Persistent infection is of particular pathological importance for the continuous release of cytokines resulting in a chronic inflammatory state responsible for tissue damage and the following severe reproductive tract sequelae **(**Scott *et al.,* 1999; Haggerty *et al.,* 2010; Lee *et al.,* 2013; Sessa *et al.,* 2015).

The main complication of persistent HPV infection is the development of cervical lesions that may progress to malignancy, recognized as the most important risk factor for cervical cancer development. Specifically, at 1 year approximately 20% of persistent infections with high risk HPV develop cervical intraepithelial neoplasia or cervical cancer within the subsequent 5 years (Oakeshott *et al.,* 2012).

In addition to genital lesions, persistent HPV infection has been suggested to have harmful effects on pregnancy, including spontaneous preterm labour and abortion, since studies have demonstrated the ability of HPV to infect the trophoblast cells of the placenta**,** impairing trophoblast-endometrial cell adhesion (You *et a*l., 2008). However, the data are still controversial and further studies are ongoing to better clarify the role of cervical HPV infection on adverse pregnancy outcomes (Niyibizi *et al*., 2017).

Concerning *C. trachomatis*, up to 26% of women with persistent infection develop pelvic inflammatory disease (PID), which, in turn, is strongly associated with subsequent reproductive sequelae. In fact, nearly 10% of wom-

en with PID has ectopic pregnancy and up to 38% of women may become infertile after recurrent episodes of PID (Haggerty *et al.,* 2010; Davies *et al.,* 2017; Wiesenfeld *et al.,* 2017). In addition, *C. trachomatis* genital infection has also been associated with spontaneous preterm labour and abortion (Lanjouw *et al*., 2016).

The evidence that *C. trachomatis* may increase the susceptibility to HPV infection or decrease the efficient clearance of an existing HPV infection, contributing to viral persistence (Zenilman *et al.*, 2001; Silins *et al.*, 2005) is particularly interesting**.** Indeed, several studies have reported an increased risk of HPV infection among *C. trachomatis*-positive women. In this regard, two potential mechanisms have been proposed: chlamydial infection may lead to epithelial disruption and facilitate the entry of HPV**,** or it may impair the immune response favouring the persistence of HPV (Silva *et al.,* 2014).

In recent years, a growing body of evidence has suggested that the cervico-vaginal microbiota plays a key role in the susceptibility as well as the outcomes of genital infections (Ma *et al.,* 2012; Lewis *et al.,* 2017). Amongst the several microorganisms colonizing the cervico-vaginal micro-environment, lactobacilli are widely known for their protective effects against HPV and *C. trachomatis* (Brotman 2011; Ma *et al.,* 2012; Mastromarino *et al.,* 2014; van de Wijgert *et al.,* 2014; Sessa *et al.,* 2017a; Sessa *et al.,* 2017b). However, the cervico-vaginal microbiota is highly complex and several bacterial species, including, for example, *Gardnerella vaginalis*, *Atopobium vaginae* and *Prevotella* spp., may, on the contrary, favour the development of HPV and/or *C. trachomatis* genital infections (Ma *et al.,* 2012; Lewis *et al.,* 2017).

The aim of our study was to undertake a metagenomic analysis of sequenced 16s rRNA gene amplicons to characterize the cervical microbiota from asymptomatic women with HPV/*C. trachomatis* co-infection. Moreover, the composition of cervical microbiota from HPV-positive or *C. trachomatis*-positive women was also analysed.

MATERIALS AND METHODS

Study design and sample collection

From July to December 2016, 35 consecutive women of Italian origin attending the Department of Gynaecology, Obstetrics and Urology at "Sapienza" University of Rome for regular check-ups were enrolled in this study.

Women with sexual activity in the week prior to sampling, recent or current antibiotic and/or hormonal medications (oral or topic), as well as use of probiotics and/or prebiotics, were excluded from the study. Cervical samples were taken seven days after menses withdrawal. All study participants gave their written informed consent prior to sampling and provided a detailed personal, medical and gynaecological history. This study design and protocol was approved by the Umberto I University Hospital ethical committee (reference number 367/16) and was conducted according to the principles expressed in the Declaration of Helsinki.

From each woman, one endo-cervical swab (FLOQ swabs, Copan) was collected for *C. trachomatis* and HPV testing as previously described (Frieden *et al.,* 2015), and for the metagenomic analysis. All the women negative to *C. trachomatis* and/or HPV infection were also screened for *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, Mycoplasma, Candida and HSV-2 infections as previously described (Frieden *et al.,* 2015) in order to include exclusively women with healthy genital conditions. All study participants were also examined for BV and for the presence of symptoms. BV was assessed using Amsel criteria and confirmed using Gram stain criteria (Nugent score). All endo-cervical samples were placed in cryovials containing 1mL of phosphate-buffered saline (PBS) and immediately stored at -80°C until further processing.

Next-generation sequencing

Cervical samples were shipped in dry ice to Microsynth AG (Balgach, Switzerland) for DNA isolation, 16s rRNA gene amplification, Illumina MiSeq sequencing and initial bioinformatics analysis, consisting of raw data processing and taxonomic classification.

DNA isolation

Cervical samples were thawed and transferred into 2 mL screwcap tubes (Sarstedt Inc, Germany) containing 10 µL of 4% Tween 80 (Downey *et al.,* 2012). Tubes were then vortexed for 2 min on a TurboMix System (Scientific Industries, USA) and 350 µL of each sample were used for DNA isolation. Total DNA was extracted using the QIAmp Investigator kit (QIAGEN, USA), according to the manufacturer's instructions. DNA was quantified by fluorescence spectroscopy (Quant-iT™ PicoGreen® dsDNA Assay Kit, Thermo Fisher, USA) and its integrity checked by agarose gel electrophoresis.

16s rRNA gene amplification and illumina MiSeq sequencing

Dual-indexed universal primers 341F (CCTACGGGNGGCWGCAG) and 802R (GACTACHVGGGTATCTAATCC; Illumina, USA) were utilized for the two-step PCR amplification of the V3-V4 hypervariable regions of the 16s rRNA gene (16S/ITS Nextera two-step PCR kit, Illumina Inc, USA), according to the manufacturer's instructions. Briefly, the first-step PCR was carried on to amplify the V3-V4 region of the 16s rRNA gene. The resulting PCR amplicons were then used for the second-step PCR for further amplification and inclusion of indexes (barcodes) as well as the Illumina sequencing adaptors. Cycling conditions were initial denaturation at 95°C for 3 min, followed by 20 cycles (for the first-step PCR) or 15 cycles (for the second-step PCR) of denaturation at 98°C for 20 s, annealing at 56°C for 30 s and elongation at 72°C for 30 s, one cycle of final elongation at 72°C for 5 min and a final cooling step to 10°C. The resulting PCR products were quantified by fluorescence spectroscopy (Quant-iT™ PicoGreen® dsDNA Assay Kit, Thermo Fisher, USA), pooled in equimolar amounts and, then, purified with Agencourt AMpure-XP magnetic beads (Beckman Coulter, USA). The final library, containing all the pooled samples, was sequenced with version 2 of MiSeq Reagent Kit, 2x250 bp output, on a MiSeq desktop sequencer (Illumina, USA). One negative control (350 µL of sterile PBS) was included and subjected to the same procedure as the samples.

Sequencing data analysis

MiSeq paired-end reads were subjected to demultiplexing and trimming of Illumina adaptor residuals using Illumina recommended parameter settings (Illumina MiSeq Reporter software, version 2.5.1.3). Sequences were aligned and joined using Fast Length Adjustment of SHort reads (FLASH, version 1.2.11) (Magoĉ and Salzberg, 2011) and

primers trimmed off the aligned sequences using cutadapt (version 1.8.1) (Martin, 2011). Chimeric sequences were identified and removed by UCHIME (version 4.2) (Edgar *et al.,* 2011) and only joined reads with an average quality score of 25 or higher were used for downstream analysis. Open reference operational taxonomic unit (OTU) clustering and taxonomic assignment were performed in QIIME (version 1.9.1) (Caporaso *et al.,* 2010) using UCLUST against the SILVA rRNA reference database (version 111) (Edgar, 2010; Quast *et al.,* 2013). An OTU was defined as a group of sequences with a similarity of 97% or more. OTUs with only one sequence (singletons) and those not found more than 10 times in any sample were excluded from the downstream analysis to minimise artefacts. OTUs that could not be identified to a species level using the reference database were searched using BLAST and assigned to the deepest taxonomical level based on available published data.

Taxa summaries were performed in QIIME and all samples were normalized to the sample with the lowest read count for alpha and beta diversity comparisons. Shannon's diversity index and Shannon's evenness were used as metrics for alpha rarefaction analysis, which was performed in QIIME. Jackknifed principal coordinates analysis (PCoA) was used so to assure that our rarefaction selection was not the cause of the observed clustering patterns. PCoA analysis was based on unweighted and weighted UniFrac distance matrixes and computed in QIIME (Lozupone and Knight, 2005; Lozupone *et al.,* 2007). For taxa comparisons, relative abundances based on all obtained reads were used.

Raw sequences were deposited into the NCBI's Sequence Read Archive (SRA) (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?), accession number SRP098954 and SRP108019.

Linear discriminant analysis with effect size measurement

Linear discriminant analysis (LDA) coupled with effect size measurement (LEfSe) (Segata *et al.,* 2011) was performed to compare taxa between *C. trachomatis*-positive and HPV-positive women as well as healthy controls. The LEfSe method is a recently developed algorithm for biomarker discovery in metagenomic data that identifies differentially abundant taxa characterizing the differences between two or more groups. Specifically, it utilizes the non-parametric factorial Kruskal-Wallis sum-rank test to compare all taxa at different taxonomic levels between groups and then the unpaired Wilcoxon rank-sum tests among subgroups. As a last step, LEfSe uses linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant taxa. Only LDA score >2 have been included in the analysis.

Statistical analysis

Nonparametric *T*-test based on Monte Carlo permutations was used for alpha diversity comparisons, Kruskal-Wallis test for taxa level comparisons, and Adonis for category comparisons of distance matrixes, all calculated in QIIME. Bonferroni correction was used to correct for multiple hypothesis testing when necessary. All remaining statistical calculations were performed in Excel (Microsoft, USA) and R 3.1.2 (R development core team). Chi-squared test was used for assessment of association of frequencies among groups (Fisher's exact test was used when any cell had expected values <5). Mann-Whitney U test for non-parametric data was used for comparison of means. The single or multiple inference significance level was set to 5%.

RESULTS

Study subject characteristics

Thirty-five women of reproductive age were enrolled in this study. Ten women were positive to *C. trachomatis*, 10 women were positive to HPV, 5 women had a HPV/*C. trachomatis* co-infection and 10 were negative to any genital pathogen (healthy controls). No women had any specific genital symptoms related to HPV and/or chlamydial infection.

All 35 samples underwent 16s rRNA amplicon-based microbiome analysis and 10 samples were excluded from downstream analysis due to a number of reads <100. Overall, the cervical microbiome analysis was performed on 18 cervical samples from women positive to *C. trachomatis* and/or HPV genital infections and 7 healthy controls. Population characteristics are summarized in *Table 1*.

A statistically significant association was observed between the behavioural risk factors (smoking, first intercourse before age 16 years, multiple partners, etc.) and either *C. trachomatis* and/or HPV infections (p=0.012). Multiple partners and a past STI was associated with HPV infection (p=0.04 and p=0.001 respectively), whereas smoking, first intercourse before age 16 years and a new partner in the last 6 months were associated with HPV/*C. trachomatis* co-infection (p=0.0083, p=0.03 and p=0.03 respectively).

Table 1 - *Characteristics of the study population.*

	Healthy (n=7)	*CT (n=7)*	*HPV/CT (n=3)*	*HPV (n=8)*
Age (mean±SD)	34.7 ± 9.7	28 ± 3.2	29.3 ± 15	35.8 ± 9.7
Smoking (%)	2 (28.6)	0 (0)	3 (100)#	4 (50)
First intercourse before age 16 years (%)	1 (14.3)	3 (42.3)	3 (100)*	3 (37.5)
New partner in the last 6 months (%)	1 (14.3)	5 (71.4)	3 (100)*	5 (62.5)
Multiple partners (%)	2 (28.6)	4 (57.1)	3 (100)	7 (87.5)*
Partners with past STIs in the last 6 months (%)	0 (0)	0 (0)	2 (66.7)	1 (12.5)
Bacterial vaginosis (%) (Nugent score 7 to 10)	0 (0)	3 (42.9)	1 (33.3)	3 (37.5)
Past STIs (%)	0 (0)	2 (28.6)	2 (66.7)	7 (87.5)*,#

SD, Standard Deviation; STI, Sexually Transmitted Infections; CT, C. trachomatis; HPV, Human Papillomavirus; *, p<0.05 vs Healthy; #, p<0.05 vs CT.

Cervical microbiota composition

An average of 121,836 [median(IQR) 135,957 (77,501)], 176,179 [median(IQR) 175,410 (20,333)], 174,082 [median(IQR) 181,587 (40,053)] and 169,328 [median(IQR) 192,916 (40,494)] paired-end Illumina reads were analysed per sample in healthy controls, *C. trachomatis*-positive, HPV-positive and co-infected women, respectively. After the removal of singletons and rare OTUs, 35 OTUs from 20 genera were identified [median(IQR) 18 (6), 25 (10), 20.5 (6) and 26 (4.5) for healthy controls, *C. trachomatis*-positive, HPV-positive and co-infected women, respectively]. The lowest read count was 29,633 and hence OTUs were randomly sub-sampled to 29,633 reads for further analysis to avoid sequencing bias.

The cervical microbiota from healthy controls was dominated by the phylum Firmicutes (median relative abundance 98%), while bacteria from the phyla Actinobacteria, Fusobacteria, Proteobacteria, Tenericutes and Bacteroidetes each accounted for less than 1% to total bacteria. On genus level, *Lactobacillus* was the single most abundant genus (median relative abundance 96%), whereas other genera (*Gardnerella*, *Atopobium*, *Bifidobacterium*, etc.) each contributed to less than 2% to total bacteria. Specifically, *L. crispatus* or *L. gasseri* were the predominant species in most healthy women (86%), while *Lactobacillus iners* was the predominant species in the remaining women (*Figure 1*).

In *C. trachomatis*-positive women, the cervical microbiota was characterized by a decrease in the phylum Firmicutes (median relative abundance 53% as compared to 98% in healthy controls) and an increase in the phyla Fusobacteria, Actinobacteria and Bacteroidetes (median relative abundances 22%, 15% and 6% as compared to 0.8%, 0.4% and 0.02% in healthy controls, respectively). Amongst the Firmicutes, *Lactobacillus* spp. was less abundant in *C. trachomatis*-positive women than in healthy controls (median relative abundance 50% and 96%, respectively). In particular, the proportion of *L. crispatus* and *L. gasseri* was almost reduced to half in *C. trachomatis*-positive women

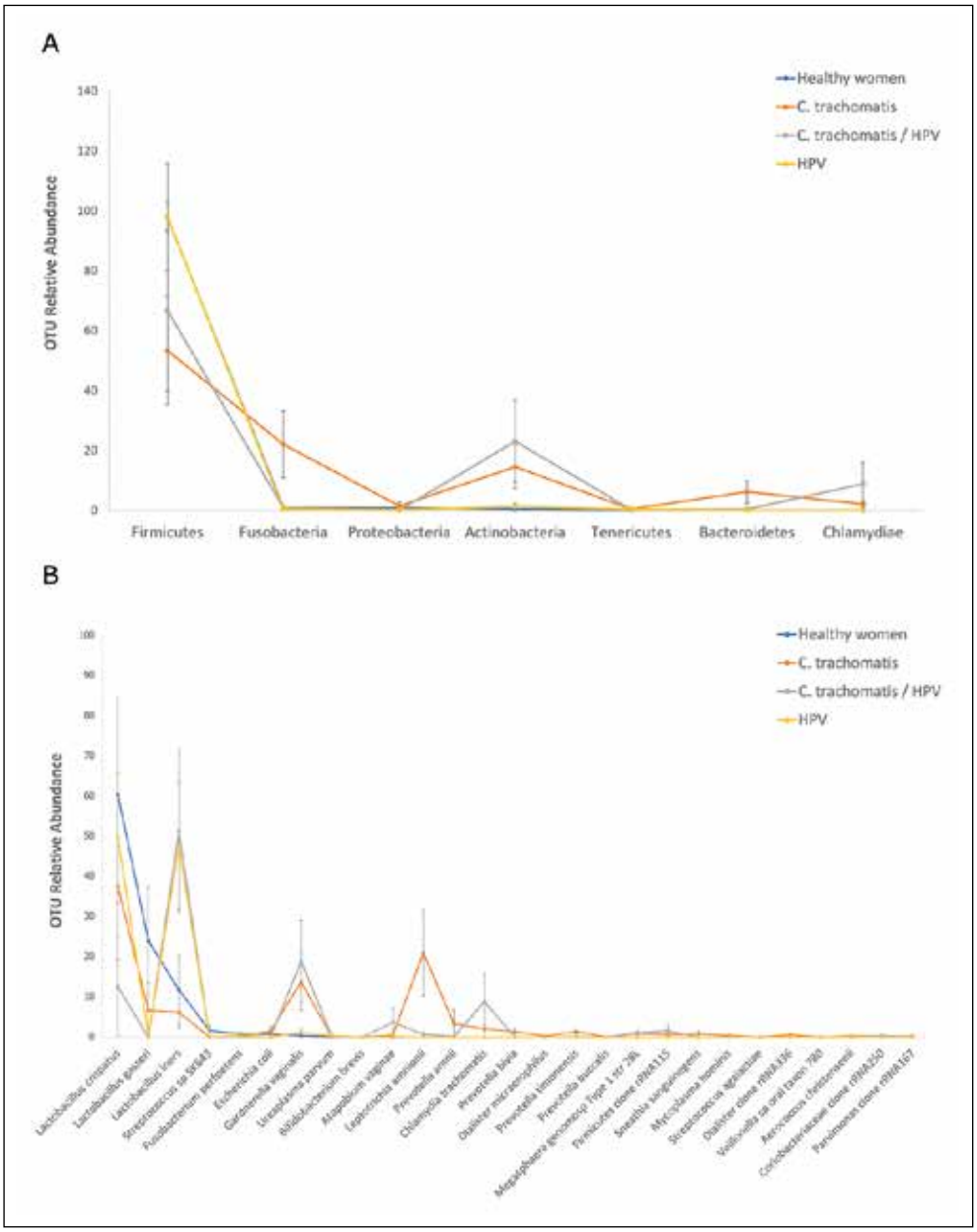

Figure 1 - *Mean relative abundance of phyla (A) and species (B) in cervical samples from C. trachomatis-positive, HPV-positive and HPV/C. trachomatis co-infected women and healthy controls.*

as compared to healthy controls (median relative abundance 84% and 44%, respectively) (*Figure 1*). Fusobacteria, Actinobacteria and Bacteroidetes were mostly represented by *Leptotrichia amnionii*, *Gardnerella vaginalis* and *Prevotella* spp. (median relative abundances 21%, 14% and 6% as compared to 0.02%, 0.4% and 0.02% in healthy controls, respectively).

In HPV-positive women, the cervical microbiota was characterized by the predominance of the phylum Firmicutes (median relative abundance 98% in both HPV group and healthy controls). Interestingly, *L. iners* was more prevalent in the cervical microbiota from HPV-positive women than from healthy controls (50% and 14%, respectively) and *L. gasseri* was absent in HPV-positive women. Bacteria from the phyla Fusobacteria, Proteobacteria, Bacteroidetes and Tenericutes each contributed to less than 1% to total bacteria, whereas the phylum Actinobacteria showed a threefold increase in HPV-positive women as compared to healthy controls (relative median abundance 1.3% and 0.4%, respectively) (*Figure 1*).

Cervical samples from HPV/*C. trachomatis* co-infected women showed a decrease in the phylum Firmicutes (median relative abundance 63% as compared to 98% in healthy controls). The phylum Actinobacteria was more abundant in HPV/*C. trachomatis* co-infected women than in *C. trachomatis*-positive women (median relative abundance 23% and 15%, respectively), whereas the phyla Fusobacteria and Bacteroidetes each contributed less than 1% to total bacteria, showing no increase compared to healthy controls. Among the Firmicutes, *L. iners* was more abundant than in either healthy controls or *C. trachomatis*-positive women (relative median abundance 51%, 12% and 6%, respectively). Actinobacteria were mostly represented by *G. vaginalis* (median relative abundance 19% as compared to 14% and 0.4% in *C. trachomatis*-positive women and healthy controls, respectively). *Atopobium vaginae* was also increased with a median relative abundance of 4% compared to 0.03% in healthy controls (*Figure 1*). However, on plylum, class, order, family, genus, and OTU levels, no statistically significant differences were observed between the different patient groups and healthy controls (p=NS).

Alpha and beta diversity analyses

Alpha diversity analysis showed that *C. trachomatis* genital infection as well as the co-infection HPV/*C. trachomatis*

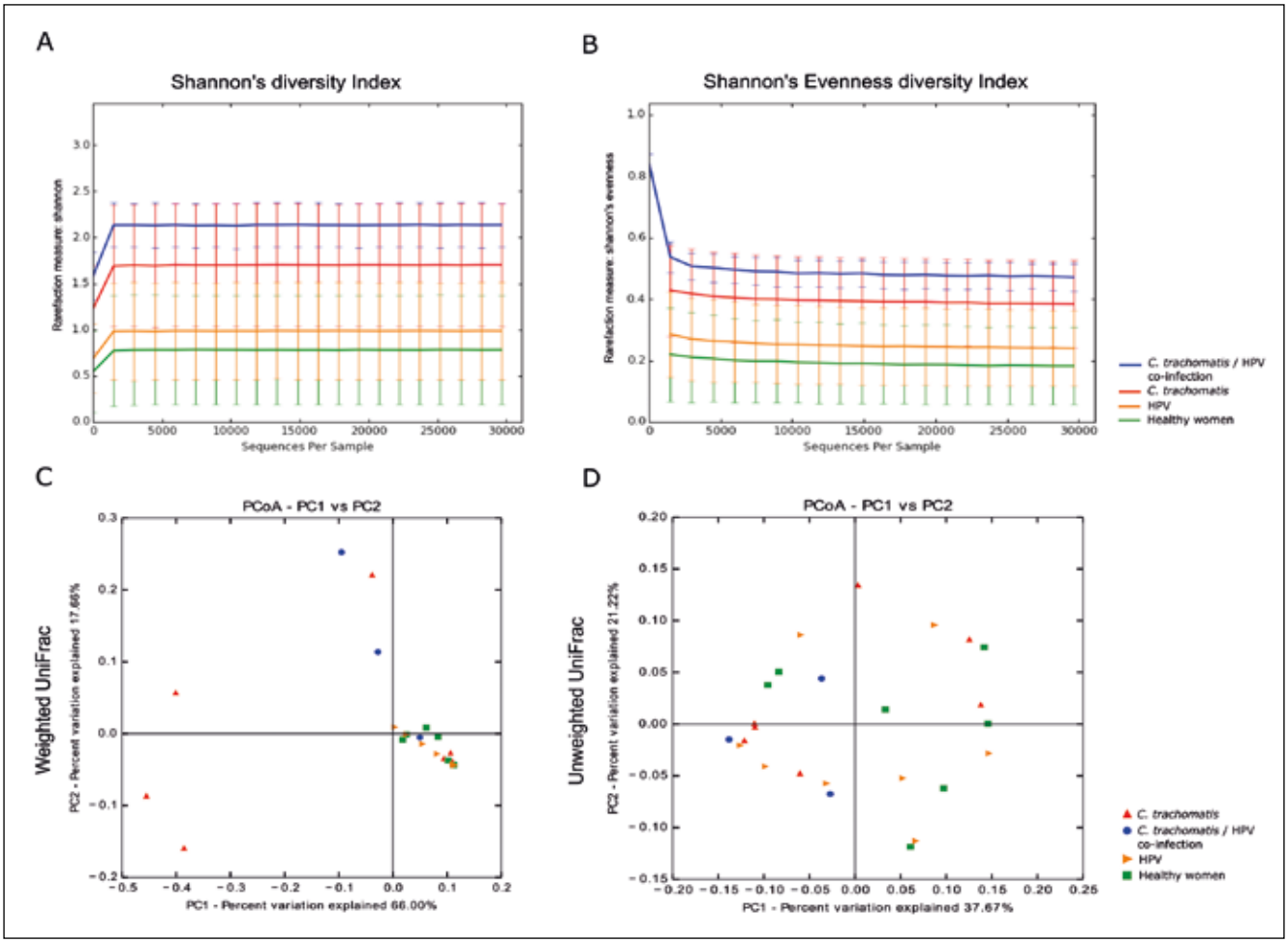

Figure 2 - *Alpha and beta diversity of the cervical microbiota from C. trachomatis-positive, HPV-positive, HPV/C. trachomatis co-infected women and healthy controls. Alpha rarefaction curves of Shannon's diversity index (A) and Shannon's evenness (B). Samples were rarefied to the smallest observed number of reads (29,613). Principal coordinate analysis (PCoA) plots of weighted (C) and unweighted (D) UniFrac distances. Each dot represents the cervical bacterial community composition of one patient or healthy control. Groups were compared using Monte-Carlo permutations for alpha diversity and Adonis for beta diversity.*

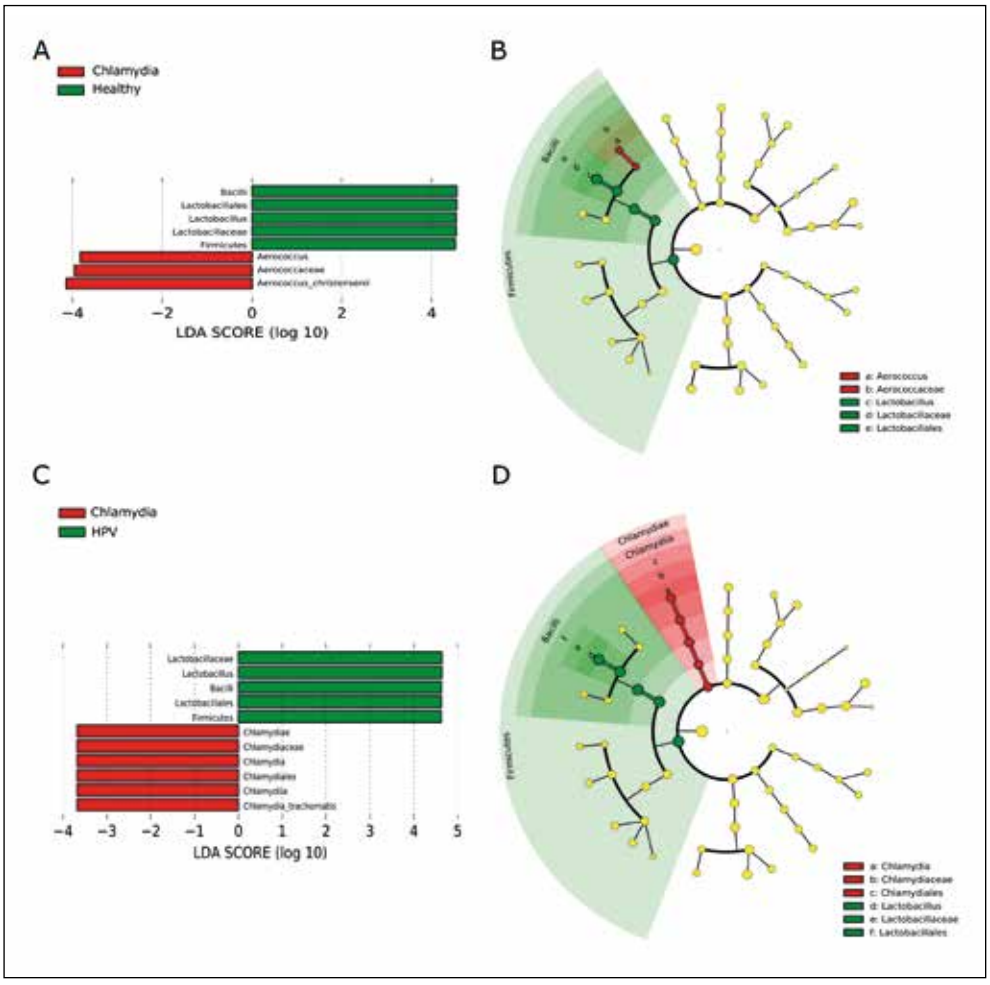

Figure 3 - *LEfSe results on cervical microbiota in C. trachomatis-positive women compared to HPV-positive women or healthy controls. Histogram of the LDA scores computed for statistically significant differentially abundant taxa between C. trachomatis-positive women and healthy controls (A) or HPV-positive women (C). LEfSe scores can be interpreted as the degree of consistent difference in relative abundance between taxa in the two classes of analysed microbial communities. The histogram thus identifies which taxa among all those detected explain the greatest differences between communities. Cladogram highlighting the relationship of significantly different taxa between C. trachomatis-positive women and healthy controls (B) or HPV-positive women (D). Differences are represented in the colour of the most abundant class. Each circle's diameter is proportional to the taxon's abundance.*

were significantly associated with an increased diversity of the cervical microbiota, as evidenced by Shannon's diversity index (*Figure 2A*, p=0.03 and p=0.02, respectively) and Shannon's evenness (*Figure 2B*, p=0.02 and p=0.01, respectively).

In the weighted UniFrac analysis, cervical samples from healthy controls as well as from HPV-positive women significantly separated in a cluster, as compared to *C. trachomatis*-positive and HPV/*C. trachomatis* co-infected women (*Figure 2C*, p<0.01), suggesting shifts in relative taxa abundance. In addition, the phylogenetic distances between *C. trachomatis*-positive and HPV/*C. trachomatis* co-infected samples were significantly higher than the distances measured between healthy controls and HPV-positive women (*Figure 2C*, p<0.01). On the contrary, no clustering was observed when comparing unweighted UniFrac distance matrixes (*Figure 2D,* p>0.05).

LEfSe analysis

Linear discriminant analysis (LDA) coupled with effect size measurement (LEfSe) (Segata *et al.,* 2011) was performed to compare taxa between *C. trachomatis*-positive, HPV-positive and co-infected women as well as healthy controls.

LEfSe revealed that the phylum Firmicutes and its derivative *Lactobacillus* spp. were significantly higher in the cervical microbiota from healthy controls (*Figure 3A*) as well as from HPV-positive women (*Figure 3C*) and, conversely, significantly lower in the cervical microbiota from *C. trachomatis*-positive women. Interestingly, *Aerococcus christensenii* was significantly associated with *C. trachomatis* infection, as evidenced by an LDA score >4 (*Figure 3A*). No significant association between taxa and HPV/*C. trachomatis* co-infected women was observed.

The taxonomic representations of the significantly different taxa (cladogram) between *C. trachomatis*-positive women and healthy controls or HPV-positive women are shown in *Figure 3B* and *Figure 3D*, highlighting the relationship between taxa at different taxonomic levels in a tree-like structure and revealing how the significantly different taxa are interrelated.

DISCUSSION

HPV and *C. trachomatis* continue to be important public health issues, because of the high risk of asymptomatic genital infections leading to serious complications like cervical cancer, ectopic pregnancy and infertility (Haggerty *et al.,* 2010; Mitra *et al.,* 2016). Furthermore, as previously mentioned, existing chlamydial genital infection has been shown to increase the risk of acquiring HPV as well as favour viral persistent infection (Silva *et al.*, 2014). Our present study is particularly interesting since it differs from previously published reports focused on vaginal or cervico-vaginal microbiota in women with a single HPV or *C. trachomatis* infection (Gao *et al.,* 2013; Lee *et al.,* 2013;

Brotman *et al.*, 2014; Dareng *et al.,* 2016; Shannon *et al*., 2017; Ma *et al.,* 2013; van der Veer *et al.,* 2017). Indeed, we undertook a metagenomic analysis of 16s rRNA gene amplicons to investigate, for the first time, the microbial composition from a specific niche of the female reproductive tract, the cervix, in asymptomatic women co-infected by HPV/*C. trachomatis*. In addition, the cervical microbiota from women with *C. trachomatis* or HPV infection, as well as from women with a healthy cervical microenvironment, was analysed.

Despite the small sample size, we found several compelling results by using different advanced statistical approaches. First, we observed that the cervical microbiota in co-infected women is notably different from the cervical microbiota in healthy controls. Indeed, the cervical microbiota in co-infected women had a higher microbial diversity than the cervical microbiota in healthy controls, as evidenced by the marked increase in Shannon's diversity index as well as in Shannon's evenness (P=0.02 and P=0.01, respectively). In particular, in co-infected women, we found a decrease in lactobacilli and an increase in anaerobic bacteria, such as *G. vaginalis* and *A. vaginae*, showing that the cervical microenvironment changed from a healthy state to a condition of dysbiosis, known to increase the susceptibility to genital infections (Lewis *et al.,* 2017).

Concerning *Lactobacillus* species, *L. iners* was more frequently found in co-infected women than in healthy controls and this finding is not surprising since previous studies showed that *C. trachomatis*-positive or HPV-positive women were more likely to have a microbiota dominated by *L. iners* (Lee *et al.,* 2013; Ma *et al.,* 2013; van der Veer *et al.,* 2017). This finding may be due to the inability of *L. iners* to produce antimicrobial compounds (e.g. hydrogen peroxide, D- lactic acid) (Mitra *et al.,* 2016; Lewis *et al.,* 2017).

Similar to HPV/*C. trachomatis* co-infection, *C. trachomatis*-positive women had a complex cervical microbiota with increased species diversity as compared to healthy controls, as evidenced by the marked increase in Shannon's diversity index as well as in Shannon's evenness (P=0.03 and P=0.02, respectively). Indeed, *C. trachomatis* infection seemed to be accompanied by a decrease in the abundance of lactobacilli and an increase in the abundance of facultative and/or strict anaerobes, like, for example, *L. amnionii*, *G. vaginalis*, *Prevotella* spp., and *Aerococcus* spp. More importantly, *Aerococcus christensenii* was significantly associated with *C. trachomatis* infection, as evidenced by LEfSe analysis (LDA score >4), a recently developed algorithm for high dimensional biomarker discovery in metagenomic data. *Aerococcus christensenii* in cervical microbiota might represent, thus, a potential biomarker of *C. trachomatis* infection, even though this needs to be further evaluated.

However, to date, the role of *A. christensenii* in the cervical environment is still not known, although this microorganism is usually found more frequently in women with bacterial vaginosis, the most common dysbiosis condition (Ling *et al.,* 2010).

Lastly, unlike HPV/*C. trachomatis* coinfection and *C. trachomatis* infection, the cervical microbiota from HPV-positive women did not show a higher species diversity as compared to cervical microbiota from healthy controls. Indeed, the cervical microbiota from HPV-positive women was similar to the cervical microbiota from healthy controls, since both were characterized by the predominance of *Lactobacillus* species. The similarity between HPV-positive women and healthy controls was further confirmed by the weighted UniFrac analysis. Specifically, we found that the cervical microbiota from HPV-infected women and healthy controls significantly defined a well separated cluster compared to the cervical microbiota from *C. trachomatis*-positive and co-infected women (p<0.01). Despite the predominance of *Lactobacillus* species, *L. gasseri* was absent in the cervical microbiota from HPV-positive women. Other studies have linked *L. gasseri* to the clearance of HPV (Brotman *et al.,* 2014; Shannon *et al.,* 2017), and our finding supports a potential protective effect of this microorganism against HPV.

As described above, HPV-positive women enrolled in our study had a low cervical microbiota diversity, in contrast to previous studies. Such discrepancy may be explained by the fact that these studies analysed the microbiota from vaginal or cervico-vaginal niches, whereas we investigated exclusively the cervical microbiota, whose composition may be significantly different from the vaginal microbiota, as suggested by Ling *et al.* (2011).

The main limitation of our study is the small sample size that precluded the identification of a specific bacterial profile linked to the different genital infections. However, the application of strict inclusion criteria in the selection of our study population strengthens the significance of our results. Indeed, we included women with a healthy genital condition and we excluded women with recent or current use of hormonal contraception and/or probiotic or prebiotic, all factors known to influence the cervical microbial composition over time.

In conclusion, the increased cervical microbial diversity observed in HPV/*C. trachomatis* co-infected women and the detection of potential microbiological biomarker of *C. trachomatis* infection will open the way to innovative approaches that may be helpful to identify women at risk of co-infection.

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