Book Chapter

Vaginal Microbiota Evaluation and Lactobacilli Quantification by qPCR in Pregnant and Non-pregnant Women: A Pilot Study

David Pacha-Herrera¹, Gabriela Vasco^{1,2}, Cecilia Cruz-Betancourt³, Juan Miguel Galarza³, Verónica Barragán¹ and António Machado^{1*}

¹Laboratorio de Bacteriología, Instituto de Microbiología, Colegio de Ciencias Biológicas y Ambientales, Universidad San Francisco de Quito, Ecuador

²Cátedra de Microbiología de la Escuela de Medicina, Facultad de Ciencias Médicas, Universidad Central del Ecuador, Ecuador

³Laboratorio de Biología Molecular, Unidad Técnica de Genética y Molecular, Hospital de Especialidades Carlos Andrade Marín del IESS, Ecuador

***Corresponding Author:** António Machado, Laboratorio de Bacteriología, Instituto de Microbiología, Colegio de Ciencias Biológicas y Ambientales, Universidad San Francisco de Quito, Quito, Ecuador

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Author Contributions: AM, GV, and DPH were responsible for modelling and experimental design. Samples collection oversaw doctors of Gynecology and Obstetrics Service of Carlos Andrade Marin Hospital (HCAM), Gynecological-Obstetric Hospital Isidro Ayora (HGOIA), and Center for Teaching Health Cipriana Dueñas. The DNA extraction was performed at Hospital Carlos Andrade Marín by CC. Molecular characterization was conducted by DPH in the Microbiology Institute at USFQ. Biostatistics analysis was developed by VB and DPH at USFQ.

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Abstract

Pregnancy outcomes and women's health are directly affected by vaginal microbiota. This microbiota consists of a dynamic ecosystem of various microbes in different ratios, which in healthy conditions protect the vaginal epithelium from infections. However, cases of vaginal infection are regularly diagnosed in women of reproductive age, contributing to more severe outcomes. Therefore, our main goal was to determine the prevalence of bacterial vaginosis (BV), aerobic vaginitis (AV), and vulvovaginal candidiasis (VVC) among Ecuadorian pregnant and non-pregnant women. A cross-sectional study was conducted among 217 women between 13 and 40 years old seeking primary healthcare in Carlos Andrade Marin Hospital (HCAM), Gynecological-Obstetric Hospital Isidro Ayora (HGOIA) and Center for Teaching Health Cipriana Dueñas during October 2018 to February 2019. The classical characterization of the vaginal microbiota was performed through microscopy by the Nugent criteria to evaluate the presence of BV, healthy and intermediate microbiota, by the criteria of Donders to determine the presence of AV and by the Marot-Leblond criteria to diagnose VVC. DNA extraction from vaginal samples and Polymerase Chain Reaction (PCR) analysis was performed to characterize the presence of *Gardnerella* spp., *Mobiluncus mulieris*, *Escherichia coli*, *Enterococcus* spp., and *Lactobacillus* spp. Finally, quantification of the lactobacilli was performed by quantitative real-time PCR (qPCR) for samples from women with normal vaginal microbiota and women with AV.

Our results showed 52% of women with healthy microbiota, 7% with intermediate microbiota, and 41% with vaginal dysbiosis, comprising 27% with AV, 8% with BV and 4% with VVC and 2% with co-infections or co-dysbiosis. Additionally, a higher amount of lactobacilli were found in pregnant women when compared to non-pregnant women, while AV cases were characterized by a significant drop of *Lactobacillus* spp., more precisely, between 1E3 and 1E5 colony forming units (CFU)/ml. Finally, women with normal vaginal microbiota showed an average load of lactobacilli between 1E6 and 1E7 CFU/ml. This

pilot study showed no statistically significant differences between pregnant and non-pregnant women, pointing to the possibility to use lactobacilli quantification for the prevention of future vaginal infections.

Keywords

Vaginal Microbiota₁, Vaginal Infection₂, Bacterial Vaginosis₃, Aerobic Vaginitis₄, Pregnant₅, Opportunistic Pathogen₆, *Lactobacillus* spp.₇ (Min.5-Max. 8)

Introduction

The normal vaginal microbiota plays a crucial role for the health of pregnant and non-pregnant women [1], preventing several urogenital diseases [2], including bacterial vaginosis (BV) [2-6], aerobic vaginitis (AV) [7-11], urinary tract infections (UTI) [12-14], yeast vaginitis [3,15,16], and sexually transmitted diseases (such as HIV) [3,6,17-19]. In the context of this study, it is also important to mention that women and teenagers in Ecuador have a wide range of health care needs, in particular, related to sexual and reproductive health [20]. In Ecuador, a major concern is the high rate of adolescent pregnancy, i.e. pregnancy between ages 10 to 19. Several studies worldwide also demonstrated a higher risk of acquiring HIV, herpes simplex virus type 2 and other sexually transmitted infections in non-pregnant women with vaginal infections or intermediate vaginal microbiota [6,18,21,22]. Thus, lactic acid-producing bacteria (such as *Lactobacillus* spp.) metabolize glycogen, increasing lactic acid and a normal acidic vaginal pH of 3.8 – 4.4 [1,12,23,24].

The vaginal microbial community is a variable econiche that fluctuates between normal and dysbiotic microbiota [1], which could be influenced by several intrinsic and extrinsic factors [24] and eventually leading to an increment of both aerobic and anaerobic microorganisms [25-28]. However, the most predominant genus in a healthy vaginal microbiota is *Lactobacillus* [1,12]. *Lactobacillus* genus is known to inhibit the adhesion and proliferation of opportunistic and primary pathogens [17]. The mechanisms by which vaginal lactobacilli

provide colonization resistance is generally considered to be through production of several antimicrobial compounds such as, hydrogen peroxide, lactic acid and/or bacteriocins [29-32], as well as acting as biosurfactant on the vaginal epithelium [12,33].

Although several species of *Lactobacillus* were already identified in vaginal microbiota, the most predominant species found in normal vaginal microbiota are *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. iners* [12,23,34]. Also, other species could be detected in low amount among healthy vaginal microbiota such as *Atopobium*, *Enterobacter*, *Escherichia*, *Gardnerella*, *Mobiluncus*, *Prevotella*, *Staphylococcus*, *Shigella* [34-37]. These species can also behave as opportunistic pathogens [32,36]. Several factors can induce disruptions of the healthy microbiota equilibrium, establishing a microbial dysbiosis and, thus, future vaginal infections [18,32,36,38].

According to previous studies, bacterial vaginosis (BV) is the most common vaginal dysbiosis among women of reproductive age [3,5,39,40], being characterized by lactobacilli replacement by anaerobes [7]. *Gardnerella* spp., *Atopobium vaginae*, *Bacteroides* spp. and *Mobiluncus* spp. are the main pathogenic anaerobes associated with BV [24], which is usually diagnosed by Nugent criteria [41] or the Amsel criteria [6]. Besides BV, a condition designated aerobic vaginitis (AV) has also been recognized, characterized by the presence of aerobic bacteria in detriment of lactobacilli and by inflammation diagnosed a yellow-green discharge [24,42]. This vaginal infection is usually dominated by *Streptococcus* sp., *Enterococcus* sp., and/or Gram-negative bacteria of enteric origin (mainly, *Escherichia coli*). Finally, vulvovaginal candidiasis (VVC) is the most prevalent cause of vaginal infection by fungi, with at least 75% of healthy women suffering one episode of VVC during lifetime [15] and whereby *Candida albicans* is the most important species [43].

Our main goal of the present study was to evaluate the presence of vaginal infection among Ecuadorian women by classical and standard microbiological techniques or criteria [25] and to determine the dominance of different types of vaginal infection among pregnant and non-pregnant women. Also, the present

study aimed to detect the presence of specific opportunistic pathogens (*Escherichia coli*, *Enterococcus faecalis*, *Gardnerella* spp., and *Mobiluncus mulieris*) by Polymerase Chain Reaction (PCR) and quantified the number of lactobacilli through quantitative real-time PCR (qPCR). The analysis of the normal amount of lactobacilli in pregnant and non-pregnant women might enable to determine the lactobacilli threshold associated with the establishment of vaginal infection.

Materials and Methods

Study Population, Design and Subject Selection

The study was conducted in the Microbiology Institute at USFQ in collaboration with Hospital Carlos Andrade Marín (HCAM) and Universidad Central del Ecuador (UCE) from October 2018 to February 2019. The research team recruited 217 Ecuadorian female volunteers of Hispanic ethnicity but in reproductive age (13 and 40 years old), of which 111 were pregnant. Applicants were excluded from the study if they reported antimicrobial treatment in the last three months or any evidence of bleeding, and also if they had sexual intercourse within the previous 48 hours. Also, a questionnaire was taken regarding demographic characteristics, sexual and health behavior of each patient, and each enrolled woman provided a usable vulvovaginal swab sample.

Ethics Statement

This study was approved by the Ethics Committee of Universidad San Francisco de Quito (USFQ) and the Ministry of Health of Ecuador (Protocol code: 2016-140M by MSP-SDM-10-2013-2019-O review board). The female participants were recruited to our study set, after having read and signed the informed consent or, in the case of underage participants, from their parents or legal representatives.

Sample Collection

Samples were taken by a gynecologist using a sterile disposable vaginal speculum. The lateral vaginal walls were swabbed with a

sterile swab to collect the cervical fluid, to prepare a smear on a microscope slide. Briefly, each vaginal smear was obtained by rolling the previous swab onto a glass slide, then heat-fixed and Gram-stained by using safranin as the counterstain. Following the Gram smear procedure, the swab was placed in 1 ml of phosphate buffer saline (PBS) and vortexed vigorously for approximately 3 minutes. The remaining vaginal material was collected by centrifugation at 16000 g for 5 minutes. The obtained pellet was suspended into an aliquot of 1 ml of saline (0.9% NaCl) which was used for culture of *Candida* spp. in different media (see section 2.4) and for wet mount microscopy for a better diagnosis of AV and VVC (see section 2.5).

A second sample was taken by a cervical brush (Rovers Cervex Brush®) through endo and exo-cervical brushing, placed immediately in Cobas® Preservative Fluid, stored at 4 °C until processing in the clinical laboratory of HCAM, and used for DNA extraction (see section 2.6). Each sample was further used to culture of *Candida* spp.

Culture of *Candida* spp.

Candida spp. was cultured on different media from the saline aliquot (see section 2.3). Briefly, 100 µl of saline solution was plated onto Petri dishes containing 5% human blood agar (HBA), chocolate agar (heated human blood agar) or Sabouraud dextrose agar (SDA). The plates were incubated at 37 °C for 48 h, under aerobic conditions, and colonies were analyzed and identified by gram staining, biochemical properties (catalase, oxidase, and hemolysis) and PCR (data not shown).

Microbiological Classification of Vaginal Infections

The Gram-stained vaginal smears were classified according to Nugent criteria for bacterial vaginosis (BV) [41], the criteria of Donders et al. for aerobic vaginitis (AV) [44] and those of Marot-Leblond et al. for vulvovaginal candidiasis (VVC) [43]. The evaluation of several cell types present in each smear was performed for 10 to 15 microscopic fields under oil immersion at 1000 X magnification [7].

After an initial evaluation of the Gram-stained smears by the Nugent criteria, all samples were evaluated by means of phase-contrast microscopy (X400 magnification) of wet smears, according to Schröders classification [8] and the Marot-Leblond *et al.* [43] criteria (see Table 1). The absence of *Lactobacillus* spp., presence of cocci or coarse bacilli in high numbers, presence of parabasal epithelial cells representing >10% of the epithelial cells, and/or presence of leucocytes were considered as indicative for AV [8]. In addition, aggravated AV diagnosis was defined as the most extreme form of aerobic vaginitis under Donders evaluation from Schröders classification [8], where AV samples showed lactobacilli severely depressed or absent because of overgrowth of other bacteria (Cocci or chains), more than 10 leukocytes per epithelial cell present in the samples and more than 50% of the leukocytes had a toxic appearance. It is important to mention that leukocytes were also evaluated on their granular appearance due to abundant lysozyme activity ('toxic leukocytes') [8]. Finally, VVC was assessed accordingly to Marot-Leblond and colleagues through at least one of the following criteria: positive Gram-stain preparation with budding yeasts in high numbers (five or more) in more than two microscopic fields, pseudohyphae, and/or hyphal forms in wet smears observation; and positive culture in Chocolate agar, HBA and/or SDA, along with negative microscopic examination results associated with eventual symptoms (thick, white vaginal discharge with no odor, vulvar and vaginal pruritus, burning, or dyspareunia) or clinical history (previous infection) obtained from the medical survey with the professional gynecologist. Absence of *Candida* cells in more than two microscopic fields and/or a low number of *Candida* spp. result on wet smears observation and culture growth was considered as normal *Candida* colonization rather than VVC [43].

Table 1: Parameters used for the diagnosis of vaginal infections.

Infection	Symptoms	Discharge	Odor	Diagnosis	References
Vulvovaginal candidiasis	Pruritus	Thick, white to yellow	Absent	Microscopic examination (Gram-stained smears and Wet mount preps), medical survey and growth culture	Carr et al., 1998; Marot-Leblond et al., 2009
Aerobic vaginitis	Inflammation	Yellow	Foul, rotten	Microscopic examination (Gram-stained smears and Wet mount preps) and medical survey	Donders et al., 2002; Donders et al., 2005
Bacterial vaginosis	Irritation, 50% asymptomatic	Thin, white to gray, homogeneous	Fishy	Microscopic examination (Gram-stained smears and Wet mount preps) and medical survey	Carr et al., 1998; Nugent et al., 1991

DNA Extraction of Vaginal Swabs

The Cobas® 4800 system (Roche Molecular Systems Inc., Pleasanton, CA) was used to extract the DNA of vaginal brushes, according to the manufacturer's instructions. DNA was quantified with a Nanovue spectrophotometer (GE Healthcare Life Science). DNA was eluted at 20 ng/μl with molecular grade water and stored at -20°C until the Polymerase Chain Reaction (PCR) analysis was performed. The quality of DNA was evaluated by measuring the concentration of phenolic compounds or the presence of salts (260/230) and protein contaminants (260/280).

Identification of the Major Bacterial Species by PCR

From 217 vaginal samples previously diagnosed by classical criteria through microscopy analysis, 97 were selected for molecular characterization by PCR in a Bio-Rad Thermocycler (Bio-Rad, Hercules, CA). Samples with scores between 0 and 1 of Nugent criteria were selected as healthy microbiota, while samples with scores between 9 and 10 of Nugent criteria (BV) and diagnosed as representing aggravated AV (see section 2.5) were used as dysbiotic microbiota. Thus, sixty samples with healthy microbiota (38 pregnant and 22 non-pregnant women), 23 samples with AV (14 pregnant and 9 non-pregnant women), and 14 samples with BV microbiota (6 pregnant and 8 non-pregnant women) were included. All samples were analyzed with a total of five primer pairs, targeting two anaerobes (*Gardnerella* species, and *Mobiluncus mulieris*), two aerobes (*Escherichia coli* and *Enterococcus faecalis*) and for the genus *Lactobacillus*. Single-template PCR assays were performed for each primer set. The sequence, amplicon size, target gene, and temperature of annealing for each primer pair are described in Table 2.

For PCR, a final volume of 20 μl was used according to the reference protocols [45-49]; which included 0.5 U of Go Taq® DNA Polymerase (Promega, Madison, WI), 1X of Green GoTaq® Flexi Buffer (Promega), 0.25 mM of MgCL₂ (Promega), 200 μM of dNTP mix (Promega), 0.5 μM of each

primer and target template DNA concentration of approximately 4 ng/μL, and the remaining volume with molecular grade H₂O. The PCR thermal cycling consisted of initial denaturation at 94 °C for 2 min; followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at each primer pair temperature (Table 2) for 30 s and extension at 72 °C for 1 min, and final extension of 5 min at 72°C. The respective use of negative (without DNA sample and samples with other related bacteria) and positive (collection of identified strains of each species through DNA sequencing) controls were used in each PCR assay. These positive controls were provided by the Microbiology Institute at USFQ. All samples were randomly performed in duplicate or triplicate with different negative and positive controls.

Table 2: PCR primers used in this study.

Set	Name	Sequence (5'-3')	Target	T (°C) of annealing	Size of fragment	Target gene	Specificity %	Validation	References
1	Primer E1	ATCAAGTACAGTTAGTCTT	<i>Enterococcus faecalis</i>	54°C	941 bp	<i>cdiI</i>	100.0%	Increase of the annealing temperature at 54°C	DTU National Food Institute, 2014
2	Primer E2	ACGATTCAAAGCTAACTG							
	ack F	ATTCTGCTTGGCGCTCCGGG	<i>Escherichia coli</i>	57°C	583 bp	<i>ackI</i>	49.0% 98.0%	Increase of the annealing temperature at 57°C	Sepehri et al., 2009
	ack R	COGTCAACTTTGCGTATTT							
3	Gard154-Fw	CTCTTGGAAACGGGTGTAA	<i>Gardnerella</i> spp.	60°C	301 bp	16S rRNA	100.0%	N/d	Henriques et al., 2012
	Gard154-Rv	TTGCTCCCAATCAAAGCGGT							
4	Lactof	TGGAAACAGRTGCTAATACCG	<i>Lactobacillus</i> spp.	62°C	233 bp	16S rRNA	47.1% 66.7%	N/d	Henriques et al., 2012
	LactoR	GTCATGTGGGAAGATTCCC							
5	Mobil-577F	GCTCTAGAGTGGTTGTGCGC	<i>Mobiluncus mulleris</i>	62°C	449 bp	16S rRNA	100.0%	N/d	Fredricks et al., 2007
	M.mulle-1026R	CCACACCATCTCTGGCATG							

N/d – Non-determined.

After PCR amplification, a volume of 4 μL from each PCR product was visualized in 1.5% (w/w) agarose (Promega) gel electrophoresis using 0.1% ethidium bromide staining. The DNA analysis was performed under permit No. MAE-DNB-CM-2016-0046.

Quantification of *Lactobacillus* sp. by Quantitative Real-Time PCR (qPCR)

To create positive controls and standard quantification solutions with a well-known *Lactobacillus* sp. concentration (CFU/mL), a sample of known concentration (also known as a calibrator) (e.g., number of CFU per mL) was obtained through a validated

calibration curve (CFU/OD) [50]. This calibrator was serially diluted tenfold and used to construct a standard curve for qPCR assays. Accordingly, *Lactobacillus gasseri* strain JCM1131 was cultured during 24 hours in Mann Rogosa Sharp Agar at 37 °C under microaerophilic conditions [50-52]. The calibrator concentration was previously proved by media growth culture counting as previously described [53]. The DNA extraction was performed from the highest CFU/ml concentration, and serial dilutions from 1E9 to 1E0 CFU/mL were used as qPCR standards. The DNA extraction of this solution with the highest concentration was performed under the same procedure already described in section 2.6. In each qPCR assay, two random controls were also used as blind samples in triplicate.

Each reaction was performed with GoTaq® Master Mix qPCR (Promega, Madison, WI, USA) in a final volume of 20 µl, 0.5 µM of each primer (LactoF-TGGAAACAGRTGCTAATACCG and LactoR-GTCCATTGTGGAAGATTCCC) and 2 µl of DNA template. Each qPCR assay was performed in a quantitative real-time PCR Thermocycler (Bio-Rad CA, USA) under the following conditions: initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, extension at 72 °C for 1 min, and a final extension for 5 minutes at 72°C. Each qPCR assay was followed by this melt curve analysis, allowing amplicon validation and identification of false positives through its profile and the specific temperature of melting (T_m). Each sample was analyzed by triplicate, and qPCR assays were realized in different days. Negative target controls and no template controls were included in all plates.

Primers used for these qPCR assays were previously described to amplify *Lactobacillus* spp. through classical PCR, but not for quantitative real-time PCR. Therefore assay metrics were determined by testing their performance across the limit of quantification (LoQ) and limit of detection (LoD), as well as linearity as previously described [54]. The optimized assay exhibited the LoQ and LoD to be 1E2 CFU per ml, while the range of linearity of the assay was from 1E9 to 1E3 CFU/mL. The load of lactobacilli in each sample was determined by

running six or five standard dilutions (1E9-1E3 CFU/mL), both in duplicate or triplicate on each qPCR assay.

For the quantification of *Lactobacillus* spp., 83 samples were selected for qPCR analysis from the initial subset of 97 vaginal samples previously characterized by PCR assays to molecular characterization of the main bacteria (see section 2.7), i.e. sixty from healthy microbiota samples and 23 from AV samples.

Statistical Analysis

Statistically significant differences in *Lactobacillus* spp. quantity among women with healthy and dysbiotic microbiota were evaluated using Kruskal Wallis one-way ANOVA and Mann Whitney tests. In addition, the same statistical analysis was carried out among pregnant and non-pregnant women. Finally, multivariable analysis was performed for sociodemographic and behavioral factors by using Minitab 2017 (Version 17, Minitab, State College, PA).

Results

Population Study

The sociodemographic characteristics for 217 women were included in the statistical analysis and presented in Table 3. Half of the women in the study were pregnant (51.2%) and approximately half (47.4%) were non-pregnant. They were between 21 and 30 years of age. Only 11 women (5.1%) identified themselves as White, Afro-Ecuadorian, or Indigenous women. So, the majority of the women in our study set (94.9%) were categorized as 'Half-blood', being of Hispanic ethnicity mixed with another background ethnicity (Caucasian, African, or Indigenous women). When performing an overall statistical analysis of age, the results do not show a significant relationship between age and the probability of having a specific diagnosis. Hence, there is no statistical evidence to determine that a woman's age is directly related to a specific vaginal disruption or having a healthy microbiota. From all sociodemographic factors analyzed, only the occupation category had a statistical significance over the diagnostic classification of vaginal

infection with a *P*-value of 0.003 through the Chi-square test (see Table 3). Similarly, the use of contraceptive methods, having different sexual partners, vaginal douches, or cigarette smoking did not show any relation to the development of any vaginal infection type during the study (see Table 4).

Table 3: Sociodemographic among women in this study with healthy vaginal microbiota, intermediate vaginal microbiota, and vaginal infections (bacterial vaginosis, aerobic vaginitis, candidiasis, and co-infections).

	Healthy microbiota <i>N</i> (%)	Intermediate microbiota <i>N</i> (%)	Candidiasis <i>N</i> (%)	Bacterial vaginosis <i>N</i> (%)	Aerobic vaginitis <i>N</i> (%)	Co-infections <i>N</i> (%)	Total <i>N</i>	<i>P</i> (X ²)
Focus group								
Non-pregnant	52 (49.1)	7 (6.6)	4 (3.8)	12 (11.3)	28 (26.4)	3 (2.8)	106	0.566 (3.9)
Pregnant	60 (54.1)	9 (8.1)	5 (4.5)	6 (5.4)	30 (27.0)	1 (0.9)	111	
Age								
≤ 20	38 (47.5)	4 (5.0)	5 (6.3)	10 (12.5)	21 (26.3)	2 (2.5)	80	0.799 (92.7)
21–25	26 (41.9)	6 (9.7)	1 (1.6)	4 (6.5)	24 (38.7)	1 (1.6)	62	
26–30	24 (58.5)	3 (7.3)	3 (7.3)	2 (4.9)	9 (21.9)	0 (0.0)	41	
31–40	24 (70.6)	3 (8.8)	0 (0.0)	2 (5.9)	4 (11.8)	1 (2.9)	34	
Global Incidence	112 (52.0)	16 (7.0)	9 (4.0)	18 (8.0)	58 (27.0)	4 (2.0)	217	0.308 (22.6)
Ethnicity								
Alto Ecuatorian	1 (25.0)	1 (25.0)	0 (0.0)	0 (0.0)	2 (50.0)	0 (0.0)	4	0.737 (11.2)
Half-blood	107 (51.9)	15 (7.3)	9 (4.4)	18 (8.7)	53 (25.7)	4 (1.9)	206	
Indigenous	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	2	
White	4 (80.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	5	
Occupation								
Housewife	31 (46.3)	1 (1.5)	1 (1.5)	11 (16.4)	22 (32.8)	1 (1.5)	67	0.003 (26.7)
Student	39 (46.4)	6 (7.1)	5 (6.0)	5 (6.0)	26 (31.0)	3 (3.6)	84	
Employee	42 (63.6)	9 (13.6)	3 (4.5)	2 (3.0)	10 (15.2)	0 (0.0)	66	
Civil Status								
Married	24 (63.2)	3 (7.9)	0 (0.0)	2 (5.3)	8 (21.1)	1 (2.6)	38	0.245 (18.4)
Divorced	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2	
Single	58 (49.6)	6 (5.1)	6 (5.1)	7 (6.0)	37 (31.6)	3 (2.6)	117	
Free Union	29 (48.3)	6 (10.0)	3 (5.0)	9 (15.0)	13 (21.7)	0 (0.0)	60	
Education Level								
None	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3	0.916 (24.2)
Basic (High-school students)	13 (41.9)	2 (6.5)	0 (0.0)	4 (12.9)	10 (32.3)	2 (6.5)	31	
Bachelor (Undergraduate students)	52 (47.7)	10 (9.2)	7 (6.4)	9 (8.3)	30 (27.5)	1 (0.9)	109	
Superior (Bachelor graduates)	30 (55.6)	3 (5.6)	1 (1.9)	5 (9.3)	14 (25.9)	1 (1.9)	54	
Higher Degrees Research (HDR) candidates (Master and Doctor's degree students)	15 (75.0)	0 (0.0)	1 (5.0)	0 (0.0)	4 (20.0)	0 (0.0)	20	

N number of women who responded in the survey within each category; % assigned percentage for each classification within each category; *P* (X²) *p*-value through the Chi-square test show any relation among each sociodemographic factor and the possibility of having a vaginal disruption or healthy microbiota.

Table 4: Behavioral variables among women in this study with healthy vaginal microbiota, intermediate vaginal microbiota, and vaginal infections (bacterial vaginosis, aerobic vaginitis, candidiasis, and co-infections).

	Healthy microbiota <i>N</i> (%)	Intermediate microbiota <i>N</i> (%)	Candidiasis <i>N</i> (%)	Bacterial vaginosis <i>N</i> (%)	Aerobic vaginitis <i>N</i> (%)	Co-infections <i>N</i> (%)	Total <i>N</i>	<i>P</i> (χ^2)
Has sexual partner								
No	16 (45.7)	3 (8.6)	3 (8.6)	2 (5.7)	10 (28.6)	1 (2.9)	35	0.707 (3.0)
Yes	96 (52.7)	13 (7.1)	6 (3.3)	16 (8.8)	48 (26.4)	3 (1.6)	182	
Different sexual partners								
No	54 (58.1)	4 (4.3)	4 (4.3)	7 (7.5)	24 (25.8)	0 (0.0)	93	0.205 (13.3)
Yes	52 (49.1)	10 (9.4)	5 (4.7)	7 (6.6)	29 (27.4)	3 (2.8)	106	
Do not answer	6 (33.3)	2 (11.1)	0 (0.0)	4 (22.2)	5 (27.8)	1 (5.6)	18	
Uses birth control method								
No	45 (46.9)	1 (25.0)	6 (6.3)	7 (7.3)	29 (30.2)	1 (1.0)	96	0.877 (5.2)
Yes	65 (56.1)	15 (7.3)	3 (2.5)	11 (9.3)	28 (23.7)	3 (2.5)	118	
Do not answer	2 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	3	
Smokes								
No	105 (51.5)	14 (6.9)	8 (3.9)	18 (8.8)	55 (27.0)	4 (2.0)	204	0.683 (3.1)
Yes	7 (53.8)	2 (15.4)	1 (7.7)	0 (0.0)	3 (23.1)	0 (0.0)	13	
Vaginal douching								
No	31 (57.4)	3 (5.6)	1 (1.9)	6 (11.1)	12 (22.2)	1 (1.9)	54	0.881 (5.2)
Yes	79 (49.7)	12 (7.5)	8 (5.0)	12 (7.5)	45 (28.3)	3 (1.9)	159	
Do not answer	2 (50.0)	1 (25.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	4	

N number of women who responded in the survey within each category; % assigned percentage for each classification within each category. *P* (χ^2) *P*-value through the Chi-square test.

Diagnosis of Vaginal Infections

The vaginal samples were evaluated in the Microbiology Institute of USFQ, according to microbiological criteria of Nugent et al. [41] to identify healthy microbiota, BV, and intermediate microbiota [41]; Schröders classification under criteria of Donders et al. [8] to characterize AV [8], and the criteria of Marot-Leblond et al. [43] to determine VVC [43]. As shown in Table 3, 112 (52.0%) vaginal samples were classified as healthy microbiota, sixteen (7.0%) were identified as intermediate microbiota, and 89 (41.0%) were diagnosed as dysbiotic (41.0%), which includes single cases of BV, AV, and VVC but also co-infections. The presence of a unique type of vaginal infection was identified in 85 vaginal samples (39.2%), whereby AV was the most prevalent infection with 26.7% of the vaginal samples, followed by BV (8.3%) and 4.1% with VVC. Furthermore, four vaginal samples were diagnosed with co-infections (1.8%), more precisely two of them with AV and BV, one with AV and VVC, and one with BV and VVC. None of the co-infection samples was further evaluated during qPCR analysis.

Prevalence of Vaginal Infections among Pregnant and Non-Pregnant Women

Each focus group (pregnant and non-pregnant women) was analyzed to identify any relation between vaginal infection and pregnancy (see Table 5). Although pregnant and non-pregnant women have similar prevalence values in the healthy microbiota, most cases of BV and co-infection were found in non-pregnant women with 67% (12/18) and 75% (3/4) of the cases, respectively, as shown in Table 5. However, these differences were not significant.

Table 5: Contingency table of vaginal samples between Focus Group and the diagnosis of vaginal infections, healthy and intermediate vaginal microbiota.

Group		Diagnostic						Total
		Aerobic vaginitis	Bacterial vaginosis	Candidiasis	Co-infection	Healthy	Intermediate	
Non-Pregnant	Number	28	12	4	3	52	7	106
	(% within the column)	(48.3)	(66.7)	(44.4)	(75.0)	(46.4)	(43.8)	(48.8)
Pregnant	Number	30	6	5	1	60	9	111
	(% within the column)	(51.7)	(33.3)	(55.6)	(25.0)	(53.6)	(56.3)	(51.2)

Number of women who responded in the survey within each category; % assigned percentage for each classification within each category. No statistically significant differences were found between pregnant and non-pregnant groups among vaginal infections, healthy and intermediate microbiota (P -value = 0.566[3.888]; see Table 3).

Presence of Opportunistic Species and *Lactobacillus* spp. in Vaginal Microbiota

The presence of *G. vaginalis* and *M. mulieris* (as BV biomarkers), *E. coli* and *E. faecalis* (as AV biomarkers), and *Lactobacillus* spp. (as healthy biomarker) were analyzed by PCR assays from the selected 97 samples (see Section 2.7). As previously mentioned, almost a half of population set was chosen by classical criteria through microscopy analysis (data not shown), more exactly, healthy microbiota samples with 0-1 and BV samples with 9-10 according to Nugent criteria [41], and the most aggravated AV samples [44].

The results still evidenced the presence of *Lactobacillus* spp. in both types of vaginal dysbiosis, although their presence decreased to 21% in BV ($P = 0.006$) and 13% in AV ($P = 0.019$) when compared to healthy microbiota samples (see Figure S1 in the *Supplementary section*). Regarding the presence of *Gardnerella* species, it was present less frequently in healthy microbiota (37%) while in BV and AV prevalence was 71% ($P =$

0.001) and 78% ($P = 0.033$), respectively. On the other hand, *M. mulieris* and *E. coli* were found in BV at 79% and 36%, respectively; while being detected in low frequency in healthy microbiota and AV cases, as shown in Figure S1. The presence of *M. mulieris* was low in frequency on AV and normal microbiota, when compared to BV cases. However, presence of *M. mulieris* was statistically different among healthy microbiota against BV ($P < 0.001$) and AV cases ($P = 0.002$), being less recurrent in healthy samples. Finally, *E. coli* did not show statistical differences among healthy microbiota and BV cases ($P = 0.062$). Also, it is important to mention that *Enterococcus faecalis* was found to be absent in the population set of the present study.

Among pregnant and non-pregnant women with healthy microbiota, we found that prevalence of *Lactobacillus* spp. was similar, as shown in Figure 1. On the other hand, pregnant women evidenced higher presence of *Gardnerella* species (39%), and *M. mulieris* (16%) when compared to non-pregnant women (32% *Gardnerella* spp., and 9% of *M. mulieris*).

In the presence of vaginal dysbiosis and infection, no statistically significant differences were found around opportunistic species between pregnant and non-pregnant women, as shown in Figure 1. However, *Lactobacillus* spp. showed statistically significant differences between pregnant and non-pregnant women in both BV and AV cases, as shown in Figure 1. In the case of BV, a higher prevalence of *Lactobacillus* spp. is shown in pregnant women (100%) compared to non-pregnant women (63%; $P = 0.028$). No significant statistically differences were found on the frequency of *Gardnerella* spp. and *M. mulieris* in these samples. In opposition, a drop of *Lactobacillus* spp. prevalence is shown in pregnant women with AV (79%; $P = 0.051$) when compared to non-pregnant women (100%).

Figure 1: Prevalence of each bacterium in pregnant and non-pregnant women diagnosed as: (A) Healthy Microbiota, (B) Aerobic Vaginitis, and (C) Bacterial Vaginosis according to the microbiological diagnosis. Statistically significant differences were evaluated by Chi-square tests.

Lactobacilli Quantification by Quantitative Real-Time PCR (qPCR)

Due to the small number of samples with BV, we restricted comparison of the lactobacilli quantification to healthy (60) versus AV (23) cases.

Due to the low number of data, a non-parametrical statistical analysis was performed by means of a Mann-Whitney. Significant differences were shown between healthy and AV groups ($P < 0.001$; see Figure 2), whereby *Lactobacillus* spp. varied between $1E6$ and $1E7$ CFU/ml in healthy microbiota decreased to between $1E3$ and $1E5$ CFU/ml in AV cases. This was confirmed by Kruskal-Wallis one-way ANOVA testing ($P < 0.001$; see Figure 2).

Mann-Whitney testing indicated no statistically significant differences between pregnant and non-pregnant women with healthy vaginal microbiota and with AV ($P = 0.330$ and $P = 0.637$), as shown in Figure 2. However, the analysis showed

statistically significant differences ($P < 0.001$) when comparing pregnant women with healthy microbiota against AV. Likewise, we found slight differences ($P = 0.006$) when comparing non-pregnant women with healthy microbiota against AV. Finally, it is worth noting that the same significance levels were also observed between healthy pregnant women against AV non-pregnant women ($P < 0.001$) and between healthy non-pregnant women against AV pregnant women ($P = 0.0041$). These preliminary results showed similar ranges of lactobacilli load in pregnant and non-pregnant women from each group set (AV and healthy vaginal microbiota).

Figure 2: Box plot of the quantification by qPCR of Lactobacillus spp. among vaginal samples: (A) Non-parametric. Statistical analysis among the overall groups (Healthy Microbiota and Aerobic Vaginitis), (B) Non-parametric. Statistical analysis among pregnant and non-pregnant women of each overall group.

Discussion

Sociodemographic and Behavioral Variables among Women

This study evaluated a possible relationship between vaginal infection, vaginal dysbiosis and sociodemographic or behavioral variables among pregnant and non-pregnant women. A disruption of the vaginal microbiota usually occurs when any cause promotes a diminution in lactobacilli levels, leading to other microorganisms' augmentation (primary or opportunistic pathogens). These causes of imbalance can be due to several intrinsic and extrinsic factors [12,17]. As intrinsic factors, the vaginal microbiota of women is driven mainly by hormonal changes during their reproductive life [23]. These intrinsic

factors were distinctively different in the two focus groups (pregnant and non-pregnant women). However, no statistically significant differences regarding composition of vaginal microbiota were detectable (see Table 3). Likewise, in this study, there was no statistically significant relationship among any extrinsic factor (behavioral variables) obtained in the questionnaire by multivariate analysis (see Table 4). This differs from other studies that established statistically significant association with some of the extrinsic factors analyzed by this study, such as the number of sexual partners [55], and ethnicity [14]. Others could not establish associations with the use of contraception, lubricant or spermicide, as well as personal hygiene habits [56]. Similarly, others did not find any effect of oral contraceptives on the vaginal microbiota of 36 women [57]. As such, several studies reported contradicting results regarding sociodemographic and behavioral variables, making conclusive comparisons difficult to achieve.

Prevalence and types of Vaginal Infection and Vaginal Dysbiosis

In our study set, 52% of women were characterized by a healthy vaginal microbiota, 7% were diagnosed with intermediate vaginal microbiota and 41% with some vaginal infection or vaginal dysbiosis (BV). Similar results were reported in the United Kingdom [56], identifying 48% of female participants with healthy vaginal microbiota and 19% with an abnormal microbiota. Similarly, Gondo et al. [5] reported that 47.5% of the women showed infection in a study enrolling 245 Brazilian women [5]. Bacterial vaginosis (BV) is usually reported as the most prevalent vaginal infection around the world [2,40,51], followed by vulvovaginal candidiasis (VVC) [15]. Another condition, aerobic vaginitis (AV), has been recently characterized by Donders and colleagues in 1999, and has been shown to play an important role for vaginal health [22,44,58]. Furthermore, Donders et al. [42] showed that this type of vaginal infection could easily be confused with an intermediate microbiota or even bacterial vaginosis [42], which may be a major reason why reliable data on the prevalence of AV in the general population are not very abundant [58].

AV can also be associated with the increased risk of preterm pre-labor rupture of membranes, chorioamnionitis, and preterm delivery [58]. Contrary to previous studies [8,13,55,59], in the present study AV was the most prevalent vaginal infection with a similar percentage of AV among pregnant (51.7%) and non-pregnant (48.3%) women. Again, the latter is in contradiction with other studies that reported low AV prevalence among pregnant women. Although Donders et al. [60] postulated that AV was not common in pregnancy, a more recently publication by Donders and colleagues [58] reported that AV could easily be confused with an intermediate microbiota and bacterial vaginosis and so reliable data on the prevalence of AV could be available in few amounts. In 2013, Jahic and colleagues diagnosed AV in 51% of the enrolled female participants, where *E. coli* and *E. faecalis* were the most prevalent bacteria [10]. In agreement, Fan et al. [9] reported the same main bacteria and *S. epidermidis* in their AV cases [9].

In non-pregnant women, several studies reported a prevalence of AV between 5 and 10.5% in symptomatic women [58,61,62], whereby the most frequently identified bacteria were *E. coli* (4 – 23%) [11], *Staphylococcus* (around 27%), *Streptococcus* (0.7 – 58.7%) and *Enterococcus* spp. (0.3 – 2.4%) [11,63,64]. These previous studies could partially explain the absence of *Enterococcus faecalis* in our study due to the low rate of detection. Finally, *E. coli* prevalence in pregnant (28.57%) and non-pregnant (11.11%), as established in our study, were within the range described by Tansarlina et al. [11] and in agreement with postulations made by Donders et al. [42].

Presence of Opportunistic Pathogens in Healthy Microbiota

The vaginal microbiota complexity in healthy and dysbiosis samples had already been described by several authors in women with AV and BV [65-67]. Similar to Zozaya-Hinchliffe et al. [66], we believed that the PCR characterization of the major bacterial species by PCR and the development of qPCR assays would be facilitated by first working with specimens whose

microbiota would be most likely to differ significantly [66]. So, we only selected vaginal samples from sixty women with normal vaginal microbiota who had Nugent scores of 0 and 1, twenty three women with aggravated AV diagnosis (see section 2.5), and fourteen women with BV who had Nugent scores of 9 and 10. These 97 women were selected to identify the major bacterial species by PCR, and then healthy and AV women (83 samples) were evaluated by qPCR (see section 4.4). However, this selection of samples could be considered a limitation of the present study.

The presence of *Gardnerella* species in a low number in the vaginal microbiota is not an indicator of BV [26,68,69], being considered as part of the healthy vaginal microbiota. Meanwhile, several studies have shown that there are almost four different groups of *Gardnerella* species (A, B, C and D), previously all considered as *Gardnerella vaginalis* [70], which not all of them are related to the development of BV [71-73]. In 2019, Vaneechoutte and colleagues amended several species of *G. vaginalis*, through Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), and described then as *Gardnerella leopoldii*, *Gardnerella Piotii* and *Gardnerella swidsinskii*. Therefore, not all *Gardnerella* species detected in several studies constituted *Gardnerella vaginalis* and could explain virulence differences between *Gardnerella* species [74-76]. Since these species could not be delineated using full-length 16S rRNA gene sequences, Hill and colleagues applied partial chaperonin 60 (cpn60) sequences to resolve these four group species [73]. Both studies showed that *G. swidsinskii* and *G. leopoldii* constituted group A, *G. Piotii* corresponded to group B, *G. vaginalis* belonged to group C, and finally, group D was the most diverse subgroup with several *Gardnerella* sp. (such as strains 101, 1500E, 6119V5 and 00703Dmash). However, this last group will require an analysis of additional isolates to establish a species differentiation [70,73]. Nonetheless, an abundance of *G. vaginalis* and *G. swidsinskii* was associated with vaginal symptoms of abnormal odor and discharge in their study set [73]. This heterogeneity and diversity within the genus *Gardnerella* may distinguish clades and how these features may impact BV development [77]. So, future studies should isolate all

Gardnerella species of the vaginal samples and further analysis could allow the qPCR methodology to quantify different species of *Gardnerella*.

Amount of *Lactobacillus* spp. among Healthy Women and Women with Vaginal Infections

As previously mentioned in results, statistically significant differences were found among the amount of *Lactobacillus* spp. between healthy and AV women ($P < 0.001$). Moreover, lactobacilli load among healthy women was established between $1E6$ and $1E7$ CFU/ml; meanwhile, the amount of *Lactobacillus* spp. in altered microbiota (AV) was defined between $1E3$ and $1E5$ CFU/ml. These results are comparable to previous studies with BV [67,78,79]. However, it is important to mention that the specificity of the lactobacilli primers (LactoF: 47.1%%; LactoR: 66.7%) was a limitation of the present study.

Amount of *Lactobacillus* spp. among Pregnant and Non-Pregnant Women

Furthermore, the results of the present study showed that both healthy and AV pregnant women have a higher concentration of *Lactobacillus* spp. when compared to non-pregnant women of the same categories. These results agree with Walther-António et al. [80]. These authors reported that lactobacilli augmentation during pregnancy and preterm birth help to prevent vaginal infection and counteract higher immune tolerance [80,81]. However, there were no statistically significant differences between the amount of *Lactobacillus* spp. of pregnant and non-pregnant women per category. Although the present study is a preliminary analysis of lactobacilli load between pregnant and non-pregnant women, these results point to the possibility to use the same lactobacilli load range to evaluate AV and healthy vaginal microbiota (whether pregnant or non-pregnant) and thus to avoid future vaginal infection establishment in women by monitoring lactobacilli load through qPCR.

These results could corroborate with several studies, which postulated an increment of lactobacilli load in pregnant women

[80,82]. However, there are some major limitations of this study: (1) with 97 participants in PCR assays and 83 participants in qPCR assays, small numbers of particular cases were retained in each subgroup, (2) in PCR assays not all possible species of aerobic bacteria could be targeted in AV samples and, (3) in qPCR assays, normalized concentrations of lactobacilli were realized through low specificity primers for *Lactobacillus* spp. Therefore, future studies must optimize lactobacilli quantification, also quantify certain *Lactobacillus* species and other aerobic bacteria among pregnant and non-pregnant women. Previous studies showed that the presence of different *Lactobacillus* species is a major determinant to the stability of the vaginal microbial community in pregnancy [27,83]. Furthermore, Verstraelen and colleagues demonstrated *L. crispatus* ability to promote and stabilize the normal microbiota while *L. gasseri* and *L. iners* predisposed to some extent to the occurrence of abnormal microbiota [83]. Future studies should be realized with a bigger and more diverse population set as well as quantification of specific *Lactobacillus* species (such as *L. crispatus*, *L. gasseri*, and *L. iners*) as postulated by others authors [1,80,83].

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Supplementary Material

Figure S1: Prevalence of each bacteria in 97 vaginal samples classified such as Healthy microbiota, Bacterial Vaginosis and Aerobic Vaginitis according to Microbiological Diagnosis. Statistically significant differences were evaluated by Chi-square tests.