Bacterial identification of the vaginal microbiota in Ecuadorian pregnant teenagers: An exploratory analysis

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Background: Bacterial vaginosis (BV) is a microbial imbalance (i.e. dysbiosis) that can produce serious medical effects in women at childbearing age. Little is known, however, about the incidence of BV or vaginal microbiota dysbiosis in pregnant teenagers in low and middle-income countries such as Ecuador. The scope of this exploratory analysis was to study the relationship between epidemiologic and microbial risk factors. Among the microbiology risk factors this study investigated five *Lactobacillus* species, two of them know in preview studies as microbiology risk factors for BV development (*Lactobacillus acidophilus* and *Lactobacillus iners*), and the last three known for being associated with a healthy vaginal tract (*Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus jensenii*). In addition, fastidious anaerobes known to be microbial risk factors for BV development in pregnant teenagers were searched as well, more exactly, *Gardnerella vaginalis*, *Atopobium vaginae* and *Mobiluncus mulieris*. *Methods*: Ninety-five healthy adolescent pregnant women, visiting a secondary level hospital in Quito - Ecuador, were enrolled into the study in 2015. The enrolled patients were between 10 to 13 weeks of pregnancy. Four epidemiological risk factors were collected in a survey: age, civil status, sexual partners and condom use. Also, vaginal pH was measured as a health risk factor. DNA was extracted from endocervical and exocervical epithelia from all the patients' samples. PCR analysis was performed in order to characterize the presence of the eight bacterial species known as risk factors for BV development, targeting three anaerobes and five *Lactobacillus* species. Univariate and multivariate analysis were performed to identify associated factors for the presence of anaerobic species using logistic regression. *Results*: The 95 vaginal microflora samples of these teenagers were analyzed. Two of the bacterial species known to cause BV: *A. vaginae* (100%) and *G. vaginalis* (93.7%) were found in high prevalence. Moreover, the most predominant bacterial *Lactobacillus* species found in the pregnant teenagers' vaginal tract were *L. crispatus* (92.6%), *L. iners* (89.5%) and *L.*

acidophilus (87.4%). In addition, the average vaginal pH measured in the study population was 5.2, and high pH was associated with the presence of the three-anaerobic species (p = 0.001). Finally, *L. jensenii*'s presence in the study decreased in 72% the occupation of the three anaerobes. *Discussion*: This work identified a high pH as a risk factor for BV anaerobes' presence in adolescent pregnant women. Moreover, this study identified *L. crispatus, L. iners* and *L. acidophilus* to be the most abundant species in our study population. From all fastidious anaerobes analyzed in this study, *A. vaginae* was present in all pregnant teenagers. To conclude, *L. jensenii* could be a potential healthy vaginal microbiota candidate in pregnant teenagers and should be further analyzed in future studies.

1 **Bacterial Identification of the Vaginal Microbiota in Ecuadorian Pregnant Teenagers: An**

2 **Exploratory Analysis**

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13 **Abstract**

14 *Background*: Bacterial vaginosis (BV) is a microbial imbalance (i.e. dysbiosis) that can produce

15 serious medical effects in women at childbearing age. Little is known, however, about the

16 incidence of BV or vaginal microbiota dysbiosis in pregnant teenagers in low and middle-income

17 countries such as Ecuador. The scope of this exploratory analysis was to study the relationship

18 between epidemiologic and microbial risk factors. Among the microbiology risk factors this study

19 investigated five *Lactobacillus* species, two of them know in preview studies as microbiology

20 risk factors for BV development (*Lactobacillus acidophilus* and *Lactobacillus iners*), and the last

21 three known for being associated with a healthy vaginal tract (*Lactobacillus crispatus*,

22 *Lactobacillus gasseri* and *Lactobacillus jensenii*). In addition, fastidious anaerobes known to be

23 microbial risk factors for BV development in pregnant teenagers were searched as well, more

24 exactly, *Gardnerella vaginalis*, *Atopobium vaginae* and *Mobiluncus mulieris*.

25 *Methods*: Ninety-five healthy adolescent pregnant women, visiting a secondary level hospital in

26 Quito - Ecuador, were enrolled into the study in 2015. The enrolled patients were between 10 to

27 13 weeks of pregnancy. Four epidemiological risk factors were collected in a survey: age, civil

28 status, sexual partners and condom use. Also, vaginal pH was measured as a health risk factor.

29 DNA was extracted from endocervical and exocervical epithelia from all the patients' samples.

30 PCR analysis was performed in order to characterize the presence of the eight bacterial species

31 known as risk factors for BV development, targeting three anaerobes and five *Lactobacillus*

32 species. Univariate and multivariate analysis were performed to identify associated factors for the

33 presence of anaerobic species using logistic regression.

34 *Results*: The 95 vaginal microflora samples of these teenagers were analyzed. Two of the

35 bacterial species known to cause BV: *A. vaginae* (100%) and *G. vaginalis* (93.7%) were found in

36 high prevalence. Moreover, the most predominant bacterial *Lactobacillus* species found in the

37 pregnant teenagers' vaginal tract were *L. crispatus* (92.6%), *L. iners* (89.5%) and *L. acidophilus*

38 (87.4%). In addition, the average vaginal pH measured in the study population was 5.2, and high

39 pH was associated with the presence of the three-anaerobic species (p = 0.001). Finally, *L.*

40 *jensenii*'s presence in the study decreased in 72% the occupation of the three anaerobes.

41 *Discussion*:   This work identified a high pH as a risk factor for BV anaerobes' presence in

42 adolescent pregnant women. Moreover, this study identified *L. crispatus, L. iners* and *L.*

43 *acidophilus* to be the most abundant species in our study population.   From all fastidious

44 anaerobes analyzed in this study, *A. vaginae* was present in all pregnant teenagers. To conclude,

45 *L. jensenii* could be a potential healthy vaginal microbiota candidate in pregnant teenagers and

46 should be further analyzed in future studies.

Introduction

47

48   The bacterial microbiota of the vagina includes a diverse set of species (Ma, Forney & Ravel,

49   2012), consisting of a balance between anaerobic and aerobic microorganisms (Alvarez-Olmos et

50   al., 2004). Commensal microorganisms in the vagina (vaginal microbiome) provide protection

51   against opportunistic and pathogenic bacteria, constituting the first line of defense against

52   invasive microorganisms (Ma, Forney & Ravel, 2012; Romero et al., 2014).

53   Several species of *Lactobacillus* sp. are dominant members of the vaginal commensal barrier and

54   are usually associated with a healthy mucous membrane. Some of the species such as *L.*

55   *crispatus*, *L. gasseri*, *L. jensenii* among others contribute in lowering pH through the production

56   of great amounts of lactic acid and bactericide compounds (Wilks et al., 2004; Husain et al.,

57   2014), such as H_2O_2 and bacteriocins (Wilks et al., 2004; Kiss et al., 2007; Tamrakar et al., 2007;

58   Nelson et al., 2009; Machado et al., 2013; O'Hanlon, Moench & Cone, 2013; Romero et al.,

59   2014). All these compounds and properties allow vaginal epithelial homeostasis and also keeping

60   away possible pathogenic bacteria. However not all *Lactobacillus* species found in the vaginal

61   tract are strong probiotic species because of their low bactericide elements production such as *L.*

62   *acidophilus* (Wilks et al., 2004), *L. iners* (Tamrakar et al., 2007) and others.

63   The vaginal microbiota of healthy women, during their reproductive age, is typically stable

64   (Romero et al., 2014). When a woman is pregnant, however, hormone levels fluctuate, causing

65   changes in the vaginal microbiota (Huang et al., 2014; Romero et al., 2014; MacIntyre et al.,

66   2015). This fluctuation can be even more dramatic in teenagers, who may be at an increased risk

67   for sexually transmitted diseases (STDs) and bacterial vaginosis (BV) (Alvarez-Olmos et al.,

68   2004). In Ecuador, teenagers have a wide range of health care needs, in particular related to

69 sexual and reproductive health, where the high rates of adolescent pregnancy and its health

70 control are a national concern (Goicolea, 2010; Loaiza & Liang, 2013; Svanemyr et al., 2017).

71 BV is a condition in which the vaginal microbiota suffers a shift associated with a reduction in

72 several species of probiotic *Lactobacillus* and an increase in the presence of anaerobes (*G.*

73 *vaginalis*, *A. vaginae* and *Mobiluncus* sp.). BV also causes an individual to be more susceptible

74 to STDs or suffer pre-term labor and late miscarriage (Wilks et al., 2004; Tamrakar et al., 2007;

75 Nelson et al., 2009; Romero et al., 2014; McMillan et al., 2015).

76 The vaginal microbiota in pregnant teenagers has been characterized in certain studies, however,

77 these studies are often conducted in high-income countries where hygiene conditions differ from

78 low-income countries (Kiss et al., 2007; Nelson et al., 2009; Verstraelen et al., 2009; Aagaard et

79 al., 2012; Huang et al., 2014; Husain et al., 2014; Hyman et al., 2014; Romero et al., 2014;

80 MacIntyre et al., 2015). There are few studies that have been conducted in low- and middle-

81 income countries (LMICs) such as Ecuador (Vaca et al., 2010) or Brazil (Ferreira et al., 2015),

82 yet those analyses relied only on Nugent scores (gram stained smears). This study was therefore

83 conducted to assess the presence of five *Lactobacillus* sp. (*L. crispatus*, *L. gasseri*, *L. jensenii*, *L.*

84 *acidophilus* and *L. iners*) and three bacterial anaerobes (*A. vaginae*, *G. vaginalis* and *M. mulieris*)

85 associated with BV in pregnant teenagers from Ecuador using PCR amplification of 16S and 23S

86 rRNA genes.

87 **Materials and Methods**

88 **Study area, design and subject selection**

89   The study was carried out in a secondary level public hospital in Quito - Ecuador that serves as a

90   national reference hospital and teaching hospital, which provides care to pregnant women,

91   including family planning and postpartum support services (Moya, 2001). The hospital also

92   provides services for pregnant teenagers (most of them from Hispanic ethnicity), and in 2014,

93   2,230 pregnant teens received assistance (Ministerio de Salud Pública el Ecuador, 2014).

94   From September 2015 to November 2015, 95 pregnant teenage women (10-19 years old) were

95   enrolled into the study. The study population involved teenagers between 10 to 13 weeks

96   pregnant and reported to have not taken antimicrobials for the previous 3 months. The enrolled

97   patients were interviewed in a private room and survey included demographic and health

98   questions, including: age, marital status, number of sexual partners in the last year and the use of

99   preservatives (condoms) during intercourse. Applicants were excluded from the study if they

100   reported to have had sexual intercourse within the last 48 hours, or in case there was any

101   evidence of macroscopic cervical bleeding or placenta and fetal disease. This investigation

102   adopted a cross-sectional study design to determine the association between the presence of

103   *Lactobacillus* sp. and BV related anaerobes.

104   **Ethics statement**

105   Ninety-five volunteers met the eligibility criteria and read and signed the informed consent (if 18

106   or 19 years old) or had their parents or legal representative sign if they were less of 18 years old.

107   The Ethics Committee of the Central University of Ecuador approved this study (Protocol code:

108   cif-cv-fcm.1).

109   **Samples collection**

110   Vaginal samples were collected by trained gynecologists. The procedure began by removing the

111   cervical mucus with a sterile swab. Furthermore, endocervical and exocervical epithelia were

112   gathered with a cervix examination brush (Rovers Medical Devices B.V.; Rovers® Cervex-

113   Brush®). Additionally, the pH of the mucus samples was determined from the vaginal swab using

114   a pH test strip (MColorpHast, Merck-Millipore). The detachable head of the cervix examination

115   brush was preserved in a liquid vial (BD, BD SurePath™ liquid-based Pap test) and transported

116   under refrigeration conditions (4°C). The samples were stored at -40°C for 5h until DNA

117   extraction.

118   **DNA extraction**

119   DNA from the vaginal brush was extracted by following commercial kit instructions

120   (NucleoSpin® Tissue 740952, Macherey-Nagel™, Düren-Germany) (Macherey-Nagel, 2014).

121   Briefly, the brush samples were placed in 1.5ml microtubes with 180µl Buffer T1 and 25µl

122   Proteinase K. Then, to homogenate the mixture, the microtubes were centrifuged for 15s at 1,500

123   x g and heated at room temperature for 5min. Next, the tubes were vortexed vigorously for 15s

124   and span for 15s at 1,500 x g. The microtubes were then incubated for 10 minutes at 70°C, and

125   followed by 5 minutes at 95°C. The tubes were centrifuged again for 15s at 1,500 x g, after which

126   200µl of Buffer B3 was added. Tubes were vortexed vigorously for 30s and heated at 70°C for 10

127   minutes. Ethanol (96-100%) was added to each sample and vigorously vortexed once more. A

128   Nucleospin®Tissue Column was placed into a collection tube and the sample was added to the

129   column. The tubes were then centrifuged for 1min at 11,000 x g and discarded and new ones were

130   placed under the column. Kit buffers (BW and B5) were added to the columns and centrifuged

131   for 1min at 11,000 x g for each buffer. The columns were dried with another centrifugation and

132   finally DNA was eluted with buffer (BE) (Macherey-Nagel, 2014). The concentration of the

133   extracted DNA was measured in a NANOVUE spectrophotometer (GE Healthcare Life Sciences)

134   to determine the DNA extraction quality. The original DNA extraction was then divided into two

135   diluted aliquots with Buffer BE to a final concentration of 20ng/µL. Finally, the remaining

136 original samples were preserved at -80°C, as well as the two aliquots of 20ng/µL, were preserved

137 at -20°C for Polymerase Chain Reaction (PCR) analysis.

138 **Polymerase Chain Reaction**

139 PCR amplification was performed for nine different primer sets, targeting three anaerobes and

140 five *Lactobacillus* species and one primer set for *Lactobacillus* genus of vaginal microbiota (see

141 Table 1). Samples of *L. jensenii* and *A. vaginae* were sequenced to confirm their identity due to

142 the lack of a strong specificity, while *L. acidophilus*, *G. vaginalis* and *M. mulieris* primer sets

143 showed a strong specificity and thus samples did not require a further sequencing confirmation.

144 The eventual confirmation step used the following universal primers for 16S rRNA sequencing

145 (27Fw-AGA GTT TGA TCM TGG CTC AG and 805Rw-GAC TAC CAG GGT ATC TAA TC;

146 Temperature of annealing: 62 ºC; Tanner et al., 1999) through a PCR assay carried out with a

147 final volume of 50-µl (adapted from the procedure below) and sent to Functional Biosciences, Inc

148 (Madison, WI, USA). The 16S rDNA sequences were compared to known sequences in GenBank

149 with the advanced gapped BLAST (basic local alignment search tool) algorithm (Camacho et al.,

150 2009).

151 The PCR assays were performed with T100™ thermal cycler (Bio-Rad Laboratories, USA) in a

152 reaction volume of 20µL. The reaction mix included 4µL 5X Green GoTaq® Flexi Buffer

153 (Promega, USA), 2µL $MgCl_2$ 2.5mM (Promega, USA), 0.50µL dNTP mix 10mM (Promega,

154 USA), 0.20µL GoTaq® Flexi DNA Polymerase (Promega, USA), 1.00µL of each primer 0,5µM,

155 2 µL from each DNA sample and the remaining volume with molecular grade H_2O.

156 PCR amplification first cycle consisted in a pre-melt phase at 94°C for 2 min and then

157 denaturation at 94°C for 30 seconds. After that, annealing at each species temperature (see Table

158   1) was realized for 30 seconds, and extension was performed at 72°C for 1 minute. This was

159   repeated for 29 more cycles to 31 for the bigger amplicons (>500 bp). An additional 5 min

160   extension was included at the end of the cycles to complete the extension of the primers. The

161   PCR products were visualized using electrophoresis in 2% agarose gels and staining with

162   ethidium bromide 0.1%, with negative and positive controls provided by the Microbiology

163   Institute at Universidad San Francisco de Quito.

164   **Statistical analysis**

165   Uni and multivariate analysis were realized in order to identify risk factors such as: age; civil

166   status; number of sexual partners; condom use; vaginal pH; and *Lactobacillus* spp. associated

167   with the presence or absence of anaerobic species (i.e. outcome) using logistic regression. Most

168   variables were treated as categorical. For testing differences in the studied factors and vaginal

169   microbiota between the groups of patients, the study was defined by the presence of three

170   anaerobic species (*A. vaginae*, *G. vaginalis* and *M. mulieris*). Chi-square statistics were computed

171   and logistic regression was used to estimate odds ratios for association between studied factors on

172   the presence of three anaerobic species (*A. vaginae*, *G. vaginalis* and *M. mulieris*). Statistically

173   significant differences were assumed when P-values were equal or less than 0.05. Statistical

174   analyses were performed using STATA version 14.0.

175   **Table 1**: Primers used in this study.

176   **Results**

177   **Characteristics of study population**

178   Most of the study participants were between 16-17 years old (53.7%) and non-married (68.4%).

179   Approximately two thirds had only one sexual partner (76.8%) and around half did not use

180 condoms (52.6%). In addition, all enrolled participants were from Hispanic ethnicity and nearly

181 one third of the study population had a vaginal pH higher than five (31.5%). The pH mean value

182 found in this study group was of 5.2 with a standard deviation (SD) of 0.7 and an interval of 4-7,

183 Table 2 provides descriptive statistics of the study population.

184 **Table 2**: Epidemiological characteristics of pregnant teenagers in this study.

185 **Commensal microbiota**

186 All samples showed the presence of *Lactobacillus* spp. The most frequently detected were *L.*

187 *crispatus* 88/95 (92.6%), *L. iners* 85/95 (89.5%) and *L. acidophilus* 83/95 (87.4%). While *L.*

188 *jensenii* and *L. gasseri* were only found in 77/95 (81.1%) and 54/95 (56.8%) of the studied

189 pregnant teenagers, respectively.

190 All study participants were colonized by at least two of the five *Lactobacillus* species, which

191 were analyzed. The most frequent combination was *L. crispatus* and *L. iners* in 82.1%. *L.*

192 *crispatus* and *L. acidophilus*, were the second most prevalent combination with 80.0%,

193 respectively. Other types of combinations were less frequent: *L. crispatus and L. jensenii*

194 (74.7%); *L. crispatus* and *L. gasseri* (55.8%); and *L. crispatus*, *L. gasseri* and *L. jensenii* (47.4%),

195 as shown in Figure 1.

196 **Figure 1:** Percentage of grouped *Lactobacillus* species found between the samples.

197 **BV associated anaerobes**

198   The main BV associated anaerobic bacteria found in our study population was *A. vaginae* (found

199   in 100% of the samples) followed by *G. vaginalis* and *M. mulieris* (in 93.7% and 34.7% of the

200   samples, respectively).

201   When we looked at the presence of anaerobic bacteria in combination with the presence of

202   *Lactobacillus* species, less than one-fifth of the samples had five *Lactobacillus* spp. and the three-

203   anaerobic species (17.5%). Nevertheless, as showed in Figure 2, no statistical significance was

204   found for the association between the number of *Lactobacillus* species and the presence of three

205   anaerobic species (P-value for Fisher's exact test > 0.05).

206   **Figure 2:** Percentage of teenagers with and without the presence of the three BV-associated

207   anaerobes by the number of *Lactobacillus* species pre-existent in the vaginal epithelium.

208   **Factors associated with presence of *Gardnerella vaginalis*, *Atopobium vaginae* and**

209   ***Mobiluncus mulieris***

210   In univariate analysis, higher pH was statistically associated with the presence of three anaerobes.

211   Having a pH higher than 5 increased the likelihood that three anaerobes would be present in the

212   samples (crude OR 4.7, 95% CI: 1.82-12.3), as shown in Table 3. In contrast, the presence of *L.*

213   *jensenii* in vaginal microbiota was associated with a decreased presence of three-anaerobic

214   species (crude OR 0.28, 95% CI: 0.1-0.81), as shown in Table 4.

215   **Table 3**: Factors associated with the presence or absence of three anaerobic bacterial species

216   (*Gardnerella vaginalis*, *Atopobium vaginae* and *Mobiluncus mulieris*) that are known to be

217   associated with bacterial vaginosis.

218 **Table 4**: Association of *Lactobacillus* spp. with the presence or absence of three anaerobic

219 bacterial species (*Gardnerella vaginalis*, *Atopobium vaginae* and *Mobiluncus mulieris*) that are

220 known to be associated with bacterial vaginosis.

221 When the associations (Table 3) were adjusted for age, condom use and number of sexual

222 partners, the only significant factor associated with presence of three anaerobes was a high pH

223 (adjusted OR=5.5; 95%CI: 2-15.2, P-value: 0.002). After adjusting for the same variables in

224 Table 4, the presence of *L. jensenii* remained to be associated with the absence of three anaerobes

225 (adjusted OR 0.16; 95% CI: 0.04-0.6, P-value: 0.006).

226 **Discussion**

227 In the current study, we found that the genital tract of pregnant Ecuadorian teenagers was

228 colonized primarily by *L. crispatus*. All samples from the studied teenagers were positive for at

229 least one analyzed *Lactobacillus* species. Additionally, from the five-species analyzed in this

230 study, colonization by the consortium formed through *L. crispatus*, *L. iners* and *L. acidophilus*

231 was the most prevalent. These results coincide with previous reports indicating that *Lactobacillus*

232 species is the most abundant commensal bacteria found in healthy vaginal microbiota of pregnant

233 and non-pregnant adult women in the world, as shown in Table 5 (Kiss et al., 2007; Tamrakar et

234 al., 2007; Verstraelen et al., 2009; Dominguez-Bello et al., 2010; Hernández-Rodríguez et al.,

235 2011; Aagaard et al., 2012; Husain et al., 2014; Hyman et al., 2014; Romero et al., 2014;

236 MacIntyre et al., 2015; McMillan et al., 2015). However, it is important to mention that other

237 *Lactobacillus* species could have been present on the vaginal epithelium of these pregnant

238 teenagers because this study did not analyze every *Lactobacillus* species but only the most

239   frequently cited in other studies (see Table 5). We found a large proportion of individuals

240   colonized by BV associated anaerobic bacteria, including: *A. vaginae* (100%), and *G. vaginalis*

241   (93.7%), which showed some discrepancies with previous studies carried out in adult women

242   (Table 5). The percentage of *A. vaginae* in this study was considerably different from other

243   published studies due to the presence of *A. vaginae* in 100% of the analyzed pregnant teenagers

244   in Ecuador. In agreement with the previous results, *G. vaginalis* was also found in more than 93%

245   of the pregnant teenagers. As referenced by previous studies, *G. vaginalis* and *A. vaginae* are the

246   main anaerobes associated with BV (Menard et al., 2008; Verstraelen & Swidsinski, 2013;

247   Bretelle et al., 2015; Machado & Cerca, 2015; Hardy et al., 2016).

248   **Table 5**: Summary of vaginal microbiota characterization studies in pregnant women (including

249   this study).

250

251

252   The association between having a certain commensal vaginal microbiota with a healthy

253   pregnancy outcome has been thoroughly studied in the past two decades (Kiss et al., 2007;

254   Nelson et al., 2009; Verstraelen & Swidsinski, 2013). It has been postulated that an optimal

255   commensal microbiota in the vaginal epithelium reduces the risk of infections with any pathogen

256   (*Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Candida* sp., and

257   others) in the reproductive track and also seems to prevent preterm birth caused by bacterial

258   vaginosis (O'Hanlon, Moench & Cone, 2013; Krauss-Silva et al., 2014). Several studies around

259   the world (see Table 5), such as in USA (Hyman et al., 2014; Romero et al., 2014), United

260   Kingdom (MacIntyre et al., 2015), Belgium (Verstraelen et al., 2009), China (Huang et al., 2014)

261   and Japan (Tamrakar et al., 2007), showed that the most abundant *Lactobacillus* species found in

262   pregnant women are *L. iners*, and *L. crispatus*. In agreement to this study, these two species were

263   the most abundant lactobacilli found in pregnant teenagers in Ecuador. The third most abundant

264   *Lactobacillus* species found in this study was *L. acidophilus*, which has only been observed in

265   studies from Mexico (Hernández-Rodríguez et al., 2011) and USA (Hyman et al., 2014). In USA,

266   it has been encountered in non-pregnant teenager's vaginal microbiota with a prevalence of 49%

267   (Alvarez-Olmos et al., 2004). *L. acidophilus* has not been analyzed or encountered in other

268   studies worldwide as shown in Table 5.

269

270   Other *Lactobacillus* species, such as *L. jensenii* and *L. gasseri*, are usually found in high

271   percentages in other studies of vaginal microbiota present in pregnant women (Tamrakar et al.,

272   2007; Verstraelen et al., 2009; Husain et al., 2014; Hyman et al., 2014; MacIntyre et al., 2015).

273   However, *Lactobacillus* species, such as *L. delbruecki, L. rhamosus, L. reuteri, L. casei. L.*

274   *paracasei, L. vaginalis, L. coleohominis, L. fermentum, L. fornicalis, L. gallinarum, L. helveticus,*

275   *L. kefiranofaciens, L. kitasatonis, and L. ultunensis,* have been analyzed as well but are not

276   commonly found in studies of pregnant women's vaginal microbiota (Hyman et al., 2014).

277   Nonetheless, *L. jhonsonii* was detected by 16S rRNA pyrosequencing characterization of vaginal

278   microbiota in pregnant women in USA (Aagaard et al., 2012), although this species was not

279   analyzed in the current study. Therefore, a more extensive analysis may be necessary in the

280   future.

281   *Atopobium vaginae* was the most frequent anaerobe observed in vaginal microbiota from

282   pregnant teenagers in our study (Table 5), and it has been detected in USA (Hyman et al., 2014;

283   Romero et al., 2014), Mexico (Hernández-Rodríguez et al., 2011), China (Huang et al., 2014),

284   Europe (Bretelle et al., 2015; MacIntyre et al., 2015) and Africa (McMillan et al., 2015). This

285   anaerobe is usually found in association with *G. vaginalis* and *M. mulieris* (Hernández-Rodríguez

286   et al., 2011) in the development of BV (Verstraelen & Swidsinski, 2013). As similar as *G.*

287   *vaginalis*, *A. vaginae* is usually found in minor percentages around the world (Nelson et al., 2009;

288   Dominguez-Bello et al., 2010; Huang et al., 2014; Romero et al., 2014; MacIntyre et al., 2015).

289   The percentage of *A. vaginae* in this study was markedly different from other published studies.

290   In our study, *A. vaginae* was present in 100% of the analyzed pregnant teenagers in Ecuador. In

291   agreement with previous results, *G. vaginalis* was also found in more than 93% of pregnant

292   teenagers. Although this study did not quantify the abundance of *G. vaginalis* or *A. vaginae*, the

293   prevalence of these anaerobes is not concordant with the normal prevalence in healthy pregnant

294   women in other studies (Nelson et al., 2009; Dominguez-Bello et al., 2010; Huang et al., 2014;

295   Romero et al., 2014), such as Africa where 66.7% of healthy pregnant women had *G. vaginalis*

296   (Nelson et al., 2009) or the USA where 2.2% of healthy pregnant women had *G. vaginalis* or *A.*

297   *vaginae* (Romero et al., 2014), which is low in comparison with the results from this study.

298   Finally, only 34.5% of the pregnant teenagers were positive for *M. mulieris,* which is high in

299   comparison to other studies performed in pregnant women such as 2.0% in Mexico (Hernández-

300   Rodríguez et al., 2011), 0.37% in Rwanda (Tchelougou et al., 2013) and 18.2% in USA (Waters et

301   al., 2008).

302   The major drawback of this study was the lack of quantitative data which may allow us to assess

303   the status of the colonization of the distinct bacterial taxa. However, BV is a very common cause

304   for high pH in the vagina, associated with the shifting of microbiota from protective

305   *Lactobacillus* sp. to anaerobes, such as *G. vaginalis* and *A. vaginae* (De Backer et al., 2007).

306   When this occurs, the pH rises from 4.2 that is considered normal to 4.5 or higher, which is now

307   used as a positive indicator for BV (O'Hanlon, Moench & Cone, 2013). The pH in pregnant

308   women has been analyzed in several studies, such as China (Huang et al., 2014) and Rwanda

309   (McMillan et al., 2015) where the pH media was 4.5 and 4.6, respectively. In this study, however,

310   the observed pH was 5.2, which is a marker for the shift in dominance from *Lactobacillus* to

311   anaerobic microbiota on the vaginal epithelium. The presence of the three anaerobes analyzed in

312   this study appeared to increase the vaginal pH, which is in agreement with other studies

313   (McMillan et al., 2015; Donders et al., 2016) that evaluated the establishment of BV in women.

314   This exploratory study was able to characterize the heterogeneity of the commensal

315   *Lactobacillus* species in these pregnant teenagers, where all study participants had more than one

316   *Lactobacillus* species colonizing the vagina. As shown in Figure 2, the reproductive track of our

317   study participants revealed two to five *Lactobacillus* species. Even though there is no statistical

318   significance with the absence or presence of the three anaerobes, our results differed from what

319   was published in other studies (Hernández-Rodríguez et al., 2011; Hyman et al., 2014). In

320   Mexico, it has been reported that the vagina was colonized by one to four species (Hernández-

321   Rodríguez et al., 2011), meanwhile in the USA by one to two species (Hyman et al., 2014). *L.*

322   *crispatus*, *L. gasseri* and *L. jensenii* (Wilks et al., 2004; Husain et al., 2014) are known as strong

323   beneficial species, however *L. acidophilus* (Wilks et al., 2004), and *L. iners* (Tamrakar et al.,

324   2007) are known as weaker protective species. Therefore, the high prevalence of these two

325   species in the vaginal microbiota could partially explain the high prevalence of *A. vaginae* and *G.*

326   *vaginalis* in Ecuadorian pregnant teenagers. Even without a statistical difference, the high

327   prevalence of *L. iners* and *L. acidophilus* could imply some sort of bewilderment in the

328   commensal bacteria of the vaginal microbiota, which could allow the proliferation of anaerobic

329   bacteria. Further study is necessary to quantify each *Lactobacillus* species and anaerobic bacteria

330   in each sample in order to determine the exact load of the detected vaginal microbiota in pregnant

331   teenagers. Also, the amount of the beneficial substances produced by *Lactobacillus* species found

332   in Ecuadorian women, such as H_2O_2 or lactic acid, should be quantified to determine their

333   protective ability.

334   Finally, this study limitations were principally caused by the low number of samples, and the lack

335   of bacterial abundance detection.

336   **Conclusion**

337   Our study identified *L. crispatus, L. iners* and *L. acidophilus* to be the most abundant species in

338   our study population of pregnant teenagers. *L. jensenii* could be a potential microbiota protective

339   candidate in the vaginal microbiota in pregnant Ecuadorian teenagers. Meanwhile, *A. vaginae*, *G.*

340   *vaginalis* and *M. mulieris* were found in 100%, 93.7% and 34.7% of the analyzed pregnant

341   teenagers, respectively.

342   To the authors' knowledge, this is the first study of vaginal microbiota in pregnant teenagers in

343   Ecuador. Furthermore, this investigation was only realized in pregnant teenagers and there was no

344   control group (non-pregnant teenagers). Further studies are necessary in pregnant women to

345   quantify the main *Lactobacillus* species and also the BV associated anaerobes identified in this

346   pilot study, as well as, to statistical determine a possible correlation with preterm birth or BV in

347   Ecuadorian pregnant women, which it was not possible to characterize in this exploratory study.

348

349   **List of abbreviations**

350   BV: Bacterial vaginosis

351   STDs: Sexually Transmitted Diseases

352   LMICs: Low and Middle-Income Countries

353 **Availability of data and material**

354 See additional database in supplementary files (Additional file 1)

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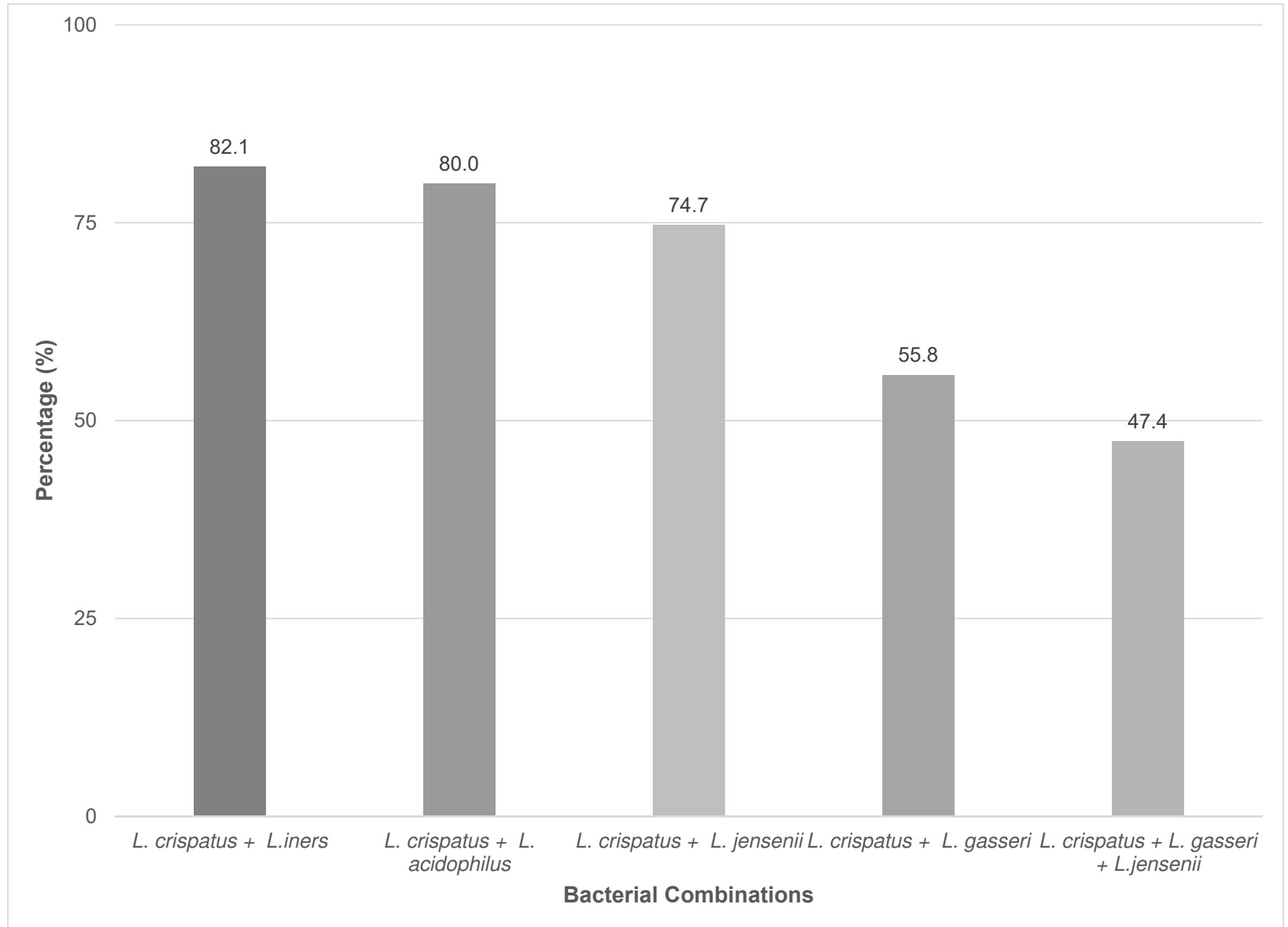
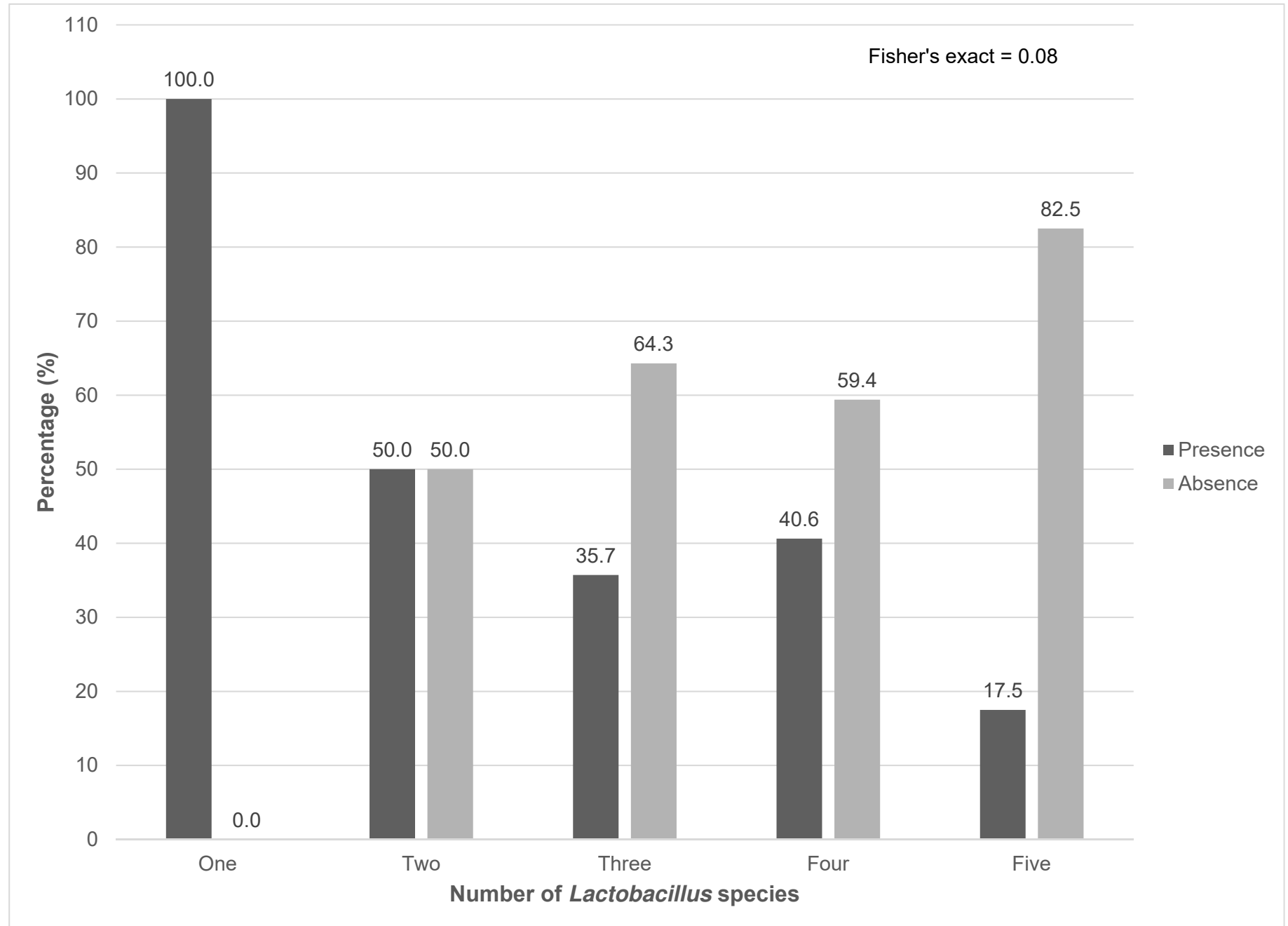
Percentage of grouped *Lactobacillus* species found between the samples.

Percentage of teenagers with and without the presence of the three BV-associated anaerobes by the number of *Lactobacillus* species pre-existent in the vaginal epithelium.

Presence or absence of three anaerobic species by number of *Lactobacillus* species.

Primers used in this study.

1   **Table 1**: Primers used in this study

Set	Name	Sequence (5′-3′)	Target	T (°C) of annealing	Size of fragment	Target	Specificity %	Cross reaction with microorganisms	Validation	Reference
1	LactoF	TGGAAACAGRTGCTAATACCG	*Lactobacillus* spp.	55 °C	233 bp	16S rRNA	100%	Negative	Samples sequenced to confirm identity	(Byun et al., 2004; Zhang et al., 2012)
	LactoR	GTCCATTGTGGAAGATTCCC								
2	LinersF	GTCTGCCTTGAAGATCGG	*L. iners*	55 °C	158 bp	16S rRNA	100%	Negative	Samples sequenced to confirm identity	(De Backer et al., 2007; Zhang et al., 2012)
	LinersR	ACAGTTGATAGGCATCATC								
3	LcrispatusF	TTACTTCGGTAATGACGTTA	*L. crispatus*	55 °C	966 bp	16S rRNA	100%	Negative	Samples sequenced to confirm identity	(De Backer et al., 2007; Zhang et al., 2012)
	LcrispatusR	GGAACTTTGTATCTCTACAA								
4	LgasseriF	TCGAGCGAGCTTGCCTAGATGAA	*L. gasseri*	60 °C	372 bp	16S rRNA	100%	n/d	Samples sequenced to confirm identity	(Zhang et al., 2012)
	LgasseriR	CGCGGCGTTGCTCCATCAGA								
5	LjenseniiF	AGTCGAGCGAGCTTGCCTATAGAAG	*L. jensenii*	57 °C	342 bp	16S rRNA	100%	Negative	Samples sequenced to confirm identity in this study	(Garg et al., 2009; Zhang et al., 2012)
	LactoR	GTCCATTGTGGAAGATTCCC								
6	LA-1 Fw	TCAATCAAAGGAAGACGCAG	*L. acidophilus*	56 °C	221 bp	23S rRNA	100%	Negative	n/d	(Tsai et al., 2010)
	LA-2 Rw	CGCTCGCAATTTCGCTTA								
7	Gard154-Fw	CTCTTGGAAACGGGTGGTAA	*Gardnerella vaginalis*	60 °C	301 bp	16S rRNA	100%	Negative	n/d	(Henriques et al., 2012)
	Gard154-Rv	TTGCTCCCAATCAAAAGCGGT								
8	Mobil-577F	GCTCGTAGGTGGTTCGTCGC	*Mobiluncus mulieris*	62 °C	449 bp	16S rRNA	100%	Negative	n/d	(Fredricks et al., 2007)
	M.mulie-1026R	CCACACCATCTCTGGCATG								
9	Atop109-Fw	GAGTAACACGTGGGCAACCT	*Atopobium vaginae*	62 °C	221 bp	16S rRNA	16.7%	5 from 6	Samples sequenced to confirm identity in this study	(Henriques et al., 2012)
	Atop109-Rv	CCGTGTCTCAGTCCCAATCT						37.5%	15 from 24		

2   **N/d – non determined**

Epidemiological characteristics of pregnant teenagers in this study.

1     **Table 2**: Epidemiological characteristics of pregnant teenagers in this study.

Epidemiological Data	N°	%
Age		
12 to 15 years	18	19.0
16 to 17 years	51	53.7
18 to 19 years	26	27.4
Civil status		
Single	65	68.4
Free union	29	30.5
Married	1	1.1
Sexual partners		
One	73	76.8
More than one	22	23.2
Condom use		
No	50	52.6
Sometimes (Occasionally, yes)	45	47.4
Vaginal pH		
4 to 5	63	68.5
6 to 7	29	31.5

Factors associated with the presence or absence of three anaerobic bacterial species (*Gardnerella vaginalis*, *Atopobium vaginae* and *Mobiluncus mulieris*) that are known to be associated with bacterial vaginosis.

1   **Table 3**: Factors associated with the presence or absence of three anaerobic bacterial species

2   (*Gardnerella vaginalis*, *Atopobium vaginae* and *Mobiluncus mulieris*) that are known to be

3   associated with bacterial vaginosis

	Absent	Present	P-value	OR	P-value
Age					
12 to 15 years	10 (15.4)	8 (26.7)		1	
16 to 17 years	34 (52.3)	17 (56.7)		0.62 (0.21-1.87)	0.400
18 to 19 years	21 (32.3)	5 (16.7)	0.21	0.3 (0.08-1.14)	0.078
Civil status					
Single	44 (67.7)	21 (70)		1	
Free union	20 (30.8)	9 (30)		0.94 (0.37- 2.42)	0.900
Married	1 (1.5)	0 (0.0)	0.99	-	
Condom use					
No	33 (50.8)	17 (56.7)		1	
Sometimes (occasionally, yes)	32 (49.2)	13 (43.3)	0.59	0.79 (0.33-1.88)	0.593
Sexual partners					
One	48 (73.9)	25 (83.3)		1	
More than one	17 (26.1)	5 (16.7)	0.31	0.56 (0.19-1.71)	0.312
pH					
4 to 5	50 (79.4)	13 (44.8)		1	
6 to 7	13 (20.6)	16 (55.2)	0.001	4.7 (1.82-12.3)	**0.001**

Association of *Lactobacillus* spp. with the presence or absence of three anaerobic bacterial species (*Gardnerella vaginalis*, *Atopobium vaginae* and *Mobiluncus mulieris*) that are known to be associated with bacterial vaginosis.

1   **Table 4**: Association of *Lactobacillus* spp. with the presence or absence of three anaerobic

2   bacterial species (*Gardnerella vaginalis*, *Atopobium vaginae* and *Mobiluncus mulieris*) that are

3   known to be associated with bacterial vaginosis.

	Absent	Present	P-value	OR	P-value
Bacterial species					
Lactobacillus **sp.**					
Absence	(0.0)	0 (0.0)		1	
Presence	65 (100.0)	30 (100.0)		does not apply	
Lactobacillus iners					
Absence	6 (9.2)	4 (13.3)		1	
Presence	59 (90.8)	26 (86.7)	0.54	0.66 (0.17-2.5)	0.547
Lactobacillus crispatus					
Absence	4 (6.1)	3 (10)		1	
Presence	61 (93.9)	27 (90)	0.51	0.59 (0.12-2.8)	0.509
Lactobacillus gasseri					
Absence	25 (38.5)	16 (53.3)		1	
Presence	40 (61.5)	14 (46.7)	0.17	0.55 (0.23-1.3)	0.176
Lactobacillus jensenii					
Absence	8 (12.3)	10 (33.3)		1	
Presence	57 (87.7)	20 (66.6)	0.01	0.28 (0.1-0.81)	**0.02**
Lactobacillus acidophilus					
Absence	6 (9.2)	6 (20)		1	
Presence	59 (90.8)	24 (80)	0.14	0.41 (0.12-1.38)	0.151

7   women/state; Nd - Not detected.

Summary of vaginal microbiota characterization studies in pregnant women (including this study).

Table 5: Summary of vaginal microbiota characterization studies in pregnant women (including this study)

N°	Population Description	Study Group (n)	Place	Methodology	pH (mean)	Bacterial species detected (%)										Author
						Lactobacillus sp.	L. iners	L. crispatus	L. acidophilus	L. jensenii	L. gasseri	G. vaginalis	A. vaginae	M. mulieris	Other Lactobacillus sp.	
1	Pregnant women (Age range 18-35)	126	Austria	Multiplex PCR by Culture	Nd	57.1	Nd	19.8	Nd	11.9	Nd	Nd	Nd	Nd	YES	(Kiss et al., 2007)
2	Pregnalen women (Age range Nd)	100	Belgium	tRFLP - PCR	Nd	X	40.3	23.4	Nd	3.9	40.3	Nd	Nd	Nd	NO	(Verstraelen et al., 2009)
3	Pregnant and non-pregnant women (Age range 19.4-39.2)	34	China	PCR and 16s region sequencing	4.5	X	55	26.9	Nd	Nd	6.3	11.8	11.8	Nd	NO	(Huang et al., 2014)
4	Pregnant teenagers (Age range 12-19)	95	Ecuador	PCR Electrophoresis gel	5.2	100	89.5	92.6	87,4	48.4	56.8	93.7	100	34.7	NO	This study (2016)
5	Pregnar women (Age range 19-44)	132	Japan	PCR Culture	Nd	98.5	41.7	51.5	Nd	25	31.8	Nd	Nd	Nd	NO	(Tamrakar et al., 2007)
6	Pregnant women (Age range 13-43)	140	Mexico	PCR (DGGE)	Nd	98.4	56.7	Nd	78,1	Nd	20.3	Nd	Nd	2.0	YES	(Hernández-Rodríguez et al., 2011)
7	Pregnant and non-pregnant women (Age range 18-55)	131	Rwanda	16s region pyrosequencing	4.6	X	X	X	Nd	X	X	X	X	Nd	NO	(McMillan et al., 2015)
8	Pregnant women (Age range Nd)	42	United Kingdom	16s region pyrosequencing	Nd	X	30*	43*	Nd	14*	9*	Nd	2*	Nd	YES	(MacIntyre et al., 2015)
9	Pregnant women (Age range 28-31)	293	United Kingdom	Culture MALDI-TOF	Nd	75	Nd	37	Nd	45	34	Nd	Nd	Nd	YES	(Husain et al., 2014)
10	Pregnant and non-pregnant women (Age range Nd)	54	USA	16s region pyrosequencing	Nd	X	58.5*	38.1*	Nd	X	4.3*	2.2*	2.2*	Nd	YES	(Romero et al., 2014)
11	Pregnant and non-pregnant women (Age mean 31,4)	84	USA	16s region pyrosequencing	Nd	X	X	X	Nd	X	Nd	Nd	Nd	Nd	YES	(Aagaard et al., 2012)
12	Pregnant women (Age ≥18)	88	USA	PCR Sanger sequencing	Nd	X	76.9 African 21.7 Asian 23.1 Caucasian 68.4 Hispanic	30.8 African 41.5 Caucasian 15.8 Hispanic	21,7 Asian 1,5 Caucasian	7.7 African 27.7 Caucasian 31.6 Hispanic	7.7 African 17.4 Asian 41.5 Caucasian 10.5 Hispanic	Nd	X	Nd	YES	(Hyman et al., 2014)
13	Pregnant women (Age mean 24)	50	USA	qPCR	Nd	Nd	Nd	X	Nd	Nd	Nd	66.7	Nd	Nd	NO	(Nelson et al., 2009)
14	Pregnant women (Age range 21-33)	9	Venezuela	PCR Pyrosequencing	Nd	88-94	Nd	Nd	Nd	Nd	Nd	0.1-2	4-34	Nd	NO	(Dominguez-Bello et al.)

2   Legend: x - species analyzed but no percentage reported; * - results only from pregnant women/state; Nd - Not detected.