

The Presence of *Lactobacillus* spp. and its Effect on the Occurrence of Other Microorganisms in the Reproductive Tract of Polish Women

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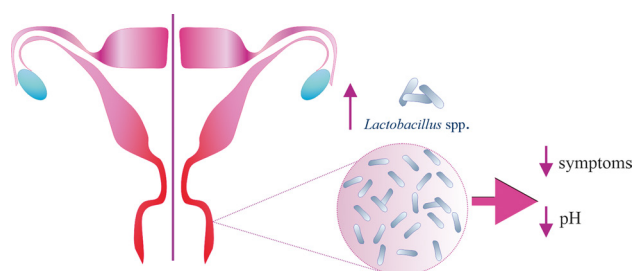
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Abstract

Disorders of the vaginal microbiota can lead to many complications and affect fertility. This study evaluates the role of *Lactobacillus* in the vagina and its impact on the incidence of colonization by pathogenic microorganisms, analyzing the results of 1,039 women of reproductive age (18–49 years) who underwent a microbiological examination of the reproductive tract in 2020. Samples were examined by microscopy, culture, and NAAT. As the number of *Lactobacillus* increases, the chance of developing symptoms decreases. In fact, it has been shown that the higher the number of *Lactobacillus* spp. the less frequently *Gardnerella vaginalis* and *Streptococcus* group B are observed. As the concentration of *Lactobacillus* spp. increases by one category, the risk of *G. vaginalis* after adjustment to age and pH decreases by 80% ($p < 0.001$). Similarly, the correla-



tion between *Lactobacillus* spp. and vaginal pH was shown. After adjustment to age, the odds of prevalence pH > 4.5 for people with *Lactobacillus* category higher 1 is 76% lower.

Key words: female reproductive tract microbiota, *Lactobacillus*, bacterial vaginosis, genital infections

Introduction

The vaginal microbiota of healthy women of reproductive age is characterized by low biodiversity and a dominance of species from the genus *Lactobacillus* (Cadieux et al. 2002; Ravel et al. 2011; Kaspróicz and Białecka 2016). The most common species of lactic acid bacilli inhabiting the vagina include *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactobacillus iners* (Witkin et al. 2021). Due to the prevalence of individual lactobacilli species, Ravel et al. (2011) developed the community state types (CST) classification, which distinguishes several types of vaginal microbiota. CST I, CST II, CST III, and CST V are dominated successively by *L. crispatus*, *L. gasseri*, *L. iners*,

and *L. jensenii*. CST IV represents a group in which *Lactobacillus* spp. is not the dominant species. There is a distinction between CST IV-A characterized by the presence of a small number of *L. iners* and CST IV-B with a predominance of bacteria such as *Fannyhessea*, *Gardnerella*, *Mobiluncus*, *Prevotella*, and *Sneathia* (Ravel et al. 2011). France et al. (2020) used an extensive database of approximately 13,160 vaginal microbiota profiles obtained by the 16S rRNA gene amplicon sequencing. Researchers have divided vaginal microbiota profiles into CST I, II, III, and V which are dominated successively by *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*. CST I and CST III were then divided into A and B, version A had a higher number of bacteria, while version B had a lower number of bacteria. CST-IV was divided

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into CST IV-A, IV-B, and IV-C dominated by “*Candidatus* Lachnocurva vaginae”, *Gardnerella vaginalis*, a group with a predominance of facultative and strict anaerobic species. CST-IV was further divided into five subtypes: CST IV-C0, CST IV-C1, CST IV-C2, CST IV-C3, and CST IV-C4 which are dominated successively by *Prevotella*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, and *Staphylococcus*. Then, collected reference data was used to train a nearest centroid classifier VALENCIA (VaginaL community state type Nearest Centroid classifier) to appropriately classify the vaginal microbiota profile. The VALENCIA closest center of gravity classification algorithm was created, allowing for the repeatable and accurate assignment of vaginal microbiota to appropriate CSTs. VALENCIA is a tool that uses the closest centroid for analysis, classifying samples based on similarity to a set of reference centroids. VALENCIA provides reliable and reproducible CST assignment by constructing a reference centroid and nearest centroid classification algorithm (France et al. 2020).

Lactic acid bacilli in the vagina perform protective functions through the production of lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins (Gajer et al. 2012; Tachedjian et al. 2018; Zheng et al. 2021). Lactic acid is found in two varieties L-lactic acid and D-lactic acid, of which D-lactic acid has a stronger protective effect. L-lactic acid blocks histone deacetylase, stimulating gene transcription and DNA repair. H_2O_2 *in vitro* shows high activity against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*. Lack of H_2O_2 in the vagina is hypothesized to increase the number of catalase-negative bacteria, which may contribute to the risk of bacterial vaginosis and human immunodeficiency virus (HIV) infection (Knezević et al. 2005). Bacteriocins, on the other hand, kill pathogens by blocking DNA synthesis. Not every *Lactobacillus* species provides a protective barrier. It concerns, for example, the species of *L. iners* (Aroutcheva et al. 2001; Vallor et al. 2001; Amabebe and Anumba 2018). Witkin et al. (2021) showed that the presence of *L. iners* was associated with more frequent colonization by pathogenic bacteria (Witkin et al. 2021) and does not protect against infection by *Chlamydia trachomatis* (Edwards et al. 2019). Lactobacilli, due to their protective properties, inhibit the proliferation of pathogenic microorganisms, counteracting the occurrence of bacterial vaginosis, among others. Bacterial vaginosis can contribute to serious health problems, including an increased risk of infertility, *C. trachomatis* infection, gonorrhea, human papilloma virus (HPV) and HIV, as well as pelvic organ inflammation (PID) and fallopian tube inflammation (Ravel et al. 2021). In addition, studies show that there is a relationship between decreased number of lactobacilli and the incidence of preterm labor, miscarriage and postpartum bleeding, and reduced fertility (Newton

et al. 2001; Ness et al. 2004; Galhardo et al. 2006; Witkin and Linhares 2017; Kiecka et al. 2021). Hence, information on the incidence of specific etiological agents of genital tract infections is valuable from a clinical perspective (Mączyńska 2008).

Women of reproductive age were selected as the study group. The aim of this study was to determine whether the number of *Lactobacillus* spp. can contribute to the inhibition of reproductive tract infections among women who report to the microbiology laboratory for check-ups or report due to emerging symptoms. Additionally, the aim was to determine how the presence of *Lactobacillus* spp. affects the occurrence of symptoms and the pH value.

Experimental

Materials and Methods

Study population. The study was a retrospective analysis of the results of 1,039 microbiological tests of the genital tract among women of reproductive age from 18 to 49 years of age at the Center of Microbiology and Autovaccines in Cracow, Poland, from April to August 2020. The study was carried out with the consent of the Bioethics Committee No. 118.6120.32.2023. The analysis included women who underwent a complete package of tests, including vaginal culture, evaluation of microscopic preparation of vaginal biocenosis, evaluation of pH value of vaginal contents, testing for *C. trachomatis*, *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, and *Mycoplasma genitalium*. In addition, a detailed medical history was recorded (all symptoms and treatment reported by the patients, including antibiotic therapy, were noted), and these data were obtained from the patient's medical records and attending physicians. The data used for the analysis were age, symptoms/absence, pH of vaginal contents, microbiological culture results, and molecular testing results (NAAT). Exclusion criteria were as follows: age criteria not met, under 18 and over 49 years of age, pregnant women, and antibiotic therapy in the last 2 months.

Collection of test material. Samples were taken from vagina and the cervical canal. Test material consisted of two vaginal swabs (one for microscopic preparation, pH and culture and the other for *Trichomonas vaginalis* culture) and one cervical swab for NAAT.

Determination of the pH value of vaginal contents. During the collection of vaginal swabs, 0.1 g of vaginal content found in the vaginal speculum was taken to determine the pH value of vaginal content. The pH of vaginal contents was measured using indicator strips in the range of 2.0–9.0 pH (Merck KGaA, Germany). The pH was then recorded in the patient's record. Normal

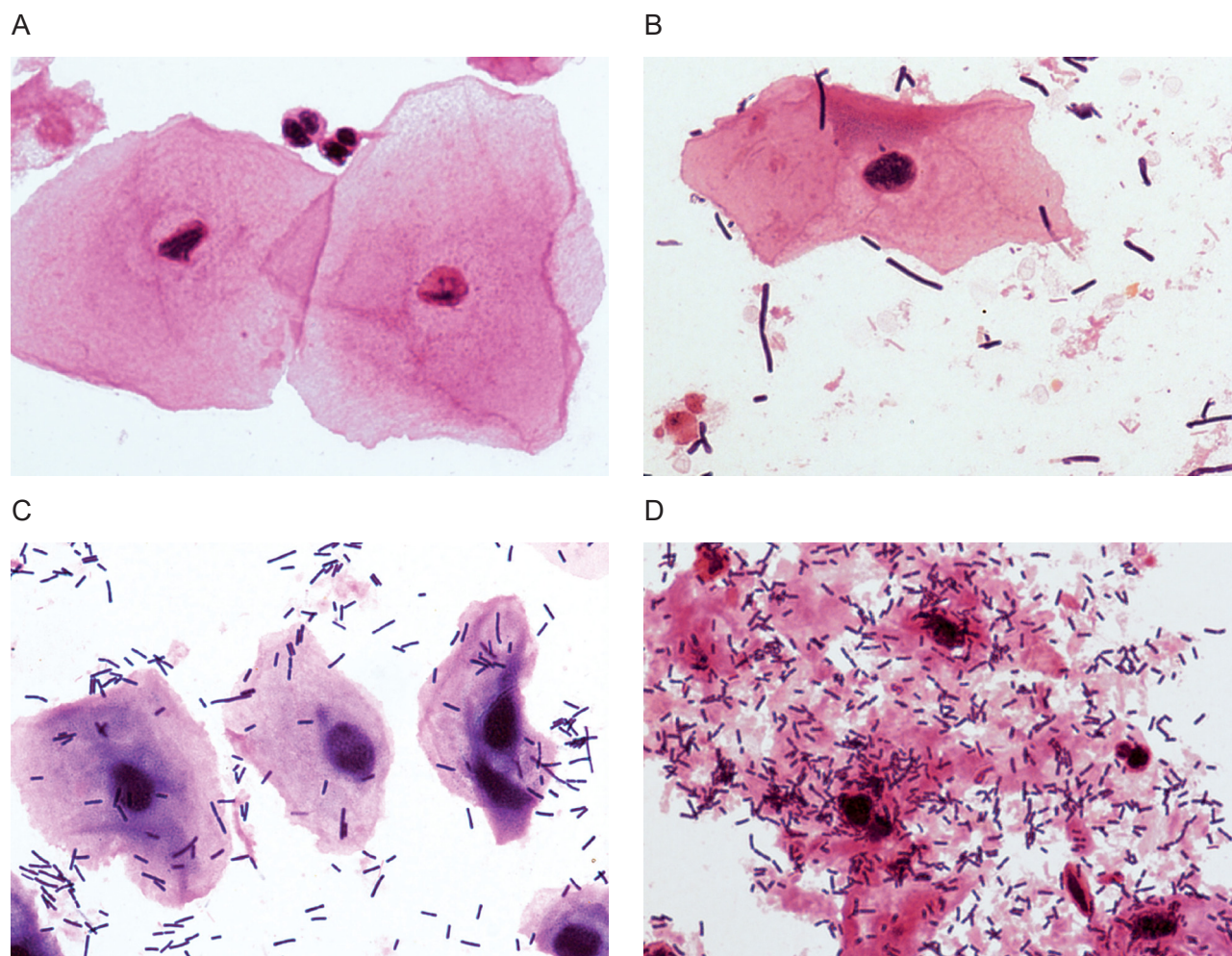


Fig. 1. Microscope photos showing *Lactobacillus* categories of 0, 1, 2, and 3.

A) 0 – means none, B) 1 – a single bacterium (up to 10 in the field of view), C) 2 – few (11–50 in the field of view), D) 3 – many (> 50 in the field of view) cells of *Lactobacillus*.

vaginal pH ranges from 3.5 to 4.5. A pH above 4.5 indicates an abnormal picture of vaginal biocenosis.

Evaluation of microscopic preparation. One of the swabs with vaginal contents was used for preparation. The collected vaginal contents were carefully spread on a microscope slide. The slide was then stained by the Gram method using a PREVI® Color Gram apparatus (bioMerieux, France). Microscopic preparations were evaluated using the Kuczyński scale (Kuczyńska 2003) with Kasprowicz modification (Kasprowicz and Białecka 2016). The presence of *Lactobacillus* spp. was evaluated on a scale of 0–3, where 0 means none, 1 – a single bacterium (up to 10 in the field of view), 2 – few (11–50 in the field of view), and 3 – many bacteria (> 50 in the field of view) (Fig. 1). The presence of leukocytes, bacteria, and fungi on a scale of 0–1 was also determined.

Detecting the presence of *T. vaginalis*. The swab was examined in 2 ml of Trichomedium (GRASO® Biotech, Poland). 48 hours later, a direct preparation was made. The preparation was observed under a microscope for identification of *T. vaginalis*.

Culture. A swab of vaginal contents was used to culture by direct plating the Petri dish on TSA media with 5% sheep blood (GRASO® Biotech, Poland), Gardnerella Agar (bioMerieux, France), CANDISelect (Bio-Rad, USA), and Chocolate Agar + P.V.S. + VCAT (Bio-Rad, USA). Then, the media was incubated for 24 hours or 48 hours at 37°C. After this time, microorganisms were identified using MALDI-TOF (Bruker Daltonics GmbH & Co., Germany) or VITEK® 2 (BioMerieux, France).

NAAT. A cervical contents swab was placed in 3 ml of UTM® medium (Copan Italia s.p.a., Italy). DNA was isolated using croBEE 201A (GeneProof a.s., Czech Republic) and croBEE NA16 (GeneProof a.s., Czech Republic) for nucleic acid extraction. NAAT assay was conducted using AmpliSens PCR assay in a Cobas® Z480 real-time thermocycler (Roche, Switzerland). NAAT was used to detect *C. trachomatis*, *U. parvum*, *U. urealyticum*, *M. hominis*, and *M. genitalium*.

Statistical analysis. An R language, version 4.0.4 (R Core Team 2021), an environment for statistical computing, was used for all statistical analysis. The

two-way p -value = 0.05 was set as a level of significance. The pH distribution was characterized by the median and interquartile range and was compared between women with or without the prevalence of particular microorganisms using the Mann-Whitney U test. The occurrence of microorganisms, symptoms, or pH > 5 was expressed in percentages and counts and compared across independent groups with the Fisher exact test. Additionally, univariable and multivariable logistic regression was applied for dichotomous outcomes (such as symptoms, pH > 4.5, prevalence of microorganisms). Due to the frequent occurrence of small cell counts in contingency tables showing the joint distribution of microorganisms and particular outcomes, a penalized likelihood Firth's method was applied to calculate odds ratios with 95% confidence intervals (ORs and CIs). p -Values were based on the Wald test. Variables used for adjustment were based on the literature and were limited only to the most important confounders.

Results

Study patients' population. The study group consisted of 1,039 women aged 18–49, among whom 287 showed burning, itching, pain, discomfort, and

discharge symptoms, while 752 patients showed none. *Lactobacillus* spp. (borderline 1–3) was found in 993 women, of which 132 women had *Lactobacillus* spp. with a score of 1, 611 women with a score of 2, and 250 women – a score of 3 (for reference here, see paragraph Evaluation of microscopic preparation) *Lactobacillus* spp. was absent in 46 patients. *G. vaginalis* was found in 51 women in the study group. Enterobacterales was detected in 55 women, *Streptococcus* group B in 81, and yeast-like fungi in 177 patients. The presence of *Ureaplasma* spp. was found in 245 women, of which *U. parvum* was identified in 165 cases, while *U. urealyticum* in 80 cases. *M. hominis* was reported in 19 women and *M. genitalium* in 1, while *C. trachomatis* was reported in eight women. This study did not show *Neisseria gonorrhoeae* and *T. vaginalis* in in any patient. Complete data on infections present in women are shown in Table I.

Microorganisms and clinical symptoms. Our data show that symptoms in the form of itching, burning, discharge, pain, and discomfort are associated with the presence of various microorganisms in the genital tract of women. Tables I and II show univariable and multivariable logistic regression models. In the crude model, women who had more *Lactobacillus* spp. were less likely to have symptoms. Specifically, the chance of clinical

Table I
Occurrence of individual microorganisms in women with and without symptoms qualified for the study and the microbiological methods used.

Microorganisms		Method	Total	Without symptoms (n = 752)	With symptoms (n = 287)	p -value*	OR# (95% CI)	p -value**
			n (%)	n (%)	n (%)			
<i>Lactobacillus</i> spp.†	0	microscopic	46 (4.4%)	19 (2.5%)	27 (9.4%)	< 0.001	1 (REF.)	–
	1		132 (12.7%)	90 (12.0%)	42 (14.6%)		0.33 (0.17; 0.66)	0.002
	2		611 (58.8%)	451 (60.0%)	160 (55.7%)		0.25 (0.14; 0.46)	< 0.001
	3		250 (24.1%)	192 (25.5%)	58 (20.2%)		0.22 (0.11; 0.41)	< 0.001
<i>Gardnerella vaginalis</i>		culture, microscopic	51 (4.9%)	11 (1.5%)	40 (13.9%)	< 0.001	10.55 (5.40; 20.63)	< 0.001
<i>Ureaplasma parvum</i>		PCR	165 (15.9%)	81 (10.8%)	84 (29.3%)	< 0.001	3.42 (2.43; 4.82)	< 0.001
<i>Ureaplasma urealyticum</i>		PCR	80 (7.7%)	27 (3.6%)	53 (18.5%)	< 0.001	6.02 (3.71; 9.75)	< 0.001
<i>Mycoplasma hominis</i>		PCR	19 (1.8%)	0 (0%)	19 (6.6%)	< 0.001	109.30 (6.58; 1,816.53)	0.001
<i>Mycoplasma genitalium</i>		PCR	1 (0.1%)	0 (0%)	1 (0.3%)	0.276	7.88 (0.32; 193.98)	0.207
<i>Chlamydia trachomatis</i>		PCR	8 (0.8%)	1 (0.1%)	7 (2.4%)	< 0.001	13.40 (2.31; 77.74)	0.004
Enterobacterales		culture	55 (5.3%)	35 (4.7%)	20 (7.0%)	0.162	1.55 (0.88; 2.72)	0.127
<i>Streptococcus</i> group B		culture	81 (7.8%)	57 (7.6%)	24 (8.4%)	0.698	1.12 (0.69; 1.84)	0.641
Yeast		culture, microscopic	177 (17.0%)	100 (13.3%)	77 (26.8%)	< 0.001	2.39 (1.71; 3.34)	< 0.001
<i>Neisseria gonorrhoeae</i>		culture	0 (0.0%)	0 (0.0%)	0 (0.0%)	–	–	–
<i>Trichomonas vaginalis</i>		culture	0 (0.0%)	0 (0.0%)	0 (0.0%)	–	–	–

* – all p -values are examined on Fisher's test
** – p -values measured by Wald method using Firth's logistic regression
– OR of occurrence symptoms in women with or without detected microorganisms, reference category in all microorganism is "undetected"
† – 0 means none, 1 single (up to 10 in the field of view), 2 few (11–50 in the field of view), 3 many (> 50 in the field of view) cells of *Lactobacillus*

Table II
Relationship between the occurrence microorganisms and symptoms.

Microorganisms		Model 1		Model 2		Model 3	
		OR* (95% CI)	p-value**	OR* (95% CI)	p-value**	OR* (95% CI)	p-value**
<i>Lactobacillus</i> spp. [†]	1	0.31 (0.15; 0.61)	<0.001	0.42 (0.20; 0.87)	0.019	0.33 (0.16; 0.69)	0.003
	2	0.23 (0.12; 0.43)	<0.001	0.38 (0.19; 0.76)	0.006	0.27 (0.13; 0.54)	<0.001
	3	0.20 (0.10; 0.38)	<0.001	0.38 (0.18; 0.81)	0.012	0.23 (0.10; 0.50)	<0.001
<i>Gardnerella vaginalis</i>		10.47 (5.36; 20.47)	<0.001	7.83 (3.93; 15.61)	<0.001	10.03 (4.92; 20.47)	<0.001
<i>Ureaplasma parvum</i>		3.40 (2.42; 4.79)	<0.001	3.18 (2.25; 4.50)	<0.001	2.93 (2.06; 4.16)	<0.001
<i>Ureaplasma urealyticum</i>		5.98 (3.69; 9.69)	<0.001	5.69 (3.49; 9.28)	<0.001	5.56 (3.40; 9.09)	<0.001
<i>Mycoplasma hominis</i>		111.18 (6.71; 1,842.58)	0.001	98.35 (5.93; 1,631.18)	0.001	94.56 (5.72; 1,563.34)	0.001
<i>Chlamydia trachomatis</i>		13.21 (2.28; 76.60)	0.004	13.56 (2.31; 79.70)	0.004	10.12 (1.69; 60.80)	0.011
Yeast		2.38 (1.70; 3.32)	<0.001	2.24 (1.59; 3.14)	<0.001	1.93 (1.36; 2.74)	<0.001

Model 1 adjusted to age, Model 2 adjusted to age and pH, Model 3 adjusted to age, pH and leucocytes
* – OR of occurrence symptoms in women with microorganisms (except of *Lactobacillus*) compared with those without microorganisms, in the case *Lactobacillus* OR refers to prevalence of symptoms in women with detected
† – *Lactobacillus* at the level 1 single (up to 10 in the field of view), 2 few (11–50 in the field of view), 3 many (> 50 in the field of view) respectively, compared with 0 (lack)
** – p-values testing by Wald method and Firth's logistic regression

symptoms in women with *Lactobacillus* spp. scores equal to 1, 2 and 3 decreased by 67%, 75%, and 78%, respectively, compared to women with no *Lactobacillus* spp. Furthermore, it has been shown that when *G. vaginalis* colonize the vagina, there is a 10-fold increased risk of symptoms (OR = 10.55, 95% CI: 5.40; 20.63). All women in whom *M. hominis* was identified were symptomatic (OR = 109.30 6 95% CI: 6.58; 1,816.53). There was no significant relationship between the presence of *M. genitalium* and the onset of symptoms ($p = 0.207$), and the bacterium was diagnosed in only one patient. The results showed a positive correlation between the prevalence of fungi and symptoms (OR = 2.39, 95% CI: 1.71; 3.34). The prevalence of *Streptococcus* group B was not statistically different ($p = 0.698$) in symptomatic versus asymptomatic women, which was also confirmed by logistic regression (OR = 1.12, 95% CI: 0.69; 1.84). Multivariable analysis showed that the vaginal colonization by *G. vaginalis*, fungi, *Mycoplasma* spp., and *C. trachomatis* is corelated with symptoms. These results showed that *Lactobacillus* spp. have a protective role. After adjustment to age, pH and leucocytes the odds of clinical symptoms for women with *Lactobacillus* spp. score = 1 is lower by 67% compared to women with *Lactobacillus* spp. with a score = 0 (OR = 0.33, 95% CI: 0.16; 0.69, $p = 0.002$). The result for women with a higher number of *Lactobacillus* was at a similar level, with the trend of even stronger protective effect (the odds decreased by 73% and 77%, respectively).

***Lactobacillus* spp. and other microorganisms.** Distribution of the prevalence of each microorganism according to the number of *Lactobacillus* spp. (0, 1, 2, 3). Table III shows statistically significant differences for *G. vaginalis*, Enterobacterales, and *Strep-*

tococcus group B, with a decreasing prevalence trend among women with higher levels of *Lactobacillus* spp. In a multivariate analysis, it was confirmed that lactobacilli provide a protective barrier against colonization of the vagina by pathogenic microorganisms. Our study showed a correlation between the number of *Lactobacillus* spp. expressed as an ordinal variable (0, 1, 2, 3) and the incidence of *G. vaginalis*. As the concentration of *Lactobacillus* spp. increases by one category (on a scale of 0–3 – as defined in paragraph Evaluation of microscopic preparation), the risk of *G. vaginalis*, after adjustment to age and pH decreases by 80% ($p < 0.001$). Similarly, as the number of *Lactobacillus* spp. increases by 1 category, the chance of vaginal colonization by Enterobacterales decreases by 46% and by 37% by *Streptococcus* group B respectively after adjustment to age and pH (Table IV). When we considered *Lactobacillus* spp. as a dichotomous variable, without taking into account its number, a statistically significant result was observed only for *G. vaginalis* with a 79% reduction of odds prevalent of GV for women with the presence of *Lactobacillus* (at any number) compared to with those without *Lactobacillus* detected (Table IV).

***Lactobacillus* spp. and pH value.** Out of 1,039 vaginal swabs, in 791 (76.1%) cases the pH was normal and in the range of 3.5–4.5. In 248 (23.9%) samples, the pH was above 4.5. Statistical analysis showed a strong relationship between the prevalence of high vaginal pH and the presence of *G. vaginalis*, *U. urealyticum*, Enterobacterales and *Streptococcus* group B, and yeast-like fungi (Table V). After adjustment to age, odds of prevalence pH > 4.5 for people with *Lactobacillus* category higher 1 is 76% lower. Distribution of pH between *Lactobacillus* categories was presented in Fig. 2.

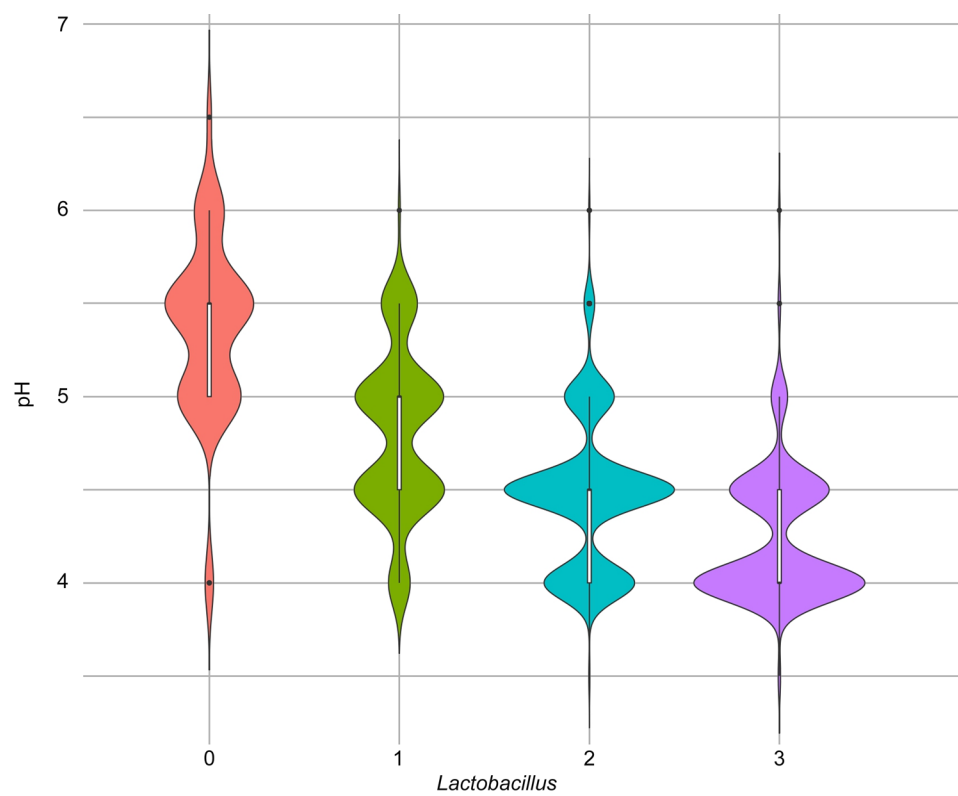


Fig. 2. Distribution of pH between *Lactobacillus* categories.

Table III
Relationship between the presence of *Lactobacillus* spp. and other microorganisms.

Microorganisms	Presence	n (%)	<i>Lactobacillus</i> spp. [†]				<i>p</i> -value*
			0	1	2	3	
<i>Gardnerella vaginalis</i>	No	988 (95.1%)	30 (65.2%)	108 (81.8%)	600 (98.2%)	250 (100%)	<0.001
	Yes	51 (4.9%)	16 (34.8%)	24 (18.2%)	11 (1.8%)	0 (0%)	–
<i>Ureaplasma parvum</i>	No	874 (84.1%)	36 (78.3%)	109 (82.6%)	520 (85.1%)	209 (83.6%)	0.542
	Yes	165 (15.9%)	10 (21.7%)	23 (17.4%)	91 (14.9%)	41 (16.4%)	–
<i>Ureaplasma urealyticum</i>	No	959 (92.3%)	40 (87.0%)	119 (90.2%)	566 (92.6%)	234 (93.6%)	0.299
	Yes	80 (7.7%)	6 (13.0%)	13 (9.8%)	45 (7.4%)	16 (6.4%)	–
<i>Mycoplasma hominis</i>	No	1020 (98.2%)	42 (91.3%)	129 (97.7%)	601 (98.4%)	248 (99.2%)	0.0153
	Yes	19 (1.8%)	4 (8.7%)	3 (2.3%)	10 (1.6%)	2 (0.8%)	–
<i>Mycoplasma genitalium</i>	No	1038 (99.9%)	45 (97.8%)	132 (100%)	611 (100%)	250 (100%)	0.0443
	Yes	1 (0.1%)	1 (2.2%)	0 (0%)	0 (0%)	0 (0%)	–
<i>Chlamydia trachomatis</i>	No	1031 (99.2%)	46 (100%)	131 (99.2%)	606 (99.2%)	248 (99.2%)	1.000
	Yes	8 (0.8%)	0 (0%)	1 (0.8%)	5 (0.8%)	2 (0.8%)	–
Enterobacterales	No	984 (94.7%)	36 (78.3%)	112 (84.8%)	590 (96.6%)	246 (98.4%)	<0.001
	Yes	55 (5.3%)	10 (21.7%)	20 (15.2%)	21 (3.4%)	4 (1.6%)	–
<i>Streptococcus</i> group B	No	958 (92.2%)	34 (73.9%)	108 (81.8%)	571 (93.5%)	245 (98.0%)	<0.001
	Yes	81 (7.8%)	12 (26.1%)	24 (18.2%)	40 (6.5%)	5 (2.0%)	–
Yeast	No	862 (83.0%)	39 (84.8%)	111 (84.1%)	506 (82.8%)	206 (82.4%)	0.976
	Yes	177 (17.0%)	7 (15.2%)	21 (15.9%)	105 (17.2%)	44 (17.6%)	–
<i>Neisseria gonorrhoeae</i>	No	1039 (100%)	46 (100%)	132 (100%)	611 (100%)	250 (100%)	–
	Yes	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	–
<i>Trichomonas vaginalis</i>	No	1039 (100%)	46 (100%)	132 (100%)	611 (100%)	250 (100%)	–
	Yes	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	–

* – all *p*-values are testing on Fisher’s exact test
† – 0 means none, 1 single (up to 10 in the field of view), 2 few (11–50 in the field of view), 3 many (> 50 in the field of view)

Table IV
The association between presence of *Lactobacillus* and other microorganisms – multivariable logistic regression models.

Microorganisms	<i>Lactobacillus</i> as ordered variable†		<i>Lactobacillus</i> as dichotomous variable††			
	Per 1 category level increase		Not present	Present	Not present	Present
	Model 1	Model 2	Model 1		Model 2	
Yeast	1.04 (0.83; 1.30)	1.32 (1.02; 1.71)	1 (REF.)	0.97 (0.43; 2.18)	1 (REF.)	1.69 (0.70; 4.07)
Enterobacterales	0.35 (0.25; 0.48)	0.54 (0.37; 0.81)	1 (REF.)	0.19 (0.09; 0.40)	1 (REF.)	0.78 (0.31; 1.97)
<i>Streptococcus</i> group B	0.40 (0.30; 0.52)	0.63 (0.45; 0.89)	1 (REF.)	0.23 (0.11; 0.47)	1 (REF.)	0.99 (0.42; 2.29)
<i>Gardnerella vaginalis</i>	0.14 (0.09; 0.21)	0.20 (0.12; 0.32)	1 (REF.)	0.05 (0.02; 0.11)	1 (REF.)	0.21 (0.09; 0.51)
<i>Chlamydia trachomatis</i>	1.12 (0.45; 2.79)	1.39 (0.52; 3.75)	1 (REF.)	0.67 (0.04; 11.0)	1 (REF.)	1.19 (0.06; 24.33)
<i>Mycoplasma hominis</i>	0.47 (0.28; 0.78)	0.61 (0.32; 1.16)	1 (REF.)	0.16 (0.05; 0.49)	1 (REF.)	0.32 (0.08; 1.32)
<i>Ureaplasma parvum</i>	0.91 (0.72; 1.13)	1.16 (0.89; 1.50)	1 (REF.)	0.58 (0.28; 1.19)	1 (REF.)	1.02 (0.46; 2.26)
<i>Ureaplasma urealyticum</i>	0.75 (0.56; 1.01)	0.88 (0.62; 1.25)	1 (REF.)	0.45 (0.19; 1.07)	1 (REF.)	0.70 (0.26; 1.90)

* – model 1 adjusted to age
** – model 2 adjusted to age and pH
† – *Lactobacillus* was coded as 0, 1, 2, 3, where 0 means none, 1 single (up to 10 in the field of view), few (11–50 in the field of view), 3 many (> 50 in the field of view) cells of *Lactobacillus*
†† – result for presence of *Lactobacillus* (1 + 2 + 3) compared to 0

Table V
The pH values in women with and without the occurrence of microorganisms.

Microorganisms	Not prevalent		Prevalent		<i>p</i> *	pH > 4.5	<i>p</i> **
	n	Q2 (Q1–Q3)	n	Q2 (Q1–Q3)		OR (95% CI)**	
<i>Lactobacillus</i> spp.	46	5.5 (5.0; 5.5)	993	4.5 (4.0; 4.5)	<0.001	0.01 (0.00; 0.05)	<0.001
<i>Gardnerella vaginalis</i>	988	4.5 (4.0; 4.5)	51	5.0 (5.0; 5.5)	<0.001	12.18 (6.31; 23.48)	<0.001
<i>Ureaplasma parvum</i>	874	4.5 (4.0; 4.5)	165	4.5 (4.5; 5.0)	<0.001	1.74 (1.21; 2.50)	0.003
<i>Ureaplasma urealyticum</i>	959	4.5 (4.0; 4.5)	80	4.5 (4.4; 5.0)	0.009	2.36 (1.47; 3.78)	<0.001
<i>Mycoplasma hominis</i>	1020	4.5 (4.0; 4.5)	19	4.5 (4.5; 5.0)	0.014	2.88 (1.18; 7.01)	0.020
<i>Mycoplasma genitalium</i>	1038	4.5 (4.0; 4.5)	1	5.0 (5.0; 5.0)	0.203	11.50 (0.46; 284.64)	0.136
<i>Chlamydia trachomatis</i>	1031	4.5 (4.0; 4.5)	8	4.5 (4.4; 4.6)	0.698	1.27 (0.29; 5.52)	0.748
Enterobacterales	984	4.5 (4.0; 4.5)	55	5.0 (4.5; 5.0)	<0.001	6.10 (3.47; 10.74)	<0.001
<i>Streptococcus</i> group B	958	4.5 (4.0; 4.5)	81	5.0 (4.5; 5.0)	<0.001	5.06 (3.17; 8.06)	<0.001
Yeast	862	4.5 (4.0; 4.5)	177	4.5 (4.5; 5.0)	0.001	1.66 (1.16; 2.37)	0.005

* – based on Mann-Whitney *U* test
** – based on logistic regression, Firth's method, crude models, OR of prevalence pH > 4.5 in women specific microorganisms compared with those without them; Q2 (Q1–Q3) denotes median (interquartile range)

Discussion

This retrospective study presents the results of women of reproductive age (18–49 years old) diagnosed at the Centre for Microbiology and Autovaccines in Cracow, Poland. The relationship between vaginal colonization by specific microorganisms and the occurrence of clinical symptoms in the form of burning, itching, discomfort, discharge and pain was investigated. The number of *Lactobacillus* spp. increases, and the risk of symptoms decrease. It confirms the protective role of *Lactobacillus* spp. (Boskey et al. 2001; Ravel et al. 2011). The occurrence of candidiasis was observed to influence the appearance of symptoms. According to

Mendling et al. (2015) candidiasis is associated with itching, discharge, burning sensation, and redness in only half of the cases. Often, women have asymptomatic *Candida* colonization, which requires treatment only before delivery to avoid obstetric complications (Mendling et al. 2015). In addition, women with symptomatic reproductive tract infections with *C. trachomatis* were found to be diagnosed more than 13 times more frequently. This may be related to the fact that *C. trachomatis* infection is associated with pelvic organ inflammation (Gottlieb et al. 2013). Additionally, colonization by *U. parvum*, *M. hominis*, and *U. urealyticum* was associated with symptoms in women of reproductive age. Our previous study showed that ureaplasmas are

closely associated with the onset of symptoms among women (Rak et al. 2022). However, symptoms do not always indicate infection with pathogenic microorganisms (Mitchell et al. 2020). This can also be seen in this data analysis. Among the 1,039 women studied, 88 were symptomatic, and there was no colonization by pathogenic microorganisms. Among these 88 symptomatic women, 10 had *Lactobacillus* spp. on 0–1, which, in theory, could explain the appearance of itching, burning, and pain. However, up to 78 symptomatic women had *Lactobacillus* score of 2 or 3 in the vagina, which is a good result, and the vaginal pH was normal. However, women reported discomfort, pain, and burning. It may be due to other factors that may influence the symptoms, including hormonal fluctuations, allergies, environmental factors, phase of the menstrual cycle, and sexual intercourse.

It is well known that lactic acid bacteria can adhere and compete for adhesion sites in the vaginal epithelium. In addition, they produce several antimicrobial compounds (lactic acid, bacteriocins, and hydrogen peroxide) that inhibit vaginal colonization by pathogenic microorganisms (Mei and Li 2022). Similar to Schwebke et al. (2014), it was shown that in women, the number of *Lactobacillus* spp. increases, colonization by *G. vaginalis* is less frequent and more lactic acid bacilli, the less frequent the infection by *Streptococcus* group B and Enterobacterales. However, this relationship was not observed for ureaplasma and mycoplasma.

According to Amsel's criteria (Amsel et al. 1983), pH is one of the predictive factors of bacterial vaginosis. A relationship between *Ureaplasma* spp. and elevated vaginal pH was demonstrated in our previous study (Rak et al. 2022). This retrospective analysis also showed a correlation between increased vaginal pH and colonization by *U. urealyticum*, *G. vaginalis*, Enterobacterales, and *Streptococcus* group B. In addition, a trend was identified: with an increase in *Lactobacillus*, there is a decrease in pH.

The strengths of this retrospective analysis were the large number of patients and the knowledge of microbiological composition of the reproductive organs of each patient. This study showed that even the use of classical diagnostic microbiology can expand our knowledge of the prevalence of microorganisms in the genital tract and affect the health of women of reproductive age.

Limitations of this study include the fact that the species of *Lactobacillus* spp. found in the vagina have not been identified. Since their identification could shed new light on the relationship between lactobacilli and vaginal health. Future research should focus on determining which species of *Lactobacillus* spp. provide a protective function. For example, in recent years, it has been increasingly reported that *L. iners*, among others, may represent a transitional species between

a healthy vagina and a tendency to vaginal infections (Kumar et al. 2023). A limitation of this study is that complete information about the patients is not known, such as sexual behavior, complete medical history, and socio-economic characteristics. An important fact is that in our study, asymptomatic women with *Lactobacillus* spp. defined out of 3 sometimes have a vagina colonized by pathogenic organisms. It shows how important it is to perform preventive examinations. Undiagnosed infections of the reproductive organs can cause obstetric complications in the form of miscarriages, premature births, and ectopic pregnancies. Farr et al. (2015) showed that a lack of *Lactobacillus* spp. in the vagina and colonization with pathogenic bacteria increases the risk of preterm delivery and low birth weight in women in early pregnancy.

In conclusion, the relationship between *Lactobacillus* spp. and the inhibition of colonization of reproductive organs by pathogenic microorganisms was found. It was shown that lactobacilli significantly reduce the risk of symptoms among women. However, further analysis of co-infections and associations between symptom occurrence in female reproductive tract infections is needed.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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