Received: 24 February 2023 | Accepted: 12 April 2023

DOI: 10.1002/jmv.28762

RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

Impact of cervicovaginal microbiome on the risk of cervical abnormalities development

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Abstract

The vaginal microbiome has emerged as potentially influencing the natural history of Human Papillomavirus (HPV) infections and their clinical impact. We aimed to characterize the vaginal microbiome in samples from 807 high-risk HPVs (Hr-HPV) positive women with a mean age of 41.45 ± 10.79 years who participated in the Regional Cervical Cancer Screening Program from the Northern Region of Portugal. Microbiome analysis was performed with commercial kits for the detection of 21 microorganisms. The most frequent microorganisms were *Ureaplasma parvum* (52.5%), *Gardnerella vaginalis* (GV) (34.5%), *Atopobium vaginae* (AV) (32.6%), Lacto (30.7%), and *Mycoplasma hominis* (MH) (23.5%). The distribution according to age reveals that MH, Mega1, GV, BVab2, AV, and Mob were more prevalent in women older than 41 years of age ($p < 0.050$), while Lacto is significantly decreased in this group (23.5% vs. 39.4%, $p < 0.001$; RR = 0.47). The risk analysis showed that Hr-HPV-16/-18 and Hr-HPV-9val genotypes are associated with an increased risk of developing cervical abnormalities, while Lacto ($p < 0.001$; odd ratio [OR] = 0.33), GV ($p = 0.0111$; OR = 0.41), AV ($p = 0.033$; OR = 0.53) and Mob ($p = 0.022$; OR = 0.29) are associated with protection. Similar results were found for the risk of development atypical squamous cells cannot exclude HSIL/high-grade squamous intraepithelial lesion. Overall, the multivariate analysis confirmed that lactobacillus

and bacteria associated with bacterial vaginosis (GV, AV, and Mob) are associated with protection against the development of cervical abnormalities. This study provides important data to be included in the future management of risk stratification for Hr-HPV-positive women.

KEYWORDS
cervical cancer, human papillomavirus (HPV), vaginal microbiome

1 | INTRODUCTION

Cervical Cancer (CC) is responsible for 604 127 new cases and 341 831 related deaths worldwide.[1] Persistent infection by Human Papillomavirus (HPV) is considered to be the etiological factor for the development of high-grade cervical lesions that can evolve into CC.[2,3] Despite existing over 150 different HPV genotypes, only certain subtypes of HPVs (HPV-16, −18, −31, −33, −35, −39, −45, −51, −52, −56, −58, −59, −66, and −68) are described as carcinogenic, also known as High-risk HPVs (Hr-HPVs).[2,4–6] Of these genotypes, HPV-16 and −18 are responsible for the majority of CC cases worldwide, and conjointly with HPV-31, −33, −45, −52, and −58, represent over 90% of all cases.[3,7–10]

HPV infection is considered the commonest sexually transmitted infection, with over 80% of all sexually active women being at risk of having at least one contact with HPV.[11,12] In Portugal, data from the Regional cervical cancer Screening Program (RCCU) from the Northern Region of Portugal indicates a prevalence of 12.5% for Hr-HPV in women aged 25–64 years. Nevertheless, despite the relatively high prevalence in women, only a subset will develop cancer, depending on the impact of other cofactors.[12,13]

In a previous report from our group, genital *Ureaplasma urealyticum* (UU), *Ureaplasma parvum* (UP), and *Mycoplasma hominis* (MH) were described as recurrent in Portuguese women at reproductive age (<30 years old), while *Mycoplasma genitalium* (MG) is infrequent.[14] Literature suggests that the vaginal microbiome might play an important role in HPV-induced diseases.[15–18] Additionally, several authors are describing the existence of different vaginal microbial profiles, designated as community state types (CSTs), according to the presence of different microorganisms, which may impact cervical disease development.[19] The predominance of different *Lactobacillus spp*. (Lacto) is referred to be an important determinant for the definition of different CSTs.[19,20] Patients with cervical lesions appear to be characterized by Lacto depletion and predominance of anaerobic bacteria such as *Gardnerella vaginalis* (GV), *Megasphera type 1* (Mega1), *Sneathia spp*., or *Prevotella spp*.[21–24] Some studies refer that *Sneathia spp*. is frequently found in cervical lesions.[23,25]

In the current study, we investigate the vaginal microbiome and correlate its presence with Hr-HPV status and cervical cytology classification, in a population attending CC screening.

2 | MATERIALS AND METHODS

2.1 | STUDY POPULATION

This study was performed in cervicovaginal samples of women that participate in the RCCU. The RCCU is an organized screening program performed in all women from the Northern Region of Portugal aged 25–60 years old with extension up to 64 years of age, at 5-year intervals. All cervical samples from the RCCU were collected in ThinPrep™ Pap Test vials containing PreservCyt Solution™ (HOLOGIC™ Inc.) and sent to the reference laboratory at the Portuguese Oncology Institute of Porto (IPO Porto). Samples were tested for Hr-HPV using the *Anyplex II HPV HR Detection kit* (Seegene®) as previously described.[26] Hr-HPV positive samples were then separated and processed with cytological triage for the detection of cell abnormalities[27] being classified by a pathologist according to the *Bethesda* Classification: Negative for Intraepithelial Lesion or Malignancy (NILM), Atypical Squamous Cells of Undetermined Significance (ASC-US), Atypical Squamous Cells cannot exclude HSIL (ASC-H), Low-Grade Squamous Intraepithelial Lesion (LSIL), High-Grade Squamous Intraepithelial Lesion (HSIL), Squamous Cell Carcinoma (SCC), Atypical Glandular Cells and Adenocarcinoma.[28]

Consecutive samples processed in the RCCU between September to November 2021 with a positive result for Hr-HPV and cytopathological classification were recruited. A total of 807 women with a mean age of 41.45 ± 10.79 years old (median age 41; range 24–64) were included—Table 1. Samples were categorized into different groups according to cytology including all consecutive cases with ASC-H ($n = 114$), LSIL ($n = 215$), HSIL ($n = 47$) and randomly selected samples with NILM ($n = 319$) and ASC-US ($n = 111$).

This study was approved by the Institutional Ethical Committee (*Comissão de Ética para a Saúde*) of IPO Porto (ref. CES-IPO:146/022).

2.2 | MICROBIOME ANALYSIS

The microbiome analysis was performed in the reminiscent deoxyribonucleic acid (DNA), stored at −80°C, from the selected samples using 3 commercial kits: *Allplex*™ STI Essential Assay Q (Seegene®), *Allplex*™ Genital Ulcer Assay (Seegene®) and *Allplex*™ Bacterian

TABLE 1 Description of the groups included in the study population.

Cytological classification	Number of cases, *n* (%)	Age (mean ± SD), years old
NILM	319 (39.5)	43.1 ± 11.3
ASC-US	111 (13.8)	41.6 ± 11.2
ASC-H	114 (14.1)	41.4 ± 9.8
LSIL	215 (26.6)	38.8 ± 10.4
HSIL	47 (5.8)	41.9 ± 8.4

Abbreviations: ASC-H, Atypical Squamous Cells cannot exclude HSIL; ASC-US, Atypical Squamous Cells of Undetermined Significance; HSIL, High-Grade Squamous Intraepithelial Lesion; LSIL, Low-Grade Squamous Intraepithelial Lesion; n, number; NILM, Negative for Intraepithelial Lesion or Malignancy; SD, Standard deviation; %, percentage.

Vaginosis Assay (Seegene®). The *Allplex™ STI Essential Assay Q (MH, UU)* enables simultaneous amplification and detection of *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), *M. genitalium* (MG), *Mycoplasma hominis* (MH), *U. urealyticum* (UU), *U. parvum* (UP), and *Trichomonas vaginalis* (TV). The *Allplex™ Genital Ulcer Assay* enables simultaneous amplification and detection of *Herpes simplex virus type 1* (HSV-1), *Herpes simplex virus type 2* (HSV-2), *Haemophilus ducreyi* (HD), *Cytomegalovirus* (CMV), *Lymphogranuloma venereum* (LGV, *C. trachomatis* Serovar L), *Treponema pallidum* (TP) and *Varicella-zoster virus* (VZV). The *Allplex™ Bacterial Vaginosis Assay* amplifies and detects *Megasphaera* Tipo 1 (Mega1), *Lactobacillus* spp. (Lacto), *Bacteroids fragilis* (BF), GV, bacteria associated to bacterial vaginosis (BVAB2), *Atopobium vaginae* (AV), and *Mobiluncus* spp. (Mob).

These kits employ a MuDT™ technology which provides multi-C_t (cycle threshold) values in a single fluorescence channel, without melting curve analysis. Amplification reactions are prepared according to the manufacturer's instructions: 5 µL of RNase-free water, 5 µL of Enzyme Master (EM1), and 5 µL of specific probes/primers (MoM), for each Kit. Reactions are executed in CFX96™ qReal-time Polymerase Chain Reaction (PCR) System, a Real-Time PCR system (Bio-Rad Laboratories, Inc.) and results are processed using Seegene Viewer™ (Seegene®) data analysis software.

2.3 | STATISTICAL ANALYSIS

Statistical analysis was performed by descriptive statistics (frequencies/prevalence) and the correlation was performed using the computer software IBM SPSS Statistics for Mac, Version 27.0 (IBM). Tables and figure assembly were performed with Microsoft Excel for Mac, Version 16.65 (Microsoft). Microbiome profiles were depicted and correlated with Hr-HPV status and cytopathological information. Categorical variables were expressed using counts and percentages. The χ^2 test or Fisher-exact test (2-sided) was used to calculate the risk ratio (RR) between groups and the different variables considering a statistical significance of 5% ($p < 0.05$) and a 95% confidence interval (CI). Logistic regression was used to estimate the odds ratio (OR) and 95% CI. Univariate analysis was used to select the statistically significant variables ($p < 0.05$) as candidates for the multivariable logistic model. Multivariable logistic regression was performed adjusting for age and Hr-HPV status.

3 | RESULTS

3.1 | Hr-HPV characterization

The analysis of the Hr-HPV prevalence in NILM and different cervical abnormalities revealed that Hr-HPV multiple infections are responsible for over 40% of cases in LSIL and ASC-US (49.3% and 44.1%, respectively); HPV-16/-18 are associated with over 30% of cases in ASC-H and HSIL (38.6% and 53.2, respectively); and HPV-9 valent vaccine genotypes (HPV-9val) are responsible for over 66% of all cases, especially, in ASC-H (94.7%) and HSIL (89.4%)—Figure 1.

3.2 | Cervicovaginal microbiome characterization

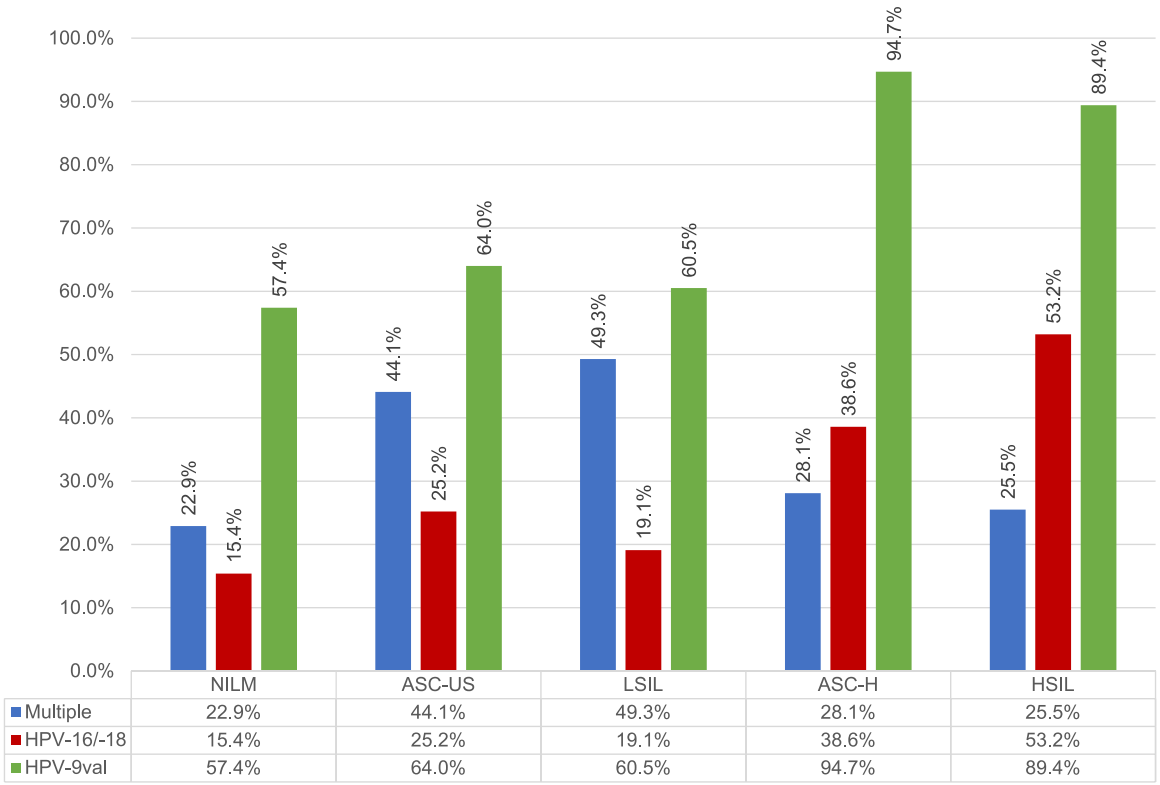
We analyzed the cervicovaginal microbiome with three different kits and the description of the results, regarding the different panels included in the kits is presented in Table 2. UP (52.5%), GV (34.5%), AV (32.6%), Lacto (30.7%), and MH (23.5%) were the dominant species among all women included in this study. Of all pathogens tested, we did not find any case of LGV. The results reveal an overall prevalence of 8.2% for microorganisms from the mGU, 68.1% for microorganisms from mSTI, and 59.1% for microorganisms from mBV.

3.3 | Cervicovaginal microbiome and age

To study the effect of age on the distribution of the different pathogens, we stratified the analysis based on the median age of women. The results showed interesting data concerning the most prevalent pathogens in the population (Table 2 and Figure 2). While UP (53.8% vs. 53.6%) and UU (14.4% vs. 18.9%%) did not differ significantly considering age, results indicate that MH ($p < 0.001$; RR = 1.97), Mega1 ($p < 0.001$; RR = 2.22), GV ($p = 0.013$; RR = 1.46), BVab2 ($p < 0.001$; RR = 2.22), AV ($p < 0.001$; RR = 1.78) e Mob ($p = 0.002$; RR = 2.17) were more prevalent in women older than 41 years of age (Supporting Information: Table I). Contrarily, Lacto is significantly decreased in the group of women older than 41 years of age (23.5% vs. 39.4%, $p < 0.001$; RR = 0.47).

3.4 | Cervicovaginal microbiome and HPV-status

The analysis of the distribution of the different pathogens from the cervicovaginal microbiome with Hr-HPV status was performed



	NILM	ASC-US	LSIL	ASC-H	HSIL
Multiple	22.9%	44.1%	49.3%	28.1%	25.5%
HPV-16/-18	15.4%	25.2%	19.1%	38.6%	53.2%
HPV-9val	57.4%	64.0%	60.5%	94.7%	89.4%

FIGURE 1 HR-HPV characterization and cervical cytology classification. ASC-H, Atypical Squamous Cells cannot exclude HSIL; ASC-US, Atypical Squamous Cells of Undetermined Significance; HSIL, High-Grade Squamous Intraepithelial Lesion; LSIL, Low-Grade Squamous Intraepithelial Lesion; NILM, Negative for Intraepithelial Lesion or Malignancy.

considering: (1) infection by multiple Hr-HPV genotypes; (2) HPV-16/-18; and (3) HPV-9val (Table 2 and Figure 3).

The relative risk analysis for the association between the microorganisms from the cervicovaginal microbiome and HPV-status revealed that UU ($p < 0.001$; RR = 1.94) and MH ($p = 0.005$; RR = 1.63) are significantly more frequent in women with multiple Hr-HPV infections (Supporting Information: Table II). On the opposite side, Lacto ($p = 0.026$; RR = 0.68) and GV ($p = 0.036$; RR = 0.71) were found to be less frequent in women with multiple Hr-HPV. Similar results were found for the analysis regarding women with HPV-16/-18 or HPV-9val infections. In addition, we found that MH ($p = 0.028$; RR = 1.51) and Mob ($p = 0.025$; RR = 1.85) are associated with infection by HPV-9val. In sum, pathogens present in the mBV are significantly unusual in women with HPV-16/-18 ($p = 0.003$; RR = 0.60) or HPV-9val infections ($p = 0.001$; RR = 0.59), while infection by pathogens present in the mSTI is more frequent in women with multiple Hr-HPV infection ($p < 0.001$; RR = 1.86).

3.5 | Cervicovaginal microbiome and cytopathological classification

When analyzing the distribution of the different pathogens across the different cytopathological findings, we have considered different cytological classifications: NILM, ASC-US, LSIL, ASC-H, and HSIL. Results demonstrated that CMV and HSV-2 are the most recurring, regarding mGU; UP, MH, and UU were the top three microorganisms from the mSTI; and AV, GV, and Lacto were the top three microorganisms from the mBV panel (Table 2). The most prevalent pathogens in NILM were GV (61%) followed by UP (54%), Lacto (46%), AV (44%), and Mega1(26%); while in ASC-US was UP (54%) followed by AV (41%), Lacto (35%), MH (24%) and GV (20%); in LSIL were UP (52%) followed by AV (21%), MH (20%), Lacto (17%), and GV (13%); in ASC-H were UP (43%) followed by MH (24%), AV (16%), Lacto (11%), and GV (11%); and in HSIL were UP (30%) followed by MH (17%), AV (15%), GV (15%), and Lacto (11%).

Risk analysis was calculated considering three outcomes: (1) risk of development of any cervical abnormality (NILM vs. ASC-US, LSIL, ASC-H, and HSIL); (2) risk of development of cervical lesions (NILM and ASC-US, vs. LSIL, ASC-H, and HSIL); and (3) risk of development of high-grade lesions or higher (NILM, ASC-US, and LSIL vs. ASC-H and HSIL (Supporting Information: Table III). Results revealed that infection by Mega1 ($p < 0.001$; RR = 0.19), Lacto ($p < 0.001$; RR = 0.29), GV ($p < 0.001$; RR = 0.11), BVab2 ($p < 0.001$; RR = 0.22), AV ($p < 0.001$; RR = 0.43) and Mob ($p < 0.001$; RR = 0.23) are significantly less frequent in women with cervical abnormalities. Similar results were identified in the analysis regarding the development of cervical lesions or ASC-H/HSIL. Overall, infection by pathogens present in the mBV is significantly less frequent in women with cervical abnormalities ($p < 0.001$; RR = 0.12), cervical lesions ($p < 0.001$; RR = 0.19) or high-grade lesions or higher ($p < 0.001$; RR =

TABLE 2 Characterization of cervicovaginal microbiome.

		Age		Cytology						HPV status		
	Positive *n*	<=41 (*n* = 381) *n*	>41 (*n* = 371) *n*	NILM (*n* = 319) *n*	ASC-US (*n* = 103) *n*	LSIL (*n* = 215) *n*	HSIL (*n* = 47) *n*	ASC-H (*n* = 114) *n*	AdC, (*n* = 1) *n*	Hr-HPV Multiple (*n* = 272) *n*	HPV-16/-18 (*n* = 188) *n*	HPV-9val (*n* = 535) *n*
mGU												
HSV-1	1	-	1	-	1	-	-	-	-	1	-	-
HSV-2	21	13	8	7	5	3	2	4	-	7	3	14
HD	1	-	1	-	1	-	-	-	-	-	-	1
CMV	39	25	14	16	5	9	4	5	-	11	9	26
TP	**1**	1	-	1	-	-	-	-	-	-	-	1
VZV	**3**	2	1	-	3	-	-	-	-	-	-	2
LGV	**0**	-	-	-	-	-	-	-	-	-	-	-
mSTI												
UU	**125**	55	70	47	21	35	3	19	-	59	25	90
NG	**6**	2	4	2	-	4	-	-	-	1	2	5
MH	**181**	68	113	78	25	43	8	27	-	77	43	132
MG	**13**	8	5	3	3	5	-	2	-	5	2	10
UP	**404**	205	199	173	56	111	14	49	1	149	88	265
CT	**39**	22	17	13	9	15	1	1	-	15	12	28
TV	**20**	11	9	9	3	5	1	2	-	8	3	16
mBV												
Mega1	**112**	38	74	83	4	11	4	10	-	33	21	76
Lacto	**237**	150	87	147	36	37	5	12	-	67	37	131
BF	**3**	1	2	1	1	-	1	-	-	2	2	3
GV	**266**	118	148	196	21	29	7	13	-	77	42	161
BVab2	**109**	37	72	78	5	13	3	10	-	35	23	75
AV	**252**	103	149	139	42	46	7	18	-	85	52	165
Mob	**80**	27	53	58	5	11	2	4	-	31	21	62

Note: Bold values indicate statistically significant values.

Abbreviations: AV, Atopobium vaginae; BF, Bacteroids fragilis; BVab2, bacteria associated to Bacterial Vaginosis; CMV, Cytomegalovirus; CT, Chlamydia trachomatis; GV, Gardnerella vaginalis; HD, Haemophilus ducreyi; HSV-2, Herpes simplex virus type 2; HSV-1, Herpes simplex virus type 1; Lacto, Lactobacillus spp; LGV, (Chlamydia trachomatis Serovar L), Lymphogranuloma venereum; mBV, Bacterial Vaginosis panel; Mega1, Megasphaera type 1; Mob, Mobiluncus spp; MG, Mycoplasma genitalium; mGU, Genital Ulcer panel; MH, Mycoplasma hominis; mSTI, Sexually Transmitted Infections panel; n, number; NG, Neisseria gonorrhoeae; TP, Treponema pallidum; TV, Trichomonas vaginalis; UP, Ureaplasma parvum; UU, Ureaplasma urealyticum; VZV, Varicella-zoster virus.

0.27). The analysis also showed that UP ($p = 0.007$; RR = 0.61) and CT ($p = 0.020$; RR = 0.22) were significantly less common in women with high-grade lesions or higher.

3.6 | Logistic regression

Univariate logistic regression analysis was executed to determine the independent contributions of different variables in the risk of developing different outcomes, by estimating the ORs (Table 3). Curiously, age older than 41 years was statistically associated with protection for the development of any cervical abnormality ($p = 0.006$; OR = 0.67) or any cervical lesion ($p < 0.001$; OR = 0.62) while for the development of ASC-H/HSIL, no statistical significance was observed ($p = 0.470$; OR = 0.88). Regarding HPV status, results showed that Hr-HPV-16/-18 is associated with increased risk for the development of cervical abnormalities ($p < 0.001$; OR = 1.92), cervical lesions ($p < 0.001$; OR = 1.90) or ASC-H/HSIL ($p < 0.001$; OR = 3.35).

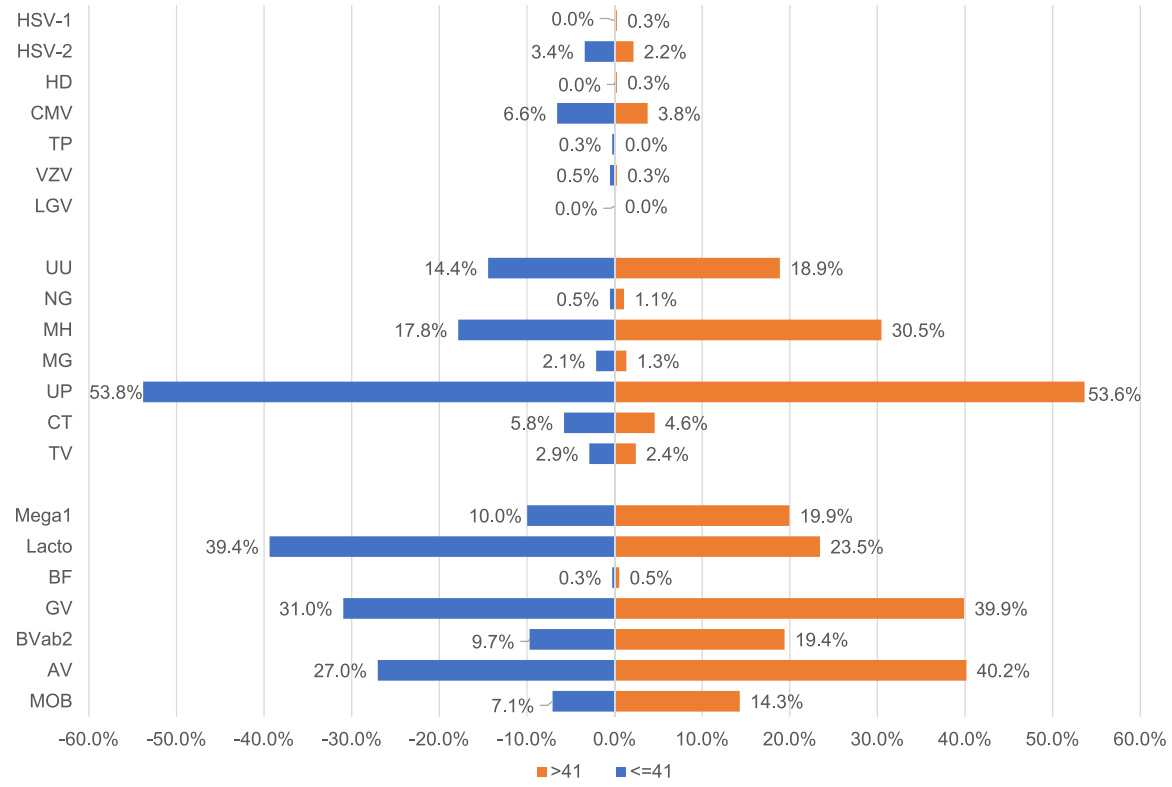


FIGURE 2 Prevalence of microorganisms according to age. AV, Atopobium vaginae; BF, Bacteroids fragilis; BVab2, bacteria associated to Bacterial Vaginosis; CMV, Cytomegalovirus; CT, Chlamydia trachomatis; GV, Gardnerella vaginalis; HD, Haemophilus ducreyi; HSV-2, Herpes simplex virus type 2; HSV-1, Herpes simplex virus type 1; Lacto, Lactobacillus spp; LGV, (Chlamydia trachomatis Serovar L), Lymphogranuloma venereum; mBV, Bacterial Vaginosis panel; Mega1, Megasphaera type 1; Mob, Mobiluncus spp; MG, Mycoplasma genitalium; mGU, Genital Ulcer panel; MH, Mycoplasma hominis; mSTI, Sexually Transmitted Infections panel; n, number; NG, Neisseria gonorrhoeae; TP, Treponema pallidum; TV, Trichomonas vaginalis; UP, Ureaplasma parvum; UU, Ureaplasma urealyticum; VZV, Varicella-zoster virus.

Similarly, infection by Hr-HPV-9val is associated with increased risk for the development of cervical abnormalities ($p < 0.001$; OR = 2.18), cervical lesions ($p < 0.001$; OR = 2.02), and more importantly ASC-H/HSIL ($p < 0.001$; OR = 9.27). Hr-HPV multiple infections were found to be associated with cervical abnormalities ($p < 0.001$; OR = 2.34) and cervical lesions ($p < 0.001$; OR = 1.69) but not for ASC-H/HSIL ($p < 0.001$; OR = 9.27). Regarding the different pathogens, results showed that the presence of Mega1, Lacto, GV, AV, and Mob are associated with protection against the development of cervical abnormalities, cervical lesions, or ASC-H/HSIL ($p < 0.050$; OR < 1.00). Results also disclosed that infection by UP and CT are also associated with protection for the development of ASC-H/HSIL ($p < 0.050$; OR < 1.00).

The variables from the univariate logistic regression that were associated with the different outcomes ($p < 0.05$) were included in the multivariate model to adjust for potential confounding factors using the manual enter model-building approach (Table 4). The multivariate logistic regression combining information regarding age, HPV-status, and the different pathogens was performed to determine the independent contributions of different variables in the risk of developing the different outcomes, by estimating the ORs. Regarding the risk of developing cervical abnormalities, Hr-HPV-16/-18 ($p = 0.008$; OR = 1.81) and Hr-HPV-9val ($p < 0.001$; OR = 7.49) are associated with an increased risk, while Lacto ($p < 0.001$; OR = 0.33), GV ($p = 0.0111$; OR = 0.41), AV ($p = 0.033$; OR = 0.53) and Mob ($p = 0.022$; OR = 0.29) are protective. Furthermore, similar results were found for the risk of development ASC-H/HSIL.

4 | DISCUSSION

Since the publication of the Human Microbiome Project in 2009, research on the impact of the microbiome in disease development has been rising.[29] Several studies have described the vaginal microbiome as being composed of multiple microbial species, although with a high predominance of Lacto.[30–32] Indeed, it was described that vaginal microbiome may be categorized into CSTs from I to V, defined by the dominance of specific Lacto: I, II, III, and V are dominated by *Lactobacillus crispatus* (Lacto C), *Lactobacillus gasseri* (Lacto G), *Lactobacillus iners* (Lacto I) and *Lactobacillus jensenii* (Lacto J), respectively; while IV is characterized by a polymicrobial state typical of Bacterial Vaginosis.[33,34] Moreover, some authors are already suggesting some variations in these CST according to association with the presence and abundance of other microorganisms such as *Megasphaera* and GV.[35]

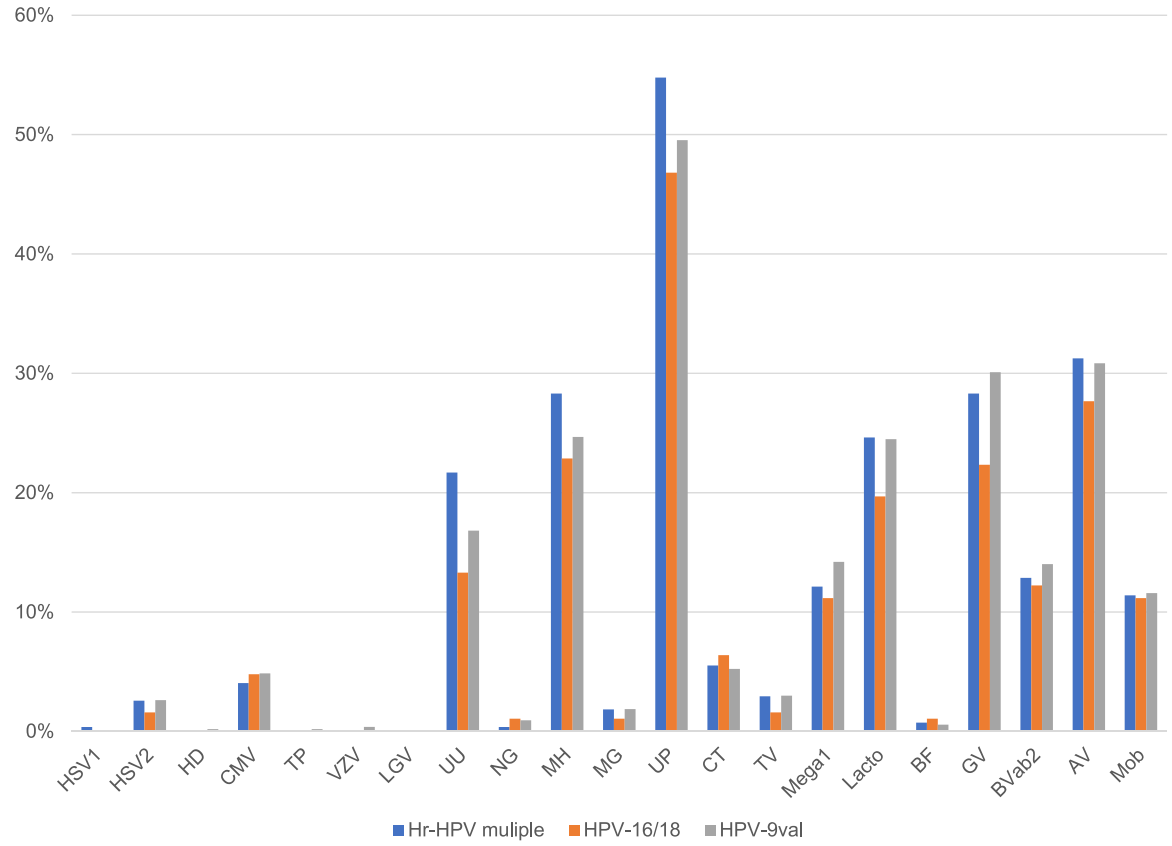


FIGURE 3 Prevalence of microorganisms according to HPV-status. AV, Atopobium vaginae; BF, Bacteroids fragilis; BVab2, bacteria associated to Bacterial Vaginosis; CMV, Cytomegalovirus; CT, Chlamydia trachomatis; GV, Gardnerella vaginalis; HD, Haemophilus ducreyi; HSV-2, Herpes simplex virus type 2; HSV-1, Herpes simplex virus type 1; Lacto, Lactobacillus spp; LGV, (Chlamydia trachomatis Serovar L), Lymphogranuloma venereum; mBV, Bacterial Vaginosis panel; Mega1, Megasphaera type 1; Mob, Mobiluncus spp; MG, Mycoplasma genitalium; mGU, Genital Ulcer panel; MH, Mycoplasma hominis; mSTI, Sexually Transmitted Infections panel; *n*, number; NG, Neisseria gonorrhoeae; TP, Treponema pallidum; TV, Trichomonas vaginalis; UP, Ureaplasma parvum; UU, Ureaplasma urealyticum; VZV, Varicella-zoster virus.

Changes in the cervicovaginal microbiome (dysbiosis) have been implicated with the development of gynecologic diseases and reproductive health.[17] Furthermore, the vaginal microbiome is affected by the different phases of women's lives (including birth, infancy, teenage, reproductive age, and menopause), ethnicity, geographical location, gestational status, use of oral contraceptives, menstrual cycle, and sexual activity.[32,36–39] The microbial vaginal flora is linked with the estrogen cycle of women, which stimulates the thickness of the vaginal epithelium, in women of childbearing age, promoting a lower vaginal pH (<4.5) sustained by *Lactobacillus spp*.[36,40] Otherwise, vaginal dysbiosis occasionally causes symptomatic conditions,[41] and the commonest clinical condition typified by vaginal dysbiosis is Bacterial Vaginosis (BV), which is linked with barely any vaginal inflammation.[42] Many studies have described that BV is followed by alterations in the microenvironment of the vagina, reduced lactate, high vaginal pH, and the presence of other bacterial populations such as GV, MH, Mob, and UU.[37,42–45]

In this study, we report the prevalence of different microorganisms in cervical samples from women of age 25–64 participating in the organized RCCU of the Northern Region of Portugal. To the best of our knowledge, we report for the first time the prevalence of microorganisms in cervicovaginal samples from women obtained in the scope of CC screening and tested their association with cytological classification and Hr-HPV status. We used commercial kits for the characterization of different groups of microorganisms that develop cervicovaginal diseases, namely Sexually Transmitted Infections, Genital Ulcers and Bacterial Vaginosis. Considering that HPV is the commonest sexually transmitted infection[11,12] and its impact on the development of cancer, the identification of other co-factors that could predit the progression from Hr-HPV infection to cancer is of great importance. Overall, we identified a high frequency of positive cases from the mSTI and mBV (68.1% and 59.1%, respectively). The five most prevalent microorganisms present in this study were UP (52.5%), GV (34.5%), AV (32.6%), Lacto (30.7%), and MH (23.5%). This study also identifies differences in the microbiome profile regarding age, cytological abnormalities patterns, and Hr-HPV status.

Data analysis manifests these women aged > 41 years old had an increased prevalence of MH, UU, AV, BVab2, GV, and Mega1, and contrary, Lacto were significantly decreased in this group (23.5% vs. 39.4% in ≤41 years). Different authors have been demonstrating that the vaginal microbiome changes through women's lifespan, and while in reproductive age, the vaginal microbiome is mainly dominated by

TABLE 3 Univariate logistic regression for the risk of development of different cervical cytopathological.

	NILM vs others			NILM + ASC-US vs cervical lesions			NILM + ASC-US + LSIL vs ASC-H/HSIL		
	p	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI
Age									
≤41 versus >41	**0.006**	**0.67**	**0.50–0.89**	**0.001**	**0.62**	**0.47–0.82**	0.470	0.88	0.62–1.24
HPV status									
Hr-HPV multiple	**<0.001**	**2.34**	**1.71–3.22**	**<0.001**	**1.69**	**1.26–2.27**	0.073	0.70	0.48–1.03
Hr-HPV-16/-18	**<0.001**	**1.92**	**1.43–2.58**	**<0.001**	**1.90**	**1.36–2.64**	**<0.001**	**3.35**	**2.31–4.85**
Hr-HPV-9val	**<0.001**	**2.18**	**1.52–3.13**	**<0.001**	**2.02**	**1.50–2.73**	**<0.001**	**9.27**	**4.93–17.4**
Pathogens									
HSV-1	1.000	NC	NC	1.000	NC	NC	1.000	NC	NC
HSV-2	0.477	1.40	0.56–3.50	0.812	0.90	0.37–2.16	0.297	1.67	0.64–4.39
HD	1.000	NC	NC	1.000	NC	NC	1.000	NC	NC
CMV	0.985	0.99	0.52–1.91	0.923	1.03	0.54–1.97	0.572	1.25	0.58–2.69
TP	1.000	NC	NC	1.000	NC	NC	1.000	NC	NC
VZV	0.999	NC	NC	0.999	NC	NC	0.999	NC	NC
UU	0.346	0.83	0.56–1.23	0.818	1.05	0.71–1.54	0.605	0.88	0.53–1.45
NG	0.688	1.42	0.26–7.81	0.293	2.49	0.45–13.7	0.999	NC	NC
MH	0.598	0.91	0.65–1.28	0.628	0.92	0.66–1.29	0.979	1.00	0.66–1.54
MG	0.189	2.39	0.65–8.77	0.504	1.46	0.48–4.37	0.721	0.76	0.17–3.46
UP	0.368	1.14	0.86–1.52	0.384	0.88	0.66–1.17	**0.008**	**0.61**	**0.43–0.88**
CT	0.296	0.70	0.35–1.37	0.890	0.96	0.50–1.83	**0.036**	**0.22**	**0.05–0.91**
TV	0.741	0.86	0.35–2.10	0.671	0.82	0.33–2.03	0.625	0.73	0.21–2.54
Mega1	**<0.001**	**0.19**	**0.12–0.30**	**<0.001**	**0.30**	**0.19–0.49**	**0.051**	**0.56**	**0.31–1.00**
Lacto	**<0.001**	**0.29**	**0.21–0.40**	**<0.001**	**0.25**	**0.17–0.35**	**<0.001**	**0.24**	**0.14–0.40**
BF	0.781	1.41	0.13–15.6	0.693	0.62	0.06–6.80	0.547	2.10	0.19–23.3
GV	**<0.001**	**0.11**	**0.08–0.16**	**<0.001**	**0.16**	**0.11–0.23**	**<0.001**	**0.24**	**0.14–0.39**
BVab2	**<0.001**	**0.23**	**0.15–0.35**	**<0.001**	**0.34**	**0.21–0.54**	**0.037**	**0.52**	**0.29–0.96**
AV	**<0.001**	**0.43**	**0.32–0.58**	**<0.001**	**0.35**	**0.25–0.48**	**<0.001**	**0.35**	**0.22–0.56**
Mob	**<0.001**	**0.23**	**0.14–0.38**	**<0.001**	**0.30**	**0.17–0.52**	**0.007**	**0.31**	**0.13–0.73**

Note: Bold values indicate statistically significant values.

Abbreviations: AV, Atopobium vaginae; BF, Bacteroids fragilis; BVab2, bacteria associated to Bacterial Vaginosis; CI, confidence interval; CMV, Cytomegalovirus; CT, Chlamydia trachomatis; GV, Gardnerella vaginalis; HD, Haemophilus ducreyi; HSIL, High-Grade Squamous Intraepithelial Lesion; HSV-2, Herpes simplex virus type 2; HSV-1, Herpes simplex virus type 1; Lacto, Lactobacillus spp; LGV, (Chlamydia trachomatis Serovar L), Lymphogranuloma venereum; LSIL, Low-Grade Squamous Intraepithelial Lesion; mBV, Bacterial Vaginosis panel; Mega1, Megasphaera type 1; Mob, Mobiluncus spp; MG, Mycoplasma genitalium; mGU, Genital Ulcer panel; MH, Mycoplasma hominis; mSTI, Sexually Transmitted Infections panel; n, number; NG, Neisseria gonorrhoeae; NILM, Negative for Intraepithelial Lesion or Malignancy; OR, odds ratio; *p*, Pearson Chi-Square; TP, Treponema pallidum; TV, Trichomonas vaginalis; UP, Ureaplasma parvum; UU, Ureaplasma urealyticum; VZV, Varicella-zoster virus.

Lacto, as women enter menopausal age, the vaginal microbiome is mainly composed of GV, UU, *Candida albicans* and *Prevotella spp*. with a progressive decrease in species of Lacto.[33,34,36,46] Kyrgiou et al. refer that healthy vaginal bacterial communities, present in pre-menopausal women, are generally populated by Lacto, and provide the first line of defense against pathogens, as they are commonly thought to ensure a low pH.[18] The exposure to estrogens and progestin allows the lowering of local pH (<4.5) due to glycogen metabolization, which restricts the growth of many pathogens and contributes to the adhesion, colonization, and survival of Lacto.[36,40,47] However, not all Lacto, are necessarily stable or healthy, for example Lacto I is present in all women, including those with

TABLE 4 Multivariate logistic regression for the risk of development of different cervical cytopathological.

	NILM versus others			NILM + ASC-US versus cervical lesions			NILM + ASC-US + LSIL versus ASC-H/HSIL		
Variables	***p***	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***	**OR**	**95% CI**
≤41 versus > 41	0.228	0.78	0.52–1.17	**<0.001**	**0.54**	**0.38-0.75**	0.177	0.751	0.50–1.14
Hr-HPV multiple	**<0.001**	**0.46**	**0.29–0.71**	**0.033**	**1.47**	**1.03-2.08**	**0.001**	**0.46**	**0.30–0.72**
Hr-HPV-16/-18	**0.008**	**1.81**	**1.17–2.82**	0.333	1.23	0.81-1.87	**0.005**	**1.89**	**1.21–2.97**
Hr-HPV-9val	**<0.001**	**7.49**	**3.80–14.73**	**0.016**	**1.61**	**1.09-2.36**	**<0.001**	**7.53**	**3.82–14.86**
UP	–	–	–	–	–	–	0.149	0.74	0.49–1.12
CT	–	–	–	–	–	–	**0.024**	**0.18**	**0.04–0.79**
Mega1	0.432	1.64	0.48–5.62	0.308	0.63	0.26–1.52	0.422	1.66	0.48–5.67
Lacto	**<0.001**	**0.33**	**0.18–0.60**	**<0.001**	**0.27**	**0.18–0.40**	**<0.001**	**0.30**	**0.17–0.55**
GV	**0.011**	**0.41**	**0.21–0.82**	**<0.001**	**0.29**	**0.19-0.46**	**0.013**	**0.42**	**0.21–0.84**
BVab2	0.736	1.25	0.34–4.58	0.667	1.22	0.50–3.00	0.742	1.24	0.34–4.55
AV	**0.033**	**0.53**	**0.30–0.95**	0.22	0.77	0.51–1.17	**0.047**	**0.55**	**0.31–0.99**
Mob	**0.022**	**0.29**	**0.10–0.83**	0.109	0.55	0.26–1.14	**0.033**	**0.31**	**0.11–0.91**

Note: Bold values indicate statistically significant values.

Abbreviations: AV, Atopobium vaginae; BF, Bacteroids fragilis; BVab2, bacteria associated to Bacterial Vaginosis; CI, confidence interval; CMV, Cytomegalovirus; CT, Chlamydia trachomatis; GV, Gardnerella vaginalis; HD, Haemophilus ducreyi; HSIL, High-Grade Squamous Intraepithelial Lesion; HSV-2, Herpes simplex virus type 2; HSV-1, Herpes simplex virus type 1; Lacto, Lactobacillus spp; LGV, (Chlamydia trachomatis Serovar L), Lymphogranuloma venereum; LSIL, Low-Grade Squamous Intraepithelial Lesion; mBV, Bacterial Vaginosis panel; Mega1, Megasphaera type 1; Mob, Mobiluncus spp; MG, Mycoplasma genitalium; mGU, Genital Ulcer panel; MH, Mycoplasma hominis; mSTI, Sexually Transmitted Infections panel; n, number; NG, Neisseria gonorrhoeae; NILM, Negative for Intraepithelial Lesion or Malignancy; OR, odds ratio; p, Pearson Chi-Square; TP, Treponema pallidum; TV, Trichomonas vaginalis; UP, Ureaplasma parvum; UU, Ureaplasma urealyticum; VZV, Varicella-zoster virus.

dysbiosis while Lacto C appears mostly in healthy women. Other studies found that a predominance of Lacto I is associated with the development of BV,[48–52] and conversely, a predominance of Lacto C seems to protect against the development of BV.[53] In our study we were not able to discriminate the individual *Lactobacillus* species which would be helpful in the characterization of the CSTs and provide further clarification regarding the correlations with age, cervical lesions, or Hr-HPV status.

Results point to an overrepresentation of Lacto in younger women and patients with NILM or ASC-US or LSIL compared to ASC-H or HSIL resulting in protection for the development of cervical lesions ($p < 0.001$; OR = 0.33). *Fan* et al described similar results confirming that Lacto infections are associated with decreased risk of developing cervical lesions.[54] Furthermore, our results showed that Lacto is less frequently associated with Hr-HPV infection, especially for HPV-9val ($p < 0.001$; RR = 0.50). Indeed, several authors described that certain species of Lacto are associated with protection against HPV infection, except for Lacto I which may be associated with increased risk.[20,55]

Ureaplasma spp. are frequent sexually transmitted infections. UP was the most prevalent pathogen in our study with over 50% positive cases amongst all women, and without significant variation regarding age. The data analysis revealed that despite its high prevalence UP was not significantly associated with Hr-HPV infection while it seems to provide a tendency for protection of the development of ASC-H/HSIL ($p = 0.074$; OR = 0.74), which seems to be consistent with reports from other authors.[56–61] Similarly, to UP, UU was distributed equally independent of age. Nevertheless, UU, which is mostly asymptomatic but described as associated with obstetric complications and HPV infection,[60,62–64] revealed to be frequently associated with Hr-HPV multiple infections ($p = 0.001$; RR = 1.94).

Not surprisingly, GV revealed to be one of the most important pathogens in the group of women older than 41 years of age. Literature states that these bacteria, although one of the most common cause of BV, is frequently present in the normal flora of women and only causes symptoms (such as vaginal discharge) under certain conditions.[65–68] Indeed, as the age of women progresses it becomes more prevalent.[33,34,36,46] Our results showed that GV is more frequently associated with age > 41 years old ($p < 0.050$) and represents a protective marker for the development of any cervical lesions ($p < 0.001$; OR = 0.41). GV is typically found at pH > 4.5 which may be a result of the decrease of estrogens and progestin favoring the infection and survival of GV.[69,70] Our result is controversial since different authors have linked a potential impact of GV in the development of cervical lesions and/or cancer while others fail to show any association.[68,71–73] Furthermore, some authors have been showing that quantification of some microorganisms such as GV may be of potential interest, which should be further explored.[74]

Regarding other common agents, we found that MH ($p = 0.028$; RR = 1.51) and Mob were highly associated with HPV-9val ($p = 0.025$; RR = 1.85). Nevertheless, Mob showed to be associated with protection against the development of any cervical lesion ($p = 0.022$; OR = 0.29). These results need further clarification since

it is unclear the true role of these agents in cervical disease development or HPV infection especially by studying healthy women.[73,75,76] Another interesting data is the results obtained for Mega1 and BVab2 which the univariate analysis revealed significant protection not only for cervical disease development ($p < 0.001$, OR = 0.19; and $p < 0.001$, OR = 0.23; respectively) but also for ASC-H/HSIL ($p = 0.051$, OR = 0.56; and $p = 0.037$, OR = 0.52; respectively). *Chen* et al argue that Mega1 infection is associated with decreased risk of developing lesions which is in agreement with our results.[16] Contrary to our findings, some studies have shown that BVab2, AV[77,78] and CT[79] infections may increase the risk of cervical lesions.

Overall, our data suggest that Lacto is the predominating pathogen in young women, while MH, UU, AV, BVab2, GV, and Mega1 are more frequent in older women. Results showed that while Lacto and GV are associated with protection for Hr-HPV infection, UU and MH are associated with increased risk for Hr-HPV infection. This results points that either Hr-HPV facilitates UU and MH infection or the contrary, which is important to clarify. Furthermore, bacteria associated with bacterial vaginosis (Mega1, Lacto, GV, BVab2, AV, and Mob) are associated with protection against the development of cervical abnormalities.

AUTHOR CONTRIBUTIONS

Conceptualization: Hugo Sousa, Ana Sousa and Rui Medeiros; methodology, Andreia Rosário, Ana Sousa, Tatiana Varandas, and Joana Marinho-Dias. *Clinicopathological data curation*: Rui Medeiros, Gabriela Martins, and Paula Monteiro. *Data analysis*: Andreia Rosário, Hugo Sousa; writing—original draft preparation, Andreia Rosário, and Hugo Sousa. *Writing—review and editing*: Andreia Rosário, Tatiana Varandas, Joana Marinho-Dias, Rui Medeiros, Paula Monteiro, and Hugo Sousa. *Supervision*: Ana Sousa and Hugo Sousa. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

The authors would like to acknowledge WERFEN® (Madrid, Spain) and Seegene® (Seoul, South Korea) for providing the kits for the experiences regarding microbiome detection.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its supplementary information files. Additional information may be provided upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Rosário A, Sousa A, Varandas T, et al. Impact of cervicovaginal microbiome on the risk of cervical abnormalities development. *J Med Virol*. 2023;95:e28762. doi:10.1002/jmv.28762