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Characterization of Infectious Vaginitis amongst Ecuadorian Women of Reproductive Age

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DEDICATORIA

Esta tesis la dedico a mi familia.

A mis padres, Eduardo y Anita, por mostrarme el camino a la superación, ustedes son mi motivación. A mis hermanas, Dany y Paty, por todo su amor y apoyo incondicional, no pude pedir mejores hermanas. A ti negrito por tu sacrificio y esfuerzo, por ser mi sostén cada día y el amor de mi vida. A mi Theo por ser la alegría que me hacía falta.

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RESUMEN

La vaginitis es un problema ginecológico común en mujeres en edad reproductiva asociado con varias condiciones de salud graves. En este estudio evaluamos la presencia de vaginosis bacteriana, candidiasis vulvovaginal y vaginitis anaeróbica en mujeres ecuatorianas en edad reproductiva.

La evaluación de la muestra vaginal se realizó de acuerdo con la presencia de síntomas, los hallazgos clínicos durante la encuesta, el análisis microscópico de los frotis vaginales y los ensayos de reacción en cadena de la polimerasa (PCR). La vaginosis bacteriana (BV) y la vaginitis aeróbica (AV) fueron diagnosticadas según criterios microbiológicos de Nugent y Donders, respectivamente, mientras que la candidiasis vulvovaginal se identificó por la preparación de tinción de Gram positiva con levaduras en formas de pseudohifas y / o de hifas; y cultivo positivo.

Las 436 muestras vaginales se analizaron de mujeres entre 18 y 56 años de edad. La mayoría de la población (66,0%) mostró una microbiota vaginal normal y sana, el 10,8% de las voluntarias tenían microbiota intermedia y el resto (23,2%) una vaginitis única o múltiple. De las 101 voluntarias con vaginitis, AV fue la principal vaginitis diagnosticada (53/101), seguida de BV (24/101) y finalmente candidiasis (7/101). Las 17 mujeres restantes mostraron coinfecciones, siendo BV y AV la coinfección más común. Otros análisis de PCR demostraron una ligera mayor prevalencia de *Gardnerella vaginalis* sobre BV y diagnóstico de flora intermedia sobre las voluntarias en comparación con la colonización microbiana restante (*Atopobium vaginae, Mobiluncus mulieris, Escherichia coli, Enterococcus faecalis* and *Candida albicans*). Mientras que *A. vaginae* fue la principal especie oportunista en mujeres con microbiota vaginal normal. En el diagnóstico de AV, se identificó *E. coli* y *E. faecalis* en menos del 25% de las mujeres con AV y *C. albicans* se detectó en el 28,6% de las mujeres diagnosticadas con candidiasis.

Este estudio identificó la vaginitis aeróbica como el principal tipo de infección vaginal bacteriana, seguido de la vaginosis bacteriana. Por otra parte, *G. vaginalis* y *A. vaginae* fueron las especies oportunistas más abundantes detectadas en nuestro conjunto poblacional. Según el mejor conocimiento de los autores, este fue el primer estudio que analizó simultáneamente tres prevalencias diferentes de vaginitis entre mujeres ecuatorianas.

Palabras clave: Microbiota vaginal; vaginosis bacteriana; vaginitis aeróbica; candidiasis vulvovaginal; análisis molecular; estudio epidemiológico; Ecuador; 16S rRNA

ABSTRACT

Vaginitis is a common gynecological problem in reproductive-age women associated with several serious health conditions. In this study we assessed the presence of bacterial vaginosis, vulvovaginal candidiasis and anaerobic vaginitis in Ecuadorian women of reproductive-age.

Vaginal sample evaluation was made according to the presence of symptoms, clinical findings during survey, microscopic analysis of vaginal swabs and Polymerase Chain Reaction (PCR) assays. Bacterial vaginosis (BV) and aerobic vaginitis (AV) were diagnosed on microbiological criteria by Nugent and Donders, respectively, while vulvovaginal candidiasis (VC) was identified by positive Gram-stain preparation with budding yeasts, pseudohyphae, and/or hyphal forms; and positive culture.

The 436 vaginal samples were analyzed from women between 18 and 56 years old. Most of the population (66.0%), showed a normal and healthy vaginal microbiota 10.8% of volunteers had intermediate microbiota and the remaining (23.2%) had a single or multiple vaginitis. From 101 volunteers with vaginitis, AV was the main diagnosed vaginitis (53/101), followed by BV (24/101) and finally VC (7/101). The remaining 17 women showed coinfections such as BV and AV which was the most common association. Further PCR analysis demonstrated a slightly higher prevalence of *Gardnerella vaginalis* over BV and intermediate flora diagnosis over the volunteers when compared to the remaining microbial colonization (*Atopobium vaginae, Mobiluncus mulieris, Escherichia coli, Enterococcus faecalis* and *Candida albicans*). While *A. vaginae* was the main opportunistic species in women with normal vaginal microbiota. In AV diagnosis, *E. coli* and *E. faecalis* were identified in less than 25% of AV women and *C. albicans* was detected in 28.6% of the women diagnosed with candidiasis.

This study identified aerobic vaginitis as the main type of bacterial vaginal infection, followed by bacterial vaginosis. Moreover, *G. vaginalis* and *A. vaginae* were the most abundant opportunistic species detected in our population set. To the best of the authors' knowledge, this was the first study to simultaneously analyze three different vaginitis prevalence among Ecuadorian women.

Keywords: Vaginal microbiota; bacterial vaginosis; aerobic vaginitis; vulvovaginal candidiasis; molecular analysis; epidemiological study; Ecuador; 16S rRNA

CONTENT INDEX

TABLE INDEX

FIGURE INDEX

PART I

GENERAL INTRODUCTION

STATE OF ART

The vaginal microbiota is an important factor in a woman´s health and pregnancy (Hellberg, Zdolsek, Nilsson, & Mårdh, 1995; Keshavarz, Duffy, Zolghadr, & Oboodi, 2016), being a dynamic ecosystem of various microbes in different quantity and ratio (Jahic, Mulavdic, Nurkic, Jahic, & Nurkic, 2013). One of the primary vaginal epithelium defense is based in a commensal microbial colonization, consisting mainly in several species of *Lactobacillus* genus (Onderdonk, Delaney, & Fichorova, 2016; Petrova, Lievens, Malik, Imholz, & Lebeer, 2015). These lactobacilli species could also influenced endogenous flora, estrogen, glycogen, and metabolic of the host by their biological products (Egan & Lipsky, 1970).

Lactobacilli species act as biological surfactant preventing the initial adhesion of potential pathogens and eventually may produce several antimicrobial substances, such as hydrogen peroxide and lactic acid, which are toxic to pathogens keeping the healthy vaginal pH between 3.8 and 4.2 and therefore preventing vaginitis and other infections (Egan & Lipsky, 1970; Ling et al., 2010). When this ecosystem is disrupted, vaginitis settles in the vaginal epithelium. Normally, vaginal infection is characterized by a microbial downward swift, in the proportion of certain *Lactobacillus* species in the presence of a pathogenic or even opportunistic microorganisms (Jahic et al., 2013; Onderdonk et al., 2016).

Several factors (such as contraceptives, antibiotics, sexual intercourse, douching, stress and hormones) can change the vaginal environment and allow pathogens to grow. In the case of BV, it is believed that some events decreases the number of *L. acidophilus* leading to an increment of the vaginal pH and the proliferation of anaerobic pathogens. Also, certain pathogens produce some products, such as amines, that increase the vaginal pH. In BV, this amines are responsible for the malodorous discharge in women.

Nevertheless BV is not associated with vaginal mucosal inflammation and rarely cause vulvar itch (Egan & Lipsky, 1970; Hainer & Gibson, 2011).

Moreover, different risk factors are associated with candidiasis, including recent antibiotic use, uncontrolled diabetes mellitus and HIV infection (Brandolt et al., 2017; Ilkit & Guzel, 2011). In pregnancy, the asymptomatic fungal colonization maybe evolve to a symptomatic infection due to altered estrogen and progesterone levels and increased glycogen production, which facility the germination of yeast and enhance the adherence of *C. albicans* to vaginal epithelial (Egan & Lipsky, 1970; Owen & Clenney, 2004).

While AV is characterized by disruption in *Lactobacillus* dominance and accompanied by more extreme inflammatory changes, leading to a red atrophic-like vaginal mucosa with numerous parabasal cells that indicates a lack of estrogenic stimulation in the vagina (Gilbert G.G. Donders et al., 2005; Kaambo, Africa, Chambuso, & Passmore, 2018). These vaginitis could be caused by different types of microorganisms, which has been studied in the last decades (G. G.G. Donders, Bellen, & Rezeberga, 2011; Gilbert G.G. Donders et al., 2005; Kaambo et al., 2018; Tansarli, Kostaras, Athanasiou, & Falagas, 2013), such as *E. coli*, *Enterococcus* sp., *Staphylococcus* sp. and *Streptococcus* sp. among others.

From several studies, bacterial vaginosis is usually described as the most common cause of vaginitis (Fethers, Fairley, Hocking, Gurrin, & Bradshaw, 2008; Oostrum, Sutter, Meys, & Verstraelen, 2018; Xia et al., 2016). In BV, the infection is caused by proliferation of several anaerobic or facultative microorganisms, such as: *G. vaginalis*; *Atopobium* sp.; *Prevotella* sp.; *Bacterioides* sp.; *Peptostreptococcus* sp.; *Mobiluncus* sp.; *Sneathia* sp.; *Leptotrichia* sp.; and genital *Mycoplasma (Mycoplasmas hominis* and *Ureaplasma urealyticum*) among the most important pathogens described in literature (Krauss-Silva et al., 2014; Onderdonk et al., 2016). Culture-independent technique

showed that *G. vaginalis* and M. *mulieris* are commonly more abundant in patients with symptomatic BV (Cardenas & Cookson, 2015). In addition, *G. vaginalis* and *A. vaginae* are commonly associated to BV biofilm formation in the vaginal mucosa and involved with recurrent BV as well as its potential to increase several sexual transmission infections (Cardenas & Cookson, 2015). On the other hand, VC is strictly associated with the proliferation of *Candida* species in vaginal epithelium in detriment of the descending lactobacilli colonization (Ilkit & Guzel, 2011). However, the *Candida* genus can also be found in the vaginal microbiota of healthy women but in low level of colonization (Ilkit & Guzel, 2011). In fact, *Candida* spp. have been found in 37% of healthy asymptomatic using culture-independent techniques (Cardenas & Cookson, 2015). In last decades, at least 16 species of *Candida* have been found in the vaginal microbiota of healthy women and women with candidiasis, where *Candida glabrata*, *Candida tropicalis* and *Candida albicans* are the most commonly detected in the vaginal epithelium of women (Cardenas & Cookson, 2015; Owen & Clenney, 2004). Finally, AV is characterized by the presence of mainly aerobic enteric commensals or pathogens including *E. coli*, *E. faecalis* Group B *Streptococcus* (*Strepcotoccus agalactiae*) and *Staphylococcus aureus* (Gilbert G.G. Donders et al., 2005; Jaiberth, Arias, Arredondo, Henao, & Herrera Posada, 2015; Kaambo et al., 2018). In relation to clinical symptoms, AV is associated with more genital inflammation increasing the activity to other opportunistic pathogens (Kaambo et al., 2018).

Vaginitis is considered by several studies as the most prevalent gynecological problem of reproductive age women, affecting millions every year and being the most common cause for gynecological medical care (Kent, 1991; Machado, Castro, Martinez-de-Oliveira, Nogueira-Silva, & Cerca, 2017; Tempera, 2005). In fact, Bacterial vaginosis (BV), even when asymptomatic, is associated with numerous health problems such as

pelvic inflammatory disease, cervicitis, preterm labor, low birth weight, miscarriages, and chorioamnionitis (Datcu, 2014; Fredricks, Fiedler, & Marrazzo, 2005; Krauss-Silva et al., 2014; Nelson et al., 2009; Tamrakar et al., 2007). Although complications of VC are rare, vulvar vestibulitis syndrome and chorioamnionitis have been reported in women during pregnancy (Egan & Lipsky, 1970). Meanwhile AV, as well as BV, is related to the increase risk of acquiring human immunodeficiency virus (HIV), Herpes simplex type 2 and other sexually transmitted infections involving *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis,* among others (Datcu, 2014; Wiesenfeld, Hillier, Krohn, Landers, & Sweet, 2003).

Several studies appointed BV as main cause of vaginitis in symptomatic women (22 – 50%), however the precise prevalence of BV is usually difficult to establish because one third to three quarters of BV women are asymptomatic (Hainer & Gibson, 2011; Onderdonk et al., 2016). VC is the second cause most common of vaginitis (17 – 19%), being estimated that 75% percent of women have vulvovaginal candidiasis at some time in life and 5% of women have recurrent episodes (Ilkit & Guzel, 2011). Finally, the prevalence of AV is reported around 8 – 11% in pregnant women and 5 – 24% of women reporting vaginal complaints (Kaambo et al., 2018). However, this type of vaginitis is the less studied among women in reproductive age.

It has been postulated that several factors may influence the prevalence of BV, such as ethnicity and geographic location. In previous studies, many authors reported lower BV prevalence in Asia and Europe but higher BV prevalence in Africa and Latin America (Onderdonk et al., 2016). In Ecuador, it has been reported that the prevalence of BV is higher in adolescents (Cuevas et al., 2010; Jaiberth et al., 2015; Vaca et al., 2010), however little is still known about BV in Ecuadorian women and even less about AV and candidiasis.

The classical and gold standard methods for vaginitis diagnosis are physical examination, clinical symptoms, pH of vaginal fluid, microscopy and whiff test (Hainer & Gibson, 2011). In relation to clinical symptoms, women who showed signals of alteration in vaginal discharge should be evaluated for vaginitis, more exactly, itching , burning, irritation, odor (Egan & Lipsky, 1970). Several criteria can be used for the diagnosis of BV, however Amsel's criteria is considered the standard approach to BV diagnosis (Mohammadzadeh, Dolatian, Jorjani, & Alavi Majd, 2014; Rao, Pindi, Rani, Sasikala, & Kawle, 2016). In fact, BV diagnosis by Amsel's criteria is made through the following criteria: milky and homogeneous adherent discharge; vaginal pH greater than 4.5; positive whiff test (detection of fishy odor upon 10% potassium hydrogen addition), this typical odor resulted from the liberation of amines and organic acids produced for the alkalization of anaerobic bacteria; and presence of clue cells (vaginal epithelial cells covered by bacteria) in the vaginal fluid. At least three from four clinical signs must be present to establish a positive BV diagnosis (Egan & Lipsky, 1970; Hainer & Gibson, 2011; Joesoef, Hillier, Josodiwondo, & Linnan, 1991; Owen & Clenney, 2004; Spiegel, Amsel, & Holmes, 1983). Despite the fact that the Amsel's criteria requires the least training and is therefore the most frequently used diagnostic procedure, it is not the most appropriate method to diagnose BV, due to its low specificity (Dickey, Nailor, & Sobel, 2009). Therefore, Nugent and colleagues attempted to improve the BV diagnosis through Gram stain of vaginal swabs. This technique enabled the observation of the existent vaginal microflora and also the preservation of the clinical sample for further medical evaluation (Nugent, Krohn, & Hillier, 1991).These authors elaborated a Gram stain scoring system based in the evaluation of the following morphotypes: large gram-positive rods (*Lactobacillus* spp. morphotypes); small gram-variable rods (*G. vaginalis* morphotypes); small Gram-

negative rods (*Bacteroides* spp. morphotypes); and curved gram-variable rods (*Mobiluncus* spp. morphotypes). Each morphotype is quantified by score from 0 to 4 regarding to the number of certain morphotype observed in the microscopic fields of the Gram-stained vaginal smear (see *Materials and Methods* section). The vaginal microflora diagnosis is then based in the sum of each morphotype score, classifying normal microflora (total score between 0 – 3), intermediate microflora (total score between 4 – 6) and BV (total score between 7 – 10) (Livengood, 2009; Nugent et al., 1991). So, based on Nugent's criteria, other microbiological criteria were established for AV and vulvovaginal candidiasis diagnosis. The criteria for microbiology diagnosis of AV is based in the absence of *Lactobacillus* species, the presence of cocci or coarse bacilli in high number as well as parabasal epithelial cells, and/or positive for leucocytes in the vaginal discharge (Gilbert G.G. Donders et al., 2005). It is important to mention that AV is commonly diagnosed by microscopy evaluation, evidencing vaginal leucocytes abundant or round parabasal cells and high number of aerobic bacteria (cocci or coarse bacilli). In relation to symptoms, 70% of AV women frequently show yellow discharge and 12% of AV women reveal dyspareunia vaginal and clinical signs of vaginitis, such as red inflammation of the vaginal epithelium (Gilbert G.G. Donders et al., 2005; Egan & Lipsky, 1970). Finally, women with vulvovaginal candidiasis usually report common symptoms, such as vulvovaginal pruritus, burning sensation, vulvovaginal swelling, dysuria and tick vaginal discharge without odor. Similar to AV, candidiasis diagnosis is also diagnosed by microscopy evaluation of the vaginal discharge, showing high number of hyphae and or pseudohyphae in more than two microscopic fields and eventually low number of lactobacilli in the positive candidiasis women (Egan & Lipsky, 1970; Marot-Leblond et al., 2009; Owen & Clenney, 2004).

Although these techniques previously described are very easy and cheap to realize in daily procedure, it is necessary an experienced diagnostic technician in the laboratory. Other disadvantage of the microscopy evaluation of vaginal discharge is the disability to identify the bacterial or fungal species in the vaginal smear leading to a low sensitivity in the classical analysis of the samples. For instance, the detection of BV by microscopic examination have a sensibility of 60% and a specificity of up to 98% (Egan & Lipsky, 1970; Hainer & Gibson, 2011). Nowadays, the molecular analysis has been applied in several studies to better understand and characterize the microbiota present in health vaginal epithelial and in vaginitis (Hong et al., 2016; Kusters, Reuland, Bouter, & Koenig, 2015; Ling et al., 2010; Obstet, 2016), such as Polymerase chain reaction (PCR), quantitative PCR (qPCR) and Next-Generation Sequencing (NGS) assays.

In this study, we applied classical and molecular techniques for the diagnosis of the different types of vaginal infection, through microscopy techniques and PCR assays, previously used in other epidemiological studies (Fredricks et al., 2005; Hainer & Gibson, 2011; Madhivanan et al., 2014). This study analyzed the frequency of three different vaginitis (BV, VC and AV) in 510 Ecuadorian women amongst reproductive age. Also, the present study evaluated the prevalence of symptomatic and asymptomatic vaginitis in population set showing the relevance for a reinforcement of sexual education and health programs to prevent high health public costs in a near future.

PART II

SCIENTIFIC PAPER

Symptomatic and Asymptomatic Vaginitis in Ecuadorian Women: An epidemiologic analysis

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Introduction

Woman's health and pregnancy is affected directly by the vaginal microbiota (Hellberg et al., 1995; Keshavarz et al., 2016). This microbiota consists in a dynamic ecosystem of various microbes in different quantity and ratio which protect the vaginal epithelium from infections (Jahic et al., 2013). One of the primal vaginal epithelium defense is based in a commensal microbial adhesion, consisting mainly in several *Lactobacillus'* species (Onderdonk et al., 2016; Petrova et al., 2015). These lactobacilli act as biological surfactant preventing the initial adhesion of potential pathogens because they produce several antimicrobial substances such as, hydrogen peroxide and lactic acid preventing vaginitis and other infections (Ling et al., 2010; O'Hanlon, Moench, & Cone, 2013).

When this ecosystem gets disrupted, vaginitis settles in the vaginal epithelium. Usually, vaginal infection is characterized by a microbial downward swift, of certain *Lactobacillus* species in the presence of pathogenic or opportunistic microorganisms (Jahic et al., 2013; Onderdonk et al., 2016), that cause certain vaginal infections, indicated as follows: BV caused by several anaerobic or facultative microorganisms, such as *G. vaginalis, Atopobium* sp., *Prevotella* sp., *Bacterioides* sp., *Peptostreptococcus* sp., *Mobiluncus* sp., *Sneathia* sp., *Leptotrichia* sp. and genital *Mycoplasma (Mycoplasmas hominis* and *Ureaplasma urealyticum*) among the most important (Krauss-Silva et al., 2014; Onderdonk et al., 2016); also there is VC commonly caused by *C. albicans, Candida glabrata* and *Candida tropicalis* (Owen & Clenney, 2004); and finally AV frequently induced by *E. coli, E. faecalis,* among other aerobic bacteria (Jahic et al., 2013).

Vaginitis is considered the most prevalent gynecological problem of reproductive-age women, affecting millions every year, and the most common cause for gynecological

medical care (Machado et al., 2017). BV is associated with numerous health problems such as pelvic inflammatory disease, cervicitis, preterm labor, low birth weight, miscarriages, and chorioamnionitis (Datcu, 2014; Fredricks et al., 2005; Krauss-Silva et al., 2014; Nelson et al., 2009; Tamrakar et al., 2007). Meanwhile AV and BV are usually associated with an increased risk of acquiring human immunodeficiency virus (HIV), Herpes simplex type 2 and other sexually transmitted infections with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis,* among others (Datcu, 2014; Wiesenfeld et al., 2003).

Previous studies reported BV as main cause of vaginitis in symptomatic women (22 – 50 %), followed by VC (17 – 19 %) and finally AV (around 11 %) (Anderson, Klink, & Cohrssen, 2004; Hainer & Gibson, 2011; Onderdonk et al., 2016; Owen & Clenney, 2004). Several and different risk factors, such as ethnicity and geographic location, could influence the prevalence of BV, as shown in previous studies, where the authors reported lower BV prevalence in Asia and Europe and higher BV prevalence in Africa and Latin America (Jaiberth et al., 2015; Onderdonk et al., 2016; Salinas et al., 2018). Meanwhile, in Ecuador, it has been reported that the prevalence of BV is higher in adolescents (Castillo et al., 2010; Cuevas et al., 2010; Salinas et al., 2018), despite of the few published studies.

The classical and gold standard methods for vaginitis diagnosis are physical examination, symptoms, pH of vaginal fluid, microscopy and the whiff test (Egan & Lipsky, 1970; Joesoef et al., 1991; Spiegel et al., 1983), which are usually used in Hospital and Clinical facilities worldwide (Owen & Clenney, 2004). Although these techniques are very easy and cheap to do daily, they show several disadvantages of sensibility (Hainer & Gibson, 2011). Nowadays, to avoid the downsides of the usual diagnosis techniques, the molecular analysis has been applied in several studies to better

understand and characterize the microbiota present in health vaginal epithelial and in vaginitis (Aagaard et al., 2012; De Backer et al., 2007; Fredricks et al., 2005; Garg, Ganguli, Das, & Talwar, 2009; Ling et al., 2010).

In this study, we applied classical and molecular techniques for the diagnosis of different types of vaginal dysbiosis, through microscopy techniques and PCR assays, as widely used in previous works (Fredricks et al., 2005; Hainer & Gibson, 2011; Madhivanan et al., 2014). This study analyzed the prevalence of BV, VC and AV in Ecuadorian women amongst reproductive age. Also, the present study aimed to elucidate the prevalence of symptomatic and asymptomatic vaginitis in population set and to demonstrate the relevance for the strengthening of health programs for sexual education, preventing potential high health public costs in a near future.

Materials and Methods

Study area, design and subject selection

This study was conducted in the Institute of Microbiology at the Universidad San Francisco de Quito (USFQ) from June 2016 to November 2017. The investigation recruited 510 Ecuadorian women from Hispanic ethnicity and reproductive age who volunteered.

The enrolled women received a kit containing an informed consent approved by the Bioethics Committee of the USFQ and the Ministry of Health of Ecuador (Contrato Marco de Acceso a los Recursos Genéticos No. MAE-DNB-CM-2016-0046); a standardized medical survey, which included demographic, sexual and health behavior related questions, as well as, information about clinical history and possible symptoms or vaginal fluid signals; and finally a vaginal swab carrying culture media (Stuart's

transport media swabs; Copan Diagnostics Inc.), which the volunteers should provide vulvovaginal swab sample. Applicants were excluded from the study if they reported to have had sexual intercourse within the last 48 hours, antimicrobial treatment in the last 3 months or any evidence of bleeding. The study was supervised by a physician, two psychologists and a full-time researcher from the USFQ.

Ethics statement

A total amount of 510 samples were initially collected, however 74 women with their respectively sample were excluded, due the previous exclusion criteria from the subject selection, the absence of a legible and full disclose survey or even by an inadequate result in DNA quantification and Gram staining procedures. Hence, only 436 samples were able to be completely processed in the present study.

The study was approved by the Ethics Committee of the USFQ (Protocol code: 2016-023IN by MSP-VGVS-2016-0244-O review board) in Quito.

Samples collection

The participants took a self-taken low vulvovaginal swab. This sterile swab was brushed against the lateral vaginal walls to collect the fluid sample, which was immediately placed in the transport media, stored at 4°C and processed within the first 12 hours to the research bacteriology laboratory of the Microbiology Institute at Universidad San Francisco de Quito (MI-USFQ). Later, the swab was used to prepare a vaginal smear for microbiological analysis of aerobic and anaerobic microorganisms with standard microbiological methods and Gram staining. After that, it was used to inoculate in several growth media for isolation of cultivable species and to extract DNA for further studies.

DNA extraction

DNA extraction was developed according to Machado et al. (2017). The swab was placed in 2 ml of phosphate buffer saline (PBS) and shaken vigorously until the solution turned cloudy, through a vortex, during approximately 3 minutes. The remaining vaginal material was collected by centrifugation at 13000 rpm for 5 minutes. The obtained pellet was suspended in 1ml of saline solution (0.9% NaCl). The aliquot of 1ml of saline solution (0.9% NaCl) were incubated at 100°C in a water bath for 15 minutes. After that, samples were immediately frozen at -20°C for 15 minutes. The samples were then centrifuged at 13000 rpm for 15 minutes, and supernatants where aliquoted into 500µl volumes, one stored at -20°C and the remaining stored at -80°C. Once completed the procedure extraction, DNA quantification was performed with a Nanovue spectrophotometer (GE Healthcare Life Science). Concentrations of DNA in ng/µl were measured, as well as the phenolic contaminants (260/230) and the protein contaminants (260/280). Finally, two aliquots of DNA concentration between 10-20 ng/µL, were preserved at -20°C for Polymerase Chain Reaction (PCR) analysis.

Polymerase Chain Reaction

Furthermore, PCR assays were performed in the 436 samples that were able to be processed on a T100 Thermal Cycler (Bio-Rad, CA, USA) using primers that have been used previously in other studies (see Table 1). The reactions for all bacteria (except *Enterococcus* sp.) were performed as singleplex PCR in a total volume of 20µl containing 0.50 units of Go Flexi Taq polymerase, 1x Green PCR Buffer with 2.5mM $MgCl_2$ (Promega, WI, USA), 0.2mM of dNTPs (Promega, WI, USA), 0.5µM of each primer and 4µl of DNA template and the remaining volume with molecular grade H_2O.

For *Enterococcus* sp., reactions were performed as singleplex PCR in a total volume of 20µL containing 0.50 units of Go Flexi Taq polymerase, 1x Green PCR Buffer with 2.5mM $MgCl_2$ (Promega, WI, USA), 0.6 mM of dNTPs, 1.6µM of each primer and 4µL of DNA template and the remaining volume with molecular grade H_2O.

PCR amplification for *A. vaginae. G. vaginalis. M. mulieris* was similar, the first cycle consisted in a pre-melt phase at 94 °C for 2 minutes and then denaturation at 94 °C for 30 s. After that, annealing at each species temperature (see Table 1) was realized for 30 s, and extension was performed at 72 °C for 1 minute. This was repeated for 29 more cycles to 32 for the bigger amplicons (>500 bp). An additional 5 minutes of extension step was included at the end of the cycles to complete the extension of the primers. For *Enterococcus* sp., the first cycle at 94 °C for 5 minutes, after the denaturation at 94 °C for 30 s, the third cycle is the annealing at 54 °C for 90 s, the extension at 72 °C for minute. This was repeated for 29 cycles, and 5 minutes of extension step was included. For *E. coli*, the pre-melt phase at 95 °C for 2 minutes, the denaturation at 95 °C for 1 minute, after that, annealing at 57 °C for 1 minute, the extension at 72 °C for 2 minutes. This was repeated for 29 cycles, and finally 5 minutes of extension step was included. In the case of *C. albicans*, the pre-melt phase at 94 °C for 3 minutes, and then denaturation at 94 °C for 40 s, after that, annealing at 60 °C for 30 s, the extension at 72 °C for 2 minutes. This was repeated for 32 cycles, and additional 5 minutes of extension step was included.

The volume of 4µL from each PCR product were visualized in 1.5% (w/w) agarose (Promega, WI, USA) gel electrophoresis using 0.1% ethidium bromide staining, with the respective use of negative and positive controls, provided by the Microbiology Institute at USFQ. All samples were randomly performed in duplicates or triplicates with different negative and positive controls.

Diagnosis of vaginal infection and normal vaginal microbiota

The vaginal sample evaluation was made according to the presence of symptoms, clinical findings during survey, a microbiological test obtained by Gram stained techniques and then PCR analysis. Briefly, the recognition of vaginal infections was assessed according to the Table 2.

Classification of vaginal smears

The vaginal smear was obtained by rolling a swab onto a glass slide, the smear was heat fixed, Gram stained and classified according to the Nugent Score. Each smear was evaluated by 10 to 15 microscopic fields under oil immersion (X1000 magnification) and evaluated for several morphotypes. The samples were assigned a score of 0 – 10, in which the criterion for normal flora vaginal was 0 to 3, intermediate vaginal flora 4 to 6 and bacterial vaginosis was 7 or higher (Nugent et al., 1991). The Nugent score evaluates and gives a total summed score depending on the number of large gram-positive rods (*Lactobacillus* morphotypes), small Gram-variable rods (*G. vaginalis* morphotypes), small Gram-negative rods (*Bacteroides* spp. morphotypes) and curved Gram-negative rods (*Mobiluncus* spp. morphotypes), as illustrated in Table 3.

Furthermore, AV was diagnosed based on criteria used by Donders and colleagues in their study; which include absence of *Lactobacillus*; positive for cocci or coarse bacilli in high number; presence of parabasal epithelial cells, and/ or positive for leucocytes (Gilbert G.G. Donders et al., 2005). Finally, VC was assessed accordingly to Marot-Leblond and colleagues through at least one of the following applicable criteria: positive Gram-stain preparation with budding yeasts in high number in more than two microscopic fields, pseudohyphae, and/or hyphal forms; and positive culture in Chocolate and Blood Agar, 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. An absence in microscopy analysis of *Candida* species in more than two microscopic fields and/or together low number of *Candida* sp. result was considered a normal *Candida* colonization rather than VC (Marot-Leblond et al., 2009).

Statistical analysis

Logistic regression analyses were conducted to examine if different factors such as age, occupation, civil status, education, and anticonception habits were associated with the presence or absence of vaginal dysbiosis. All dependent and most independent variables were treated as categorical. Logistic regression models were used in which the independent variables were: age, occupation, civil status, education and anticonception habits; while the dependent variables for each model were healthy microbiota, intermediate microbiota and presence of vaginal infection. Further analysis was done between previous independent variables and each type of vaginal infection (VC, BC and AV), as dependent variables, using logistic regression models. Statistically significant differences were assumed when *P*-values were equal or less than 0.05. Statistical analyses were performed using SPSS version 22.0.

Results

Epidemiological characteristics

From the 436 samples, which were processed as previously referred, a total of 411 women accepted to fully answer the survey after the sample recollection and 4 women partially answered the same survey, making it a total of 415 surveys. The surveys were

partially answered by the volunteers in their five sociodemographic and behavioral questions as follows: age (413/415), occupation (411/415), civil status (411/415), education level (412/415) and birth control methods (415/415).

The recruited women turned out to have between 18 and 56 years old, and most of them were in the first two ranges from ≤21 to 28 years old which represent the 80.4% (332/413) (see Table 4). Approximately, 92.2% of volunteers were undergraduate students and professionals (379/411). The professional's category divided itself as follows: health professionals (23.0%), administrative clerks (20.3%), educational fields (14.9%) and employees from the same institution with other college degrees (18.0%). Most of the volunteers were single women and constituted 83.2% (342/411) of our study set. From the single women set, 53.5% of the participants had a steady sexual partner (183/342) and 46.5% did not have any sexual partner (159/342).

Finally, in relation to birth control methods, 31.1% (129/415) of participants used condom, 29.4% (122/415) used other birth control methods and 39.5% (164/415) did not use any birth control method. Alternative birth control methods included hormonal, barrier (spermicides, diaphragm, cervical cap and sterilization), intrauterine device (IUD) and natural (abstinence, fertility awareness method (FAM) and withdrawal). In our study, the most used alternative contraceptive methods were hormonal, with 46.7% (57/122) for oral contraceptives and 6.6% (8/122) for implants.

Diagnosis of vaginal microbiota in the study population

To better understand the results in the following paragraphs, the results were divided and compared between the analysis from the 436 diagnosed (DNA/gram) samples and the 415 answered surveys. The diagnosed results were divided in three large groups:

normal flora 66.0% (288/436), intermediate flora 10.8% (47/436) and vaginal dysbiosis 23.2% (101/436) and then compared to the questions in the survey (see Table 4). However, take notice that only 411 surveys were fully answered and 4 surveys were partially answered in our study set, as previous mentioned in "epidemiological characteristics". From now on, the data will be compared with the birth control methods category because is the only question that was fully answered in all the surveys.

In relation with the normal microbiota, the findings showed that most of the population had a normal vaginal microbiota 66.7% (277/415). These healthy samples had a *Lactobacillus* dominance without presence of an asymptomatic or symptomatic infection of any kind. The age range between 43-49 years old showed the highest percentage of healthy microbiota 77.8% (7/9) while women in the ≥50 years old range had the lowest prevalence of normal vaginal microbiota 41.7% (5/12). In relation to intermediate flora, only 42 of 415 (10.1%) women with this type of microbiota filled out the standardized medical survey. Within these results, the highest percentages of intermediate flora belonged to women between 29 and 35 years old (14.7%, 5/34) and women ≥ 50 years old (16.7%, 2/12), as shown in Table 4. Finally, only 95 of 415 women showed vaginal dysbiosis (22.9%), such as VC, BV and AV. In relation with age, most vaginal dysbiosis diagnosis was concentrated in women below 29 years old (77.9%, 74/95). More exactly, 58.1% women (43/74) in the ≤ 21 years old range and 41.9% women (31/74) in the 22 and 28 years old range. Although, none of the age categories were absent of vaginal dysbiosis cases (see Table 4).

From the 95 vaginal dysbiosis cases, 16 volunteers showed coinfections, being four asymptomatic cases and one case with three vaginal infections simultaneously. The remaining 80 women (83.3%) were diagnosed with only one type of vaginal dysbiosis,

and from these women, only 41 volunteers (51.2%) showed physical symptoms during their survey.

In relation to the occupation, the student' category had the highest prevalence of normal flora (69.2%; 211/305), while the unprofessional had most vaginal infections cases (40.0%; 10/25). In addition, married women had a higher percentage of normal flora (73.1%; 38/52) whereas free union volunteers showed the highest percentage of vaginal dysbiosis (50.0%; 4/8). Furthermore, from 95 cases of vaginal dysbiosis in civil status category, 82.1% of the infections were present in single volunteers with couple (39/95) and without couple (39/95), as shown in Table 4.

Finally, by educational level, women with a college degree had more vaginal dysbiosis (26.0%) showing 20 cases from a total of 77 women, of which 11 women presented symptoms (55%). Surprisingly women with condom use habits, as birth control method, showed a superior infection rate (27.9%; 36/129), when compared to women none (22.0%; 36/164) or even other birth control methods than condom (19.7%; 24/122).

Types of vaginitis in the study population

Among the study population (436 women with and without the requested survey), 101 volunteers had a vaginal dysbiosis. From women with vaginitis, 53 women were diagnosed with AV (52.5%), 24 participants were identified with BV (23.8%) and, finally, only 7 women were established with VC (6.9%), as shown in Figure 1. Therefore, 84 women were strictly diagnosed with a single type of vaginal dysbiosis (83.2%) remaining 17 women with vaginal coinfections in our study. From these 17 women with coinfections (16.8%), the most common coinfection was BV and AV in 12 women, followed by three women with BV and VC, one women with AV and VC, and finally one women with all three infections.

As previously referred, AV was the main diagnosed vaginitis in our population set, where only 51.0% of the women with AV were symptomatic cases (see Figure 2). The 27 women with symptomatic AV showed symptoms, such as inflammation, discharge yellow and odor rotten of the vaginal fluid. Many AV women were below 29 years old (40/49 cases), although women ≥ 50 showed an AV prevalence of 41.7% (5/12 women). The highest percentages of AV were also identified in housewife (28.6%) and in divorced women (22.2%). Finally, no significant values were observed in educational level or birth control methods.

In relationship to BV, only 15 from 24 of BV women (62.5%) showed symptomatic dysbiosis (see Figure 2) demonstrating physical symptoms, such as irritation, homogeneous and gray discharge thin with fishy odor. Most BV women were below 29 years old (18/24 cases) although women between 36 and 42 years old showed the highest BV prevalence of 15.4% (4/26 women). The highest percentages of BV were also identified in student (6.2%) and in divorced women (11.1%). No significant values were observed in educational level but, in birth control methods, 11 of 22 BV women (50.0%) used condom in their sexual relationship.

As shown in Figure 2, VC was diagnosed in seven women where only four women (57.1%) had physical symptoms, such as pruritus and thick discharge with a color of white to yellow. All VC cases were detected in women under 36 years old, where 5 of the 7 cases (71.4%) were in the age category of ≤ 21 years old, student at occupation category and single without couple at civil status. Six of the VC volunteers (85.7%) only had secondary level in education while 4 of the 7 cases (57.1%) used condom as birth control methods.

In this study, we detected 17 cases of coinfections. However, only 16 women answered the survey where only 5 were diagnosed with different types of vaginal dysbiosis (see

Figure 2). From these 16 coinfections with completed survey, all cases were below 43 years old and with sexual habits. Although the category of 29-35 years had the highest prevalence of coinfections (11.8%; 4/34), it is important to mention that 7 of 16 cases of coinfections (43.8%) belonged to the age category 22 - 28 years old and they did not use any type of birth control method. Meanwhile, 8 of 16 cases of coinfections (50.0%) were student at educational level and 10 of 16 cases were single (4 and 6 women with and without couple, respectively) at civil status (see Table 4). Finally, in the population, five women of the 436 revealed to possess several sexual partners, being one of them the volunteer with a coinfection of BV, AV and VC.

Vaginal colonization of pathogenic and opportunistic species

As previously referred, among all 436 women, 288 women were classified with normal vaginal microbiota (66.0%), 47 women had intermediate microbiota (10.8%) and the remaining 101 volunteers had a single or multiple vaginal dysbiosis (23.2%). The vaginal colonization of each category by pathogenic and opportunistic species was done through PCR identification of the main dominant species from each vaginitis, more exactly: *A. vaginae*, *G. vaginalis* and *M. mulieris* for BV; *E. coli* and *E. faecalis* for AV; and *C. albicans* for VC. Although AV was the main diagnosed vaginitis among the vaginal dysbiosis (as shown in Figure 1), the most dominant species in vaginal colonization of the study set were *G. vaginalis* (184/436; see Table S1 in Supplementary Information) and *A. vaginae* (180/436), which usually are associated with BV. The microbial colonization was then followed by *E. coli* in 51 women, *M. mulieris* in 14 women and finally *E. faecalis* and *C. albicans* in 8 and 7 women, respectively.

As shown in Figure 3, the prevalence percentage of each microbial species was always superior in vaginal dysbiosis excepting for *E. faecalis* that illustrated a superior

percentage in intermediate microbiota. In fact, *E. faecalis* was the less prevalent analyzed pathogen in our study, illustrating only 2% of prevalence in the vaginal dysbiosis. In BV and intermediate flora diagnosis, it is also possible to observe a slight *G. vaginalis* prevalence over the samples when compared to *A. vaginae* prevalence and in detriment what it is previously regarded in the women diagnosed with normal flora (see Figure 3). In AV diagnosis, *E. coli* and *E. faecalis* were identified in less of 25% of AV women, demonstrating the eventual presence of other species as pathogenic and/or opportunistic microorganism in the established of AV in Ecuadorian women. It is important to mention the presence of gram positive coccus during microscopic examination of the vaginal smears. In addition, *C. albicans* was detected in 28.6% (see Table S1 in Supplementary Information) of the women diagnosed with VC, where it was assessed accordingly to Marot-Leblond and colleagues (2009) criteria to vaginitis by *Candida* sp. Finally, coinfections represented 16.8% (17/101) of the vaginal dysbiosis, where 12 of the 17 coinfections (70.6%) were BV cases colonized simultaneously by *G. vaginalis* and *A. vaginae* in association with other type of vaginal dysbiosis (AV or VC).

Regarding normal microbiota diagnosis, *A. vaginae* and *G. vaginalis* were again the dominant microbial opportunistic species being in more than three quarters of the health women, more exactly, 35.4 and 34.0% of the normal flora, respectively. On the other hand, *M. mulieris* another well-known BV-associated bacterium was only found in one healthy women (0.3%). Furthermore, *E. coli* was present in 26 healthy women (9.0%) followed by *E. faecalis* identified only in 3 healthy women, representing the colonization of well-known AV-associated bacteria as second place in healthy vaginal microbiota analysis. At last, *C. albicans* were only found in one healthy women by

PCR, although other similar *Candida* species were observed during microscopic analysis of the vaginal smear samples.

Factors associated with presence of vaginitis

As previously referred in Methods, logistic regression analyses were used to identify factors that predicted the presence or absence of vaginal dysbiosis. No association between age, marital status and the prevalence of any type of vaginal infection (BV, AV and VC) was found in our study set (see Table 4). However, regarding the occupation category, being a student increased the odds of having a normal microbiota ($p \leq 0.01$, OR=2.245) and it diminished the odds of AV ($p \leq 0.05$, OR=0.405). While, in education category, women with secondary level education showed statistically lower odds to acquire intermediate microbiota ($p \leq 0.05$, OR=0.357). Furthermore, in Birth Control Methods category, the use of contraceptives (condom OR=0.388 or another than condom OR=0.363) demonstrated significantly lowers the odds of intermediate microbiota ($p \leq 0.05$). Consequently, another method of contraception category statistically increased the odds of diagnosis of normal vaginal microbiota in women ($p \leq 0.05$ OR=1.752).

As previously shown in Figure 2, there was no significant difference between symptomatic and asymptomatic infections. However, when compared to the vaginal colonization of pathogenic and opportunistic species, the presence of *C. albicans,* ($p \leq 0.001$, OR=15.664), *M. mulieris* ($p \leq 0.001$, OR=1.828) and *G. vaginalis* ($p \leq 0.05$, OR=3.929) increased the odds ratio of vaginal dysbiosis among women in our study (see Table S1 in Supplementary Information). Therefore, *C. albicans,* ($p \leq 0.001$, OR=0.299), *M. mulieris* ($p \leq 0.01$, OR=0.56) and *G. vaginalis* ($p \leq 0.01$, OR=0.500) significantly decreased the odds diagnosis of normal vaginal microbiota.

In relation to different types of vaginal dysbiosis, *A. vaginae* statistically augmented the risk of VC ($p \leq 0.05$, OR=7.242) among our population (see Table S1 in Supplementary Information). Meanwhile, *M. mulieris* ($p \leq 0.001$, OR=11.573), *G. vaginalis* ($p \leq 0.001$, OR=4.047) and *C. albicans* ($p \leq 0.05$, OR=2.940) significantly increased the odds for BV infection. On the other hand, the presence of *E. coli* ($p \leq 0.05$, OR=0.242) diminished the odds of BV establishment in the vaginal epithelium. In addition, it is important to mention that *M. mulieris* ($p \leq 0.01$, OR=4.655) also significantly increased the odds of AV among our study. Finally, *M. mulieris* ($p \leq 0.01$, OR=7.443) and *C. albicans* ($p \leq 0.001$, OR=9.678) statistically enhanced the odds of coinfections in these volunteers.

Discussion

To the authors' best knowledge, this is the first epidemiologic study in Ecuador that evaluated simultaneously the prevalence of several vaginal dysbiosis (BV, AV and VC) in symptomatic and asymptomatic women, identifying the main pathogens associated with these infections by molecular methods. Similar to previous studies, healthy vaginal microbiota was identified in two thirds of the volunteers (66.0%) (Hernández-Rodríguez et al., 2011; Puapermpoonsiri et al., 1996; Tamrakar et al., 2007). In fact, Cauci et al. (2002) identified the prevalence of 67.8% of healthy flora in women with a mean age of 45.3 years old in peri and postmenopausal women, similar to this study, in which the category of 43-49 years old had the highest prevalence reported of normal flora (77.8%). Still, others countries, such as USA (60.6%), Chile (58.3%) and Turkey (47.7%), showed different rates of healthy vaginal microflora (Martinez.M.Ovalle.A., 2017; Schwebke, Hillier, Sobel, McGregor, & Sweet, 1996; Zarakolu et al., 2004).

Only 10.8% of the volunteers showed intermediate microbiota and the most women were between 29 and 35 years old. Similar results were also obtained in other studies

(Chawla, Bhalla, Chadha, Grover, & Garg, 2013; Martinez.M.Ovalle.A., 2017; Schwebke et al., 1996).

However, several studies reported superior rates of intermediate microbiota in women (Gondo et al., 2011; Larsson, Carlsson, Fåhraeus, Jakobsson, & Forsum, 2004; Waqqar et al., 2018), showing rates between 36.25 and 69.2%. Nonetheless, Cauci and colleagues reported a lower rate of intermediate microbiota (6.1%) (Cauci et al., 2002), when compared to the present study.

In our population set, vaginal dysbiosis was diagnosed in 101 women (23.2%), in a similar rate to the prevalence reported in USA (28%) (Kent, 1991) but lower with the prevalence detected in Syria (51%) (Yentur Doni et al., 2016). However, one of the most high prevalence of vaginitis was reported in Bosnia (96%) (Jahic et al., 2013). Moreover, 23% of the study set showed at least one well-established vaginitis where most of these women was below 29 years old. Nevertheless, other studies identified a higher risk for vaginal infection in women older than 30 years old (Na et al., 2014; Wang, Huang, Wu, Qi, & Lin, 2017). In this study, age was not a statistically significant risk factor related to the presence of vaginal dysbiosis.

In this study, women in free union had a higher percentage of vaginal dysbiosis, without any statically significance, in conflict with other studies that showed no obvious association between marital status or long term relationship and the presence of infection (Ocviyanti, Rosana, Olivia, & Darmawan, 2010; Wang et al., 2017). However, in Hainan (an island province of China), Na and colleagues reported that marriage was significantly associated with VC in their study set (689 cases and 652 controls) (Na et al., 2014). In addition, the present study showed a lower prevalence of AV in student women when compared to unprofessional women, being statistically significant ($p \leq 0,05$) and supported by previous studies that reported higher prevalence of vaginitis

in women with a lower level of education (Na et al., 2014; Wang et al., 2017). Furthermore, several studies found evidence of a negative association between BV infection and the use of condoms (Fethers et al., 2008; Hutchinson, Kip, & Ness, 2007; McClelland et al., 2008; Shoubnikova, Hellberg, Nilsson, & Mårdh, 1997). However, in this study, women that used condom as birth control method showed a slight more vaginal dysbiosis percentage without any statically significance when compared with other or even none birth control methods (see Table 4). In agreement with our study, other studies revealed that use of oral contraceptives, intrauterine device and barrier methods was not related to the risk of vaginal infection (Chiaffarino, Parazzini, De Besi, & Lavezzari, 2004; McClelland et al., 2008; Shoubnikova et al., 1997).

As previously shown in Figure 1, the most prevalent form of vaginal dysbiosis in our study was AV (52.5%; 53/101), followed by BV (23.8%; 24/101) and then VC (6.9%; 7/101). Although few studies analyzed the presence of different vaginitis in women, Jahic and colleagues reported a similar prevalence of AV (51%) in their population set, a lower rate of BV (15%) and a higher rate of VC (17%). However, another study realized by Mulu et al. (2015) showed a more similar candidiasis rate (9.2%) when compared to the present study (Jahic et al., 2013).

Aerobic vaginitis was first characterized in 2002 by Donders and colleagues in Belgium (Gilbert G.G. Donders et al., 2005; Kaambo et al., 2018). Little is still known about its global epidemiology and their implications, when compared to other types of vaginitis, such as BV and VC. Although the prevalence of AV was similar in a study reported in Bosnia (51%) (Jahic et al., 2013), when compared to the present study; other countries showed lower AV prevalence in their studies, such studies in Belgium (7.9% and 10%) (Donders et al.,2011; Donders et al., 2005), Brazil (4.9%) (Marconi et al., 2012) and USA (8-11%) (Kaambo et al., 2018). In fact, this low prevalence of AV had been

reported in several review studies (Gilbert G.G. Donders et al., 2005; Kaambo et al., 2018; Tansarli et al., 2013). In addition, Tansarli and colleagues reported a prevalence 5-10.5% of symptomatic AV women (Tansarli et al., 2013), while Kaambo and colleagues showed a rate of AV between 8 and 11% in pregnant women and also 5-24% of AV in women with symptomatic vaginitis (Kaambo et al., 2018). Furthermore, AV was the main type of vaginitis in our population set, where 49% of this vaginitis was diagnosed in asymptomatic women but showing a smaller prevalence when compared to another study realized by Gondo et al. (2011) in Brazil, where 57.1% of AV was detected in asymptomatic women.

Moreover, BV prevalence among women of reproductive age in the present study was of 23.8%, being similar to other studies in Ecuador (Vaca et al., 2010), Perú (Jones et al., 2007) and USA (Koumans et al., 2007). In Ecuador, Vaca and collegues reported 31.5% of BV while, in Perú, Jones and colleagues reported 27 %, and finally, in USA, Koumans and colleagues showed 29.2% in their population set. Though, some countries of Europe demonstrated a lower prevalence of BV in their study sets of pregnant women (Desseauve et al., 2012; Machado et al., 2017), such as France (7.1%) and Portugal (3.88%). Further, in Ethiopia, Bitew et al. (2017) described an higher BV prevalence (48.6%) in women (Bitew et al., 2017), the same happens in India with a prevalence of 44.8% (Seth, Chaitra, Vaishnavi, & R, 2017). Therefore, most epidemiological studies demonstrated a variety of BV prevalence accordingly to their geographical locations (Wang et al., 2017). In fact, this variety of BV prevalence had been also reported in several review studies (Fethers et al., 2008; Kenyon et al., 2013; Oostrum et al., 2018), where it had been normally reported a BV prevalence between 6.1% and 51.6%. Finally, in the present study, 62.5% of women with BV were classified as symptomatic infection, showing a similar prevalence as previously reported by Gondo and colleagues

(2011) in Brazil (66%). However, in USA, Koumans et al. (2007) stated only 15.7% of women symptomatic with BV.

Also, in the present study, VC was identified only in 6.9% of women with vaginitis and in a similar prevalence to a study realized in Ethiopia (8.3%) (Mulu et al., 2015). But, several countries reported a higher rate of candidiasis, more precisely, Brazil (52.4%) , Italy (43.5%), India (35%) , Nigeria (36%), Chile (43.9%) and USA (20-30%) (Aguin et al., 2015; Amouri et al., 2011; Cannobi et al., 2011; Corsello et al., 2003; Martinez et al., 2017; Olowe et al., 2014; Rathod et al., 2012). In the present study, 57.1% of VC were diagnosed in symptomatic women, demonstrating therefore a greater prevalence when compared to Mulu et al. (2015) with 6.8% of symptomatic women and lower prevalence when compared to Gondo et al. (2011) with 92% of symptomatic women. Most studies reported the presence or absence of infection, as well as their pathogen colonization; however, little is known about the epidemiological prevalence of symptomatic and asymptomatic women until nowadays.

In relation to coinfections, we detected 17 cases from the total of 101 vaginal dysbiosis, where the 70.6% of women had symptomatic vaginitis. Despite this high percentage of symptomatic coinfections, another study revealed a higher prevalence of symptomatic infection in presence of coinfections (Gondo et al., 2011), more exactly 85.7%. Also, Rivers and colleagues showed a high prevalence of symptoms (80% of abnormal vaginal discharge) in women with a coinfection for BV and candidiasis vulvovaginal (Rivers et al., 2011). Therefore, this work supported previous studies by reporting a greater number of symptomatic women with multiple vaginal infections. However, further studies are necessary to analyze asymptomatic women in each type of vaginitis. Finally, it is important to mention that the only coinfection diagnosed simultaneously with BV, AV and candidiasis was reported in a woman with several sexual partners.

Although it was not possible to establish any statistical significance, this coinfection is in agreement to consider several sexual partners as a risk factor and already reported with several previous studies (Fethers et al., 2008; Smart, Singal, & Mindel, 2004).

Further analysis was done to identify the main microbial species commonly associated to the diagnosed vaginitis in this study. This analysis was then compared with previous studies of other countries, as shown in Table 5. Although AV was the main diagnosed vaginitis in our study, only 17.8 and 2.0% of these infections were colonized by *E. coli* and *E. faecalis*, respectively. These results distinguished from previous reports that showed higher prevalence of *E. coli* and *E. faecalis* in their studies (Fan et al., 2013; Tempera et al., 2006). As shown in Table 5, studies from Bosnia and Italy showed a prevalence of *E. coli* between 55.0 and 86.7% and *E. faecalis* between 40.0 and 52.0%. Moreover, Von Gruenigen and colleagues (2000) identified rates of 28 and 44% of *E. coli* and *E. faecalis*, respectively, in their small population set in USA. In Japan, Puapermpoonsiri et al. (1996) reported a prevalence of 38.0% of *E. faecalis* in their study set. However, other studies realized in developing countries, such as Nigeria, Mexico and Iraq, detected a similar or less prevalence of *E. coli* in their diagnosed AV women (Otuonye et al., 2004; Flores-Paz et al., 2003; Razzak et al., 2011), more exactly, 16.2, 13.5 and 6.0 %, respectively. In fact, Iavazzo and colleagues (2008) reported fewer prevalence of *E. coli* and *E. faecalis* in a large population set (1.632 women) in Greece, more precisely, 4.0 and 0.3%, respectively. It is important to mention that several studies described other AV-associated aerobes than *E. coli* and *E. faecalis* (Iavazzo, Vogiatzi, & Falagas, 2008; Otuonye et al., 2004; Tansarli et al., 2013), such as *Streptococcus* and *Staphylococcus* species. This data could explain the low values of *E. coli* and *E. faecalis* prevalence in our study, and it encourages to

pursue the investigation of other species related to vaginal infections (Otuonye et al., 2004; Flores-Paz et al., 2003; Razzak et al., 2011).

In relation to BV, *G. vaginalis* was the dominant pathogenic species in this vaginitis (59.4%), followed by *A. vaginae* (55.4%) and finally by *M. mulieris* (11.9%). *M. mulieris* and *G. vaginalis* significantly increased the odds for BV infection ($p \leq 0.001$) while the presence of *E. coli* diminished the probability of BV ($p \leq 0.05$). These results were below the prevalence of *G. vaginalis* and *A. vaginae* determined in our previous study in pregnant teenagers (Salinas et al., 2018), however the present study had a greater number of volunteers and analyzed women at adult age range (18-56 years old). Nevertheless, when compared to another Latin America countries, such as Brazil, the three BV-associated anaerobes prevalence maintained the same vaginal colonization dominance but with higher percentages of detection (Malaguti et al., 2015), more precisely: *G. vaginalis* (59.4% *versus* 45.7%); *A. vaginae* (55.4% *versus* 9.3%); and *M. mulieris* (11.9% *versus* 3.7%). However, in USA, Schwebke and colleagues (2014) detected *A. vaginae* in an identical prevalence colonization (54.0%) when compared to our study (55.4%). In addition, several studies realized in Europe, such as Portugal (Machado et al., 2017), Lithuania (Janulaitiene et al., 2017) and Bulgaria (Tosheva et al., 2017), reported a greater prevalence of the same BV-associated anaerobes, when compared to the present study; but maintaining the same hierarchy order of *G. vaginalis*, *A. vaginae* and *M. mulieris* (see Table 5). Finally, in China, two studies demonstrated again the same hierarchy but greater prevalence of *G. vaginalis* and *A. vaginae* in their study sets (Xia et al., 2016; Shen et al., 2016), more exactly, 63.2-82.8% and 17.1-65.5%, respectively. In summary, these studies supported the results obtained in present study and illustrated the necessity to control the vaginitis diagnosis

in Ecuador in order to avoid the augmentation of BV registered in other epidemiological studies.

At last, VC was the least vaginal dysbiosis diagnosed in this study and moreover only 5.0% of these cases were *C. albicans* part of the vaginal microbiota dysbiosis. Thus, our results are in discrepancy with other studies worldwide, as shown in Table 5. In 2010, Vaca and colleagues reported a prevalence of 23.7% of *C. albicans* in their study set of adolescents between 13 and 17 years old in Ecuador (Vaca et al., 2010b). In other Latin-American countries, such as Brazil and Colombia, *C. albicans* prevalence in VC also fluctuated between 22.0 and 80.0% (Moreira Mascarenhas et al., 2012; Duque et al., 2009), respectively. While, studies realized in Europe (such as Italy and Belgium) reported a more constant and prevalent existence of *C. albicans* in VC (Corsello et al., 2003; De Vos et al., 2005), more precisely, around 77.1 and 78.6 %. In opposite, Masri and colleagues (2015) reported a prevalence of 17.2% of *C. albicans* in their study in pregnant women from Malaysia. Finally, Olowe and colleagues (2014) showed a higher prevalence of *C. albicans* (36%) in pregnant women but differing with the results obtained by Aubyn and Tagoe (2013) in Ghana with only 22.0% of *C. albicans*. These findings suggest the possibility of other *Candida* species being responsible for VC, as proposed by several previous studies (Amouri et al., 2011; Brandolt et al., 2017; Corsello et al., 2003; De Vos et al., 2005), such as *C. parapsilosis*, *C. tropicalis*, *C. krusei* and *C. glabrata* (Deorukhkar, Saini, & Mathew, 2014; Krcmery & Barnes, 2002; Nejat et al., 2018).

Overall, the major drawback of this study was the lack of quantitative data which may allow us to assess the status of colonization of the distinct microbial taxa. Also, DNA sequencing of the samples and its analysis could allow us to identify the species with greater reliability in vaginal microbiota and possibly analyze the clades to which these

species belong. However, this epidemiological study was able to characterize the heterogeneity of the vaginal infections in Ecuadorian women, where the majority of the population set showed a normal vaginal microbiota (66.0%) and 23.2% of the volunteers had a single or multiple vaginal dysbiosis. AV was the predominant vaginal dysbiosis (52.5%), of which 51% was symptomatic, and followed by BV, diagnosed in 23.8% of the women with vaginitis, of which 62.5% showed symptoms. Only 6.9% of this group set showed VC, of which 57.1% had symptoms. In our group set of vaginitis, 16.8% of women had coinfection, of which 70.6% was symptomatic. The most dominant species in vaginal colonization of the study set were *G. vaginalis* and *A. vaginae*, which are usually associated with BV development. While *E. coli* and *E. faecalis* were identified in less of 25% of AV women.

One of the limitations of this study was the lack of available clinical data during the surveys, as other previous epidemiological studies in Latin America, such as Ecuador (Salinas et al., 2018). This study was conducted in a representative age range of the adult reproductive women. Further studies should be realized in Ecuador to confirm the prevalence of several types of vaginitis among pregnant and no pregnant women and to clarifying the microbial colonization in vaginal infections.

Conclusion

The present study identified AV as the main cause of vaginal dysbiosis in our population set. Also, *G. vaginalis* and *A. vaginae* were the most abundant opportunistic species in our molecular analysis, being frequently detected in normal and intermediate vaginal microbiota. Although *A. vaginae* were slightly more prevalent than *G. vaginalis* in normal vaginal microbiota, *G. vaginalis* were dominant in intermediate vaginal microbiota and vaginal infection. Meanwhile, *E. coli* and *E. faecalis* were identified in low percentage of women with AV, demonstrating the eventual presence of other opportunistic pathogens in Ecuadorian women with AV, such as *Staphylococcus* or *Streptococcus* species. Finally, *C. albicans* was only detected in 28.6% of the women diagnosed with VC, suggesting the eventual involvement of other *Candida* species in the establishment of this vaginitis. To the authors' knowledge, this is the first study of vaginal microbiota in Ecuadorian women to assess the prevalence of several types of vaginal dysbiosis. Further studies should be realized on bigger population set, including pregnant women and longitudinal evaluation of the vaginal microbiota.

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TABLES

Table 1. PCR primers used in this study.

Set	Name	Sequence (5′-3′)	Target	T (°C) of annealing	Size of fragment	Target gene	Specificity %	Validation	Reference
1	Atop109-Fw	GAGTAACACGTGGGCAACCT	*Atopobium vaginae*	62 °C	221 bp	16S rRNA	16.7%	Samples sequenced to confirm identity	(Henriques et al., 2012)
	Atop109-Rv	CCGTGTCTCAGTCCCAATCT					37.5%		
2	Mobil-577F	GCTCGTAGGTGGTTCGTCGC	*Mobiluncus mulieris*	62 °C	449 bp	16S rRNA	100%	n/d	(Fredricks et al., 2007)
	M.mulie-1026R	CCACACCATCTCTGGCATG							
3	Gard154-Fw	CTCTTGGAAACGGGTGGTAA	*Gardnerella vaginalis*	60 °C	301 bp	16S rRNA	100%	n/d	(Henriques et al., 2012)
	Gard154-Rv	TTGCTCCCAATCAAAAGCGGT							
4	Primer E1	ATCAAGTACAGTTAGTCTT	*Enterococcus faecalis*	54° C	941 bp	ddl	100%	Increase of the annealing temperature at 54° C	(DTU- National Food Institute, 2014)
	Primer E2	ACGATTCAAAGCTAACTG							
5	adk F	ATTCTGCTTGGCGCTCCGGG	*Escherichia coli*	57° C	583 bp	adk	49%	Increase of the annealing temperature at 57° C	(Sepehri, Kotlowski, Bernstein, & Krause, 2009)
	adk R	CCGTCAACTTTCGCGTATTT					98 %		
6	SC1F	CGGAGATTTTCTCAATAAGGACCAC	*Candida albicans*	60° C	670 bp	KER1	100%	n/d	(Galán, Veses, Murgui, Casanova, & Martínez, 2006)
	SC1R	AGTCAATCTCTGTCTCCCCTTGC							

N/d – non determined

Table 2. Parameters used for vaginal infections diagnosis.

Infection	Symptoms	Discharge	Odor	Diagnosis	Reference
Vulvovaginal Candidiasis	Pruritus	Thick, white to yellow	Absent	Gram stain, medical survey	(Carr, Felsenstein, & Friedman, 1998)
Aerobic Vaginitis	Inflammation	Yellow	Foul, rotten	Gram stain, medical survey	(Gilbert G.G. Donders et al., 2005)
Bacterial Vaginosis	Irritation, 50% asymptomatic	Thin, white to gray, homogeneous	Fishy	Gram stain, medical survey	(Carr et al., 1998)

Table 3. Scoring system used to classify the Gram-stained smear of the recollected samples.

Score	Morphotypes		
	Lactobacillus spp.	*Gardnerella*, *Bacteroides* and *Prevotella* spp.	*Mobiluncus* spp.
0	>30	0	0
1	5-30	<1	1-5
2	1-4	1-4	>5
3	<1	5-30	-

Table 4. Sociodemographic, behavioral variables among women in this study with normal flora, intermediate vaginal flora, Bacterial vaginosis, Aerobic vaginitis, candidiasis and coinfections.

	Normal Flora *N* **(%)**	**Intermediate Flora** *N* **(%)**	**Candidiasis** *N* **(%)**	**Bacterial Vaginosis** *N* **(%)**	**Aerobic Vaginitis** *N* **(%)**	**Coinfections** *N* **(%)**	**Total** *N*
Age							
≤ 21	107 (64.1)	17 (10.2)	5 (3.0)	12 (7.2)	23 (13.8)	3 (1.8)	167
22 – 28	119 (72.1)	15 (9.1)	1 (0.6)	6 (3.6)	17 (10.3)	7 (4.2)	165
29 – 35	21 (61.8)	5 (14.7)	1 (2.9)	1 (2.9)	2 (5.9)	4 (11.8)	34
36 – 42	17 (65.4)	2 (7.7)	0 (0)	4 (15.4)	1 (3.8)	2 (7.7)	26
43 – 49	7 (77.8)	1 (11.1)	0 (0)	0 (0)	1 (11.1)	0 (0)	9
≥ 50	5 (41.7)	2 (16.6)	0 (0)	0 (0)	5 (41.7)	0 (0)	12
Occupation							
Housewife	4 (57.1)	1 (14.3)	0 (0)	0 (0)	2 (28.6)	0 (0)	7
Student	211 (69.2)**	29 (9.5)	5 (1.6)	19 (6.2)	33 (10.8) *	8 (2.6)	305
Unprofessional	11 (44)	4 (16)	1 (4)	1 (4)	5 (20)	3 (12)	25
Professional	48 (64.9)	8 (10.8)	1 (1.4)	3 (4.1)	9 (12.2)	5 (6.8)	74
Civil Status							
Married	38 (73.1)	5 (9.6)	0 (0)	2 (3.8)	5 (9.6)	2 (3.8)	52
Divorced	4 (44.4)	1 (11.1)	0 (0)	1 (11.1)	2 (22.2)	1 (11.1)	9
Single							
With Couple	132 (72.1)	12 (6.6)	2 (1.1)	11 (6)	22 (12)	4 (2.2)	183
Without Couple	97 (61)	23 (14.5)	5 (3.1)	9 (5.7)	19 (12)	6 (3.8)	159
Free Union	3 (37.5)	1 (12.5)	0 (0)	0 (0)	1 (12.5)	3 (37.5)	8
Education Level							
≤ Basic	4 (66.7)	1 (16.7)	0 (0)	0 (0)	1 (16.7)	0 (0)	6
Secondary	224 (68.1)	31 (9.4) *	6 (1.8)	19 (5.8)	39 (11.9)	10 (3)	329
≥ University	48 (62.3)	9 (11.7)	1 (1.3)	4 (5.2)	9 (11.7)	6 (7.8)	77
Birth Control Methods							
Condom	84 (65.1)	9 (7) *	4 (3.1) *	11 (8.5)	17 (13.2)	4 (3.1)	129
Other than condom	90 (73.8) *	8 (6.6) *	1 (0.8) *	5 (4.1)	13 (10.7)	5 (4.1)	122
None	103 (62.8)	25 (15.2)	2 (1.2)	7 (4.3)	20 (12.2)	7 (4.3)	164

Legend: *N* number of women who responded in the survey within each category; *%* assigned percentage for each classification within each category.
* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Table 5. Summary of vaginal infection studies in women (including this study).

N	Population description	Study group(n)	Country	Methodology	Bacterial species detected (%)						References
					A. vaginae	*G. vaginalis*	*M. mulieris*	*E. coli*	*E. faecalis*	*C. albicans*	
1	Women in reproductive age (Age range 18-56)	436	Ecuador	Microscopic examination , Nugent criteria, PCR	55.4	59.4	11.9	17.8	2.0	5.0	This study
Bacterial vaginosis											
2	Pregnant teenage (Age range 10-19)	95	Ecuador	PCR	100	93.7	35.7	Na	Na	Na	(Salinas et al., 2018)
3	Women (Age 15-54)	223	Brazil	Multiplex PCR	9.3	45.7	3.7	Na	Na	Na	(Malaguti, Bahls, Uchimura, Gimenes, & Consolaro, 2015)
4	Premenopausal women (Age 18-48)	196	USA	Microscopic examination and PCR	Na	53.0	Na	Na	Na	Na	(Haggerty et al., 2009)
5	Women (Age range 14-37)	50	USA	Clinical examination and PCR	54.0	Na	Na	Na	Na	Na	(Schwebke, Flynn, & Rivers, 2014)
6	Pregnant women (Age 19-41)	206	Portugal	PCR	Na	67.4	Na	Na	Na	Na	(Machado et al., 2017)
7	Women (Age 22-53)	116	Lithuania	Clinical and microscopic examination, PCR	89.7	100	Na	Na	Na	Na	(Janulaitiene et al., 2017)
8	Women (Age 16-45)	538	Bulgaria	Multiplex PCR	68.1	98.4	17.0	Na	Na	Na	(Tosheva et al., 2017)
9	Posmenopausal women (mean 55.6 ± 2.6 years)	52	China	16S rRNA PCR	65.5	82.8	Na	Na	Na	Na	(Shen et al., 2016)
10	Premenopausal women (Age 18-48)	196	China	Microscopic examination and PCR-DGGE	17.1	63.2	Na	Na	Na	Na	(Xia et al., 2016)
Aerobic vaginitis											
11	Women with gynecologyc cancer (Age Na)	26	USA	Microscopic examination and culture	Na	Na	Na	28	44	Na	(Von Gruenigen et al., 2000)
12	Pregnant women (Age 15-40)	326	Japan	Microscopic examination and culture	Na	100	13.0	Na	38.0	25.0	(Puapermpoonsiri et al., 1996)
13	Women (Age 18-45)	100	Bosnia	Clinical examination and culture	Na	Na	Na	55.0	52.0	17.0	(Jahic et al., 2013)

14	Women with diagnosis of AV (mean age 33.5±8.68 years)	81	Italy	Clinical examination and culture	Na	Na	Na	86.7	40	Na	(Tempera et al., 2006)
15	Cervical discharge specimens (Age Na)	6811	México	Microscopic examination and culture	Na	Na	Na	13.46	Na	Na	(Flores-Paz et al., 2003)
16	Symptomatic women (Age range 18-57)	1632	Greece	Microscopic examination, culture and API 20 methods	Na	40.4	Na	4.0	0.3	42.5	(Iavazzo et al., 2008)
17	Women (Age range 15-50)	250	Nigeria	Microscopic examination and culture	Na	Na	Na	6	Na	Na	(Otuonye et al., 2004)
18	Non pregnant women (Age Na)	80	Iraq	Microscopic examinatin an biochimical test	Na	Na	Na	16.2	Na	Na	(Razzak et al., 2011)
Candidiasis											
19	Adolescents (Age 13-17)	213	Ecuador	Microscopic examination	Na	Na	Na	Na	Na	23.7	(Vaca et al., 2010)
20	Adolescents (Age 10-19)	100	Brazil	Microscopic examination and culture	Na	Na	Na	Na	Na	22.0	(Moreira Mascarenhas et al., 2012)
21	Women with candidiasis (Age 14-51)	150	Colombia	Microscopic examination and culture	Na	Na	Na	Na	Na	80.0	(Duque et al., 2009)
22	Women with candidiasis (Age range 15–94)	951	Italy	Culture	Na	Na	Na	Na	Na	77.1	(Corsello et al., 2003)
23	Women with diagnosis of candidiasis vulvovaginal (Age Na)	77	Belgium	PCR	Na	Na	Na	Na	Na	78.6	(De Vos et al., 2005)
24	Pregnant women (Age 18-30)	1163	Malaysia	Microscopic examination and culture	Na	Na	Na	Na	Na	17.2	(Masri et al., 2015)
25	Women (Age 21-29)	100	Nigeria	Culture	Na	Na	Na	Na	Na	36	(Olowe et al., 2014)
26	University students (Age range 18-41)	50	Ghana	Culture	28.0	Na	Na	Na	Na	22.0	(Aubyn & Tagoe, 2013)

Legend: Na – Not analyzed

SUPPLEMENTARY INFORMATION

Table S1. Molecular detection of the main pathogenic species among women in this study with normal flora, intermediate vaginal, candidiasis, bacterial vaginosis, aerobic vaginitis and coinfections.

	Normal Flora (N=288) ***N* (%)**	**Intermediate Flora (N=47)** ***N* (%)**	**Candidiasis (N=7)** ***N* (%)**	**Bacterial Vaginosis (N=24)** ***N* (%)**	**Aerobic Vaginitis (N=53)** ***N* (%)**	**Coinfections (N=17)** ***N* (%)**
Atopobium vaginae	102 (35.4)	22 (46.8)	6 (85.7) *	15 (62.5)	23 (43.4)	12 (70.6)
Mobiluncus mulieris	1 (0.3) **	1 (2.1)	0 (0)	5 (20.8) ***	4 (7.5) **	3 (17.6) **
Gardnerella vaginalis	98 (34.0)**	25 (53.2)	5 (71.4)	19 (79.2) ***	24 (45.3)	12 (70.6)
Escherichia coli	26 (9.0)	7 (14.9)	2 (28.6)	3 (12.5) *	12 (22.6)	1 (5.9)
Enterococcus faecalis	3 (1.0)	2 (4.3)	0 (0)	1 (4.2)	1 (1.9)	0 (0)
Candida albicans	1 (0.3)***	2 (4.3)	2 (28.6)	1 (4.2) *	1 (1.9)	1 (5.9) ***

Legend – N: number of women with normal flora, intermediate flora or vaginitis % assigned percentage for each category.

* $P \leq 0$.

FIGURES

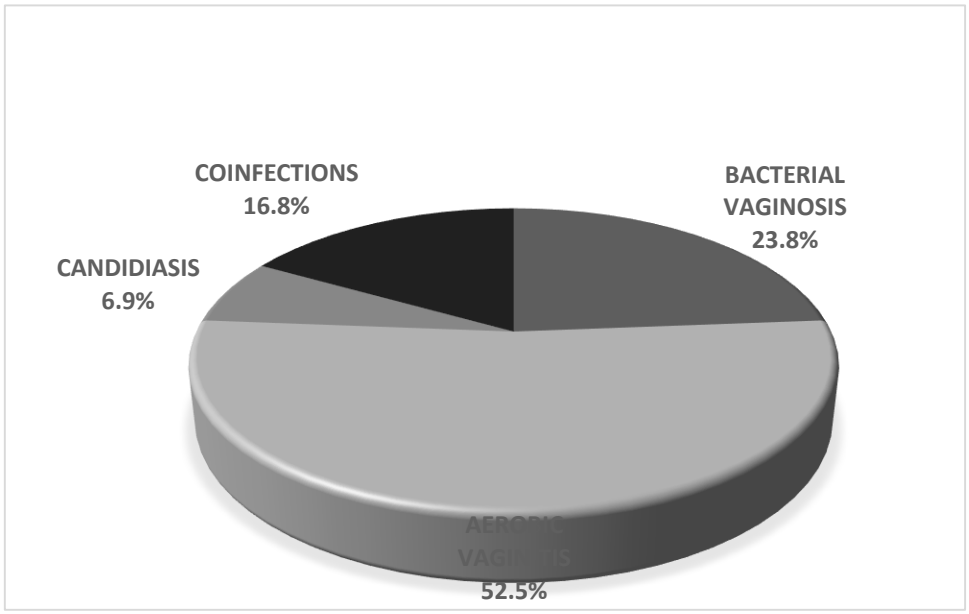

Figure 1 - Prevalence of bacterial vaginosis, aerobic vaginitis, candidiasis and coinfections in women in reproductive age.

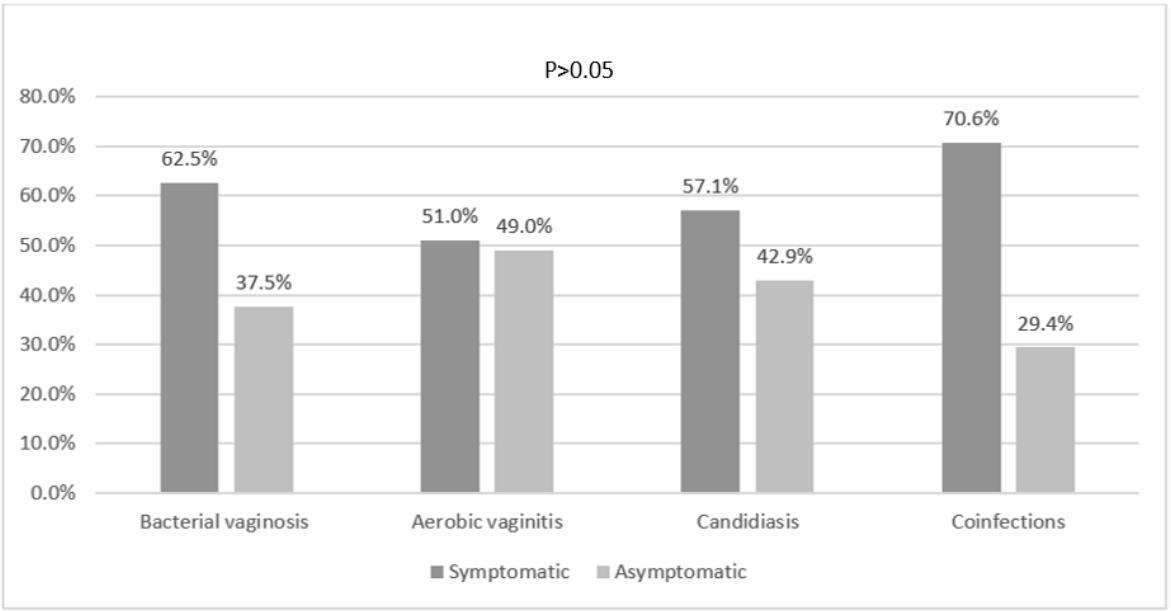

Figure 2 - Symptomatic and asymptomatic women in this study with candidiasis, bacterial vaginosis, aerobic vaginitis and coinfections.
Legend: $P > 0.05$ not statistically significant

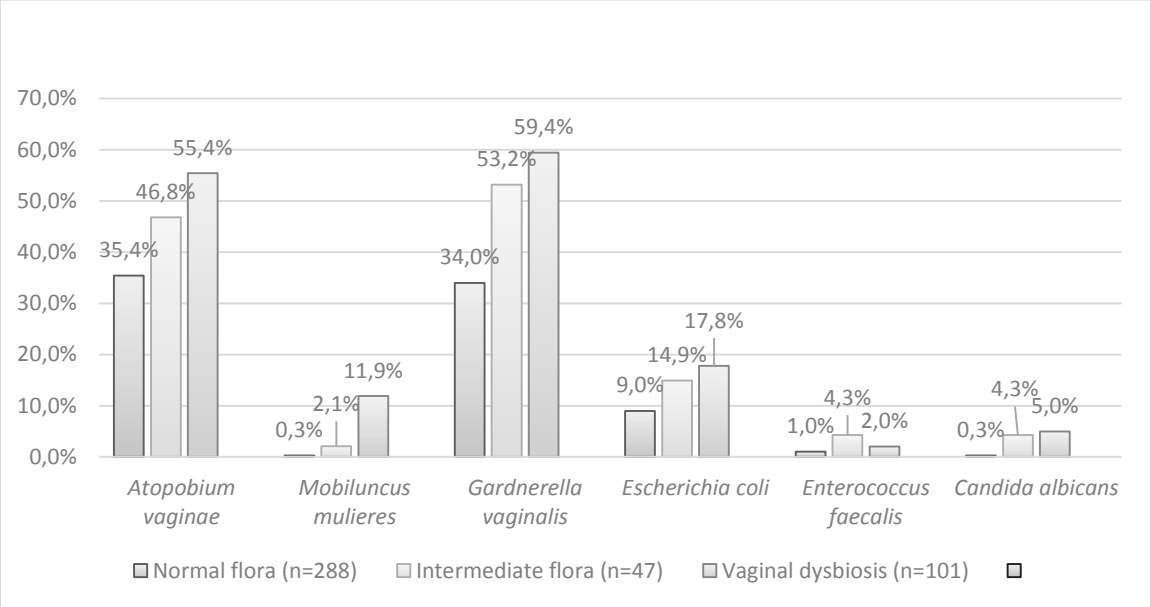

Figure 3 - Molecular detection of the main opportunistic and pathogenic species among women in this study with normal flora, intermediate vaginal and vaginal dysbiosis.
Legend: *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$