Published in final edited form as:

Curr HIV Res. 2012 September 1; 10(6): 504–512.

Bacterial Colonization and Beta Defensins in the Female Genital Tract in HIV Infection

Wei Jiang^{*1}, Santosh K. Ghosh², Rebecca Flyckt³, Magdalena Kalinowska⁴, David Starks³, Richard Jurevic², Aaron Weinberg², Michael M. Lederman¹, and Benigno Rodriguez¹

¹Division of Infectious Diseases, Department of Medicine, Center for AIDS Research, Case Western Reserve University and University Hospitals/Case Medical Center, Cleveland, USA

²Department of Biological Sciences, Case School of Dental Medicine, Case Western Reserve University, Cleveland, USA

³Department of Obstetrics and Gynecology, University Hospitals/Case Medical Center, Cleveland, USA

⁴Albert Einstein College of Medicine, New York, USA

Abstract

Beta defensins are antimicrobial peptides that serve to protect the host from microbial invasion at skin and mucosal surfaces. Here we explore the relationships among beta defensin levels, total bacterial colonization, and colonization by bacterial vaginosis (BV)-related bacteria and lactobacilli in the female genital tract in HIV infected women and healthy controls.

Cervicovaginal lavage (CVL) samples were obtained from 30 HIV-infected women and 36 uninfected controls. Quantitative PCR assays were used to measure DNA levels of bacterial 16S ribosomal DNA (reflective of total bacterial load), and levels of three BV-related bacteria, three *Lactobacillus* species (*L. crispatus*, *L. iners* and *L. jensenii*), and total *Lactobacillus* levels in CVL. Levels of human beta defensins (hBD-2 and hBD-3) were quantified by ELISA. In viremic HIV+ donors, we found that CVL levels of bacterial 16S rDNA were significantly increased, and inversely correlated with peripheral CD4+ T cell counts in HIV+ women, and inversely correlated with age in both HIV+ women and controls. Although CVL DNA levels of BV-associated bacteria tended to be increased, and CVL levels of *lactobacillus* DNAs tended to be decreased in HIV+ donors, none of these differences was significant. CVL levels of hBD-2 and hBD-3 were correlated and were not different in HIV+ women and controls. However, significant positive correlations between hBD-3 levels and total bacterial DNA levels in controls were not demonstrable in HIV+ women; the significant positive correlations of hBD2 or hBD-3 and three *Lactobacillus* species in controls were also not demonstrable in HIV+ women. These results suggest that HIV infection is associated with impaired regulation of innate defenses at mucosal sites.

Keywords

HIV; Bacterial 16S rDNA; beta defensins; Cervicovaginal lavage

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*Address correspondence to this author at the Division of Infectious Diseases, Department of Medicine, Center for AIDS Research, Case Western Reserve University and University Hospitals/Case Medical Center, 2109 Adelbert Rd, CWRU BRB1048B, 4984. Cleveland, OH, 44106, USA; Tel: 001-216-3681882; Fax: 001-216-3685415; jxw103@case.edu.

CONFLICT OF INTEREST

Declared none.

INTRODUCTION

In the lower genital tract of healthy women, *Lactobacillus spp.* promote probiotic functions by inducing an antimicrobial environment that includes induction of defensins [1, 2]. HIV infection is associated with an alteration of the vaginal bacterial flora with loss of lactobacilli and an increase in bacterial vaginosis (BV) [3, 4]. Women with BV have low levels of lactobacilli in their genital tracts, and BV may be associated with an increased risk of HIV acquisition [4, 5]. Little is known regarding total levels of bacteria in cervical vaginal lavage (CVL) and their relationship to vaginal innate immunity in HIV disease.

Antimicrobial peptides, which comprise an important arm of the innate defense system at mucosal sites, are small proteins with microbicidal activity against bacteria, fungi, and viruses [6]. They include Human β -defensins (hBDs), a family of epithelial cell-derived cationic peptides (4–5 kDa), that have both antimicrobial and immunomodulatory properties [7–10]. Among hBDs, hBD-1 is constitutively expressed, whereas hBD-2 and -3 are inducible [11–14]. We recently found that hBD-2 and -3 can inhibit HIV-1 replication *in vitro* [15]. Inhibition by hBD-3 is mediated in part through internalization of CXCR4, the coreceptor utilized by X4 tropic viruses [16].

We examined CVL obtained from otherwise healthy HIV-infected women including antiretroviral therapy (ART) treated and ART-naïve donors, and healthy controls for total levels of bacterial DNA, levels of *Lactobacillus* DNA, and levels of human beta defensin proteins at these sites. We found that levels of bacterial 16S rDNA (reflecting total bacterial load) are significantly increased in HIV+ women in whom they are inversely related to CD4 T cell counts. In the controls, but not in HIV+ women, bacterial DNA levels in CVL were positively related to hBD-3 levels, and in controls, levels of *Lactobacillus spp.* DNAs and hBD levels tended to be more strongly and positively correlated than among HIV+ subjects. In both control and HIV+ subjects, bacterial DNA levels in CVL were inversely correlated with age.

MATERIALS AND METHODS

Study Population

These studies were approved by the Institutional Review Board of Case Western Reserve University, University Hospitals/Case Medical Center. Samples were obtained from 30 women with HIV infection and from 36 healthy controls. All participants signed consent forms. The controls comprised women who were visiting the GYN clinic for routine evaluations. The HIV+ subjects were patients receiving care at the John T. Carey Special Immunology Unit who were also undergoing routine gynecologic examination. Clinical characteristics of these subjects are shown in Table 1. None of the women had clinical signs of BV or clinical or microbiological evidence of genital infection.

Ethical Issues

Before the initiation of any study procedures, subjects were informed about the study and asked to sign an IRB approved informed consent/HIPAA document. Subjects were consented in a private examination room at University Hospitals Case Medical Center (UHCMC) by the research assistant/nurses or Researchers/Investigators on the staff trained in Human Subject Protections. Subjects were given time to read the consent, to ask questions and were able to consider the risks and/or benefits to participation in this research study prior to obtaining their signature.

All laboratory specimens, evaluation forms, reports, and other records that left the site were identified by coded number only to maintain subject confidentiality. All study samples were

kept in a secure area in a limited-access laboratory facility and only the research team had access to the samples. The samples and data were identified only by code numbers.

Samples

After gynecologic examination, each CVL sample was obtained by directing a stream of 10 mL of PBS at the cervix and collecting the pooled fluid in the posterior fornix. CVL samples were aliquoted and stored at -80°C until analysis.

Genomic DNA

Genomic DNA was extracted from CVL using a Qiagen DNA isolation kit (Qiagen Inc., Valencia, Calif.). DNA concentration was determined using a NanodropTM spectrophotometer (Thermo Scientific, DE). Genomic DNA was stored at -70°C until analyzed by quantitative PCR.

Quantitative PCR for Measurement of Bacterial 16S rDNAs

Purified DNA was analyzed as follows: a 20 μL amplification reaction consisted of 2 μL of 10 $\times$ PCR buffer (100 mM Tris HCL, pH 8.3, 500 mM KCL; Invitrogen Corp., Carlsbad, CA), 3.5 mM MgCl_2 , 0.2 mM dNTP, 0.5 μM forward and reverse primers, 0.32 μM probe labeled with FAM and Black Hole Quencher® (BHQ®) dyes (338P, 5'-FAM-GCT GCC TCC CGT AGG AGT-BHQ1-3'), 0.75 units of platinum Taq polymerase (Invitrogen), and 5 μL of CVL DNA. Degenerate forward (8F) and reverse primers (Table 2) were used to amplify DNA templates encoding 16S ribosomal RNA shared by multiple bacterial genera [17]. The DNA was amplified in triplicate and mean values were calculated. A standard curve was generated using PCR amplicons from the targeted regions. The reaction conditions for amplification of DNA were 95°C for 5 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 1 min. This method allows for detection of bacterial 16S rDNA as rare as 6 copies/ μL [18].

Quantitative PCR for Measurement of *Lactobacillus* DNA and BV-Associated Bacterial DNA in CVL

Amplification reactions consisted of 10 μL of SYBR master mix (ABI, Warrington, UK), 0.5 μM forward and reverse primers, 0.75 units of platinum Taq polymerase (Invitrogen), and 5 μL of CVL DNA in a final volume of 20 μL . Reaction conditions for measurement of total *Lactobacillus* DNA or for measurement of individual species *L. crispatus*, *L. jensenii*, and *L. iners* were 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 1 min [19]. Reaction conditions using primers for measurement of three BV-related species BVAB1 [20], BVAB2 [19], and *G. vaginalis* [20] were 95°C for 10 min, followed by 45 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C 1 min, and holding at 72°C for 7 min. The DNA was amplified in triplicate and mean values were calculated. A standard curve was created using PCR amplicons from the targeted regions. Primer sequences are shown in Table 2.

Measurement of hBD-2 and hBD-3 Levels in CVL Samples

The CVL samples were spun ($1000 \times g$ for 15 min at 4°C) to remove debris, and the supernatants were acetone precipitated and resuspended in 250 mmole/L of CaCl_2 . hBD-2 and hBD-3 peptide levels were determined by ELISA [21]. We coated 96-well immunoplates (MaxiSorpTM; Nunc) with 100 μL goat anti-hBD-2 or rabbit anti-hBD-3 antibodies (Peprotech, NJ) diluted to 1 mg/L in 0.05 mol/L carbonate buffer, pH 9.6, 4°C , for 18 h. Subsequently, plates were blocked with 200 μL 1% bovine serum albumin in PBS at room temperature for 10 min. After washing 3 times with 200 μL PBS, 0.01% Tween 20, 100 μL /well of test samples were added and incubated at room temperature for 60 min. Plates were washed 3 times with PBS, 0.01% Tween 20 and wells were incubated at room

temperature with 100 μ L biotinylated goat anti-human BD-2 or biotinylated rabbit anti-human BD-3 (Peprotech, NJ) diluted to 0.2 mg/L in PBS, 0.01% Tween 20 for 30 min. Plates were washed 3 times with PBS, 0.01% Tween 20 and 100 μ L/well streptavidin-peroxidase (Roche Diagnostics; 1:10,000 in PBS, 0.01% Tween 20) was added. Plates were then incubated at room temperature for an additional 30 min, washed 3 times as described above, and incubated with 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Roche Diagnostics) in the dark at room temperature for 20 min. Absorbance was measured at 415 nm in a microplate reader (Bio-Rad Model 680). hBDs were quantified by simultaneous ELISA (in 250 mM CaCl₂) using recombinant hBDs as calibrators. Total protein content of CVL samples was measured in a NanodropTM spectrophotometer (Thermo Scientific, DE), and the defensin concentrations in the samples were expressed as ng/mg of total protein.

Statistical Analysis

Conventional measures of central location and dispersion were used to describe the data, and paired variables were compared by Mann-Whitney U test. Spearman's rank correlation was used to explore associations between pairs of continuous variables. Multiple regression analysis was used to assess the independent contribution of several explanatory variables to a continuous dependent variable. Analyses were performed using SPSS, v. 16.01 (SPSS Inc, Chicago). All tests were two-sided, and $P = 0.05$ was considered statistically significant.

RESULTS

CVL Levels of Total Bacterial 16S rDNA are Increased in HIV-Infected Donors

Total bacterial levels, as determined by 16S rDNA measurement, were lower in CVL obtained from control subjects (median 6×10^7 copies/ μ L) than in CVL from HIV+ donors (2.6×10^8 copies/ μ L, $P = 0.0075$) (Fig. 1A). Among HIV+ donors, bacterial 16S rDNA levels were higher in the 16 HIV-infected subjects with detectable viremia (median 3.1×10^8 copies/ μ L) than in the 14 subjects on HAART with viral levels in plasma below the limits of detection (median 7.2×10^7 copies/ μ L), although the difference was not significant ($P = 0.18$, Fig. 1A).

To determine whether increased total bacterial DNA was related to BV associated bacterial colonization in HIV+ donors, we investigated three bacterial species, including BV associated bacterium 1 (BVAB1), BV associated bacterium 2 (BVAB2), and *Gardnerella vaginalis* (*G. vaginalis*), microbes that are linked to bacterial vaginosis [20]. CVL levels of BVAB1 DNA (Fig. 1B, $P = 0.07$) and *G. vaginalis* DNA (Fig. 1D, $P = 0.06$) were marginally increased in HIV+ subjects compared to levels in controls. CVL levels of BVAB2 DNA were similar in HIV+ subjects and controls (Fig. 1C, $P = 0.27$).

CVL Levels of *Lactobacillus* DNA and DNA Levels of Three *Lactobacillus* Species Tended to be Lower (But Not Significantly So) in HIV-Infected Donors than in Controls

CVL DNA levels of *Lactobacillus* species that include *L. crispatus*, *L. iners*, and *L. jensenii* were slightly, but not significantly, decreased in HIV+ subjects compared to levels in controls (Fig. 2A); levels in viremic subjects were not different from levels in HIV+ aviremic subjects (Fig. 2A). Consistent with these results, CVL levels of *L. crispatus* (Fig. 2B, $P = 0.08$), *L. iners* (Fig. 2C, $P = 0.39$), and *L. jensenii* (Fig. 2D, $P = 0.12$) DNA were slightly, but not significantly, decreased in HIV+ subjects compared to levels in controls.

CVL Levels of hBD-2 are Positively Correlated with hBD-3 CVL Levels in Both Controls and HIV+ Subjects

To explore innate immune defense in the female genital tract, we measured CVL levels of hBD-2 and hBD-3. We found that hBD-2 and hBD-3 levels were not different between

controls and HIV+ subjects ($P = 0.46$ and 0.67 , respectively) (Fig. 3A, B), and although levels of hBD-2 and hBD-3 tended to be lower in viremic HIV+ donors than in aviremic patients, these differences were not significant (data not shown).

CVL levels of hBD-2 correlated positively with CVL levels of hBD-3 in both HIV- (Fig. 3C, $r = 0.48$, $P = 0.001$) and HIV+ subjects (Fig. 3D, $r = 0.36$, $P = 0.04$). Expression of hBDs can be induced by pro-inflammatory cytokines (e.g., TNF- α , IL-1 β), by certain microorganisms, their lipopolysaccharides, and by other microbial products [11, 22, 23]. The correlation between CVL levels of hBD-2 and hBD-3 suggests that expression of these two antimicrobial peptides in the female genital tract is linked, and is suggestive of a common regulatory mechanism for both hBD-2 and hBD-3.

Correlations Between CVL DNA Levels of *Lactobacillus* Species *L. crispatus*, *L. iners*, *L. jensenii* and hBD Levels

We next investigated the relationship between innate immune defenses and *Lactobacillus* colonization in HIV infection and in controls. We found that in controls the correlations between CVL levels of hBD-2 and CVL DNA levels of *L. crispatus*, *L. iners*, or *L. jensenii* were either significant or marginally significant ($r = 0.40$, $P = 0.02$, $r = 0.30$, $P = 0.08$, and $r = 0.36$, $P = 0.03$, respectively, Fig. 4A–C). The correlations between CVL levels of hBD-3 and CVL DNA levels of *L. crispatus*, *L. iners*, or *L. jensenii* were either not significant or marginally significant ($r = 0.31$, $P = 0.06$, $r = 0.31$, $P = 0.07$ and $r = 0.18$, $P = 0.30$, respectively, Fig. 4D–F). In contrast in HIV+ women, correlations between both hBD-2 and -3 and CVL DNA levels of *L. crispatus*, *L. iners*, or *L. jensenii* were not significant (Fig. 4H–L, $P > 0.10$), suggesting that if expression of mucosal antibacterial defenses in the female genital tract is induced in response to *Lactobacillus*, this response may be dysregulated in HIV disease.

CVL Bacterial Levels and Defensin-3 Levels Correlate in Controls But Not in HIV+ Subjects

We also examined the relationship between CVL levels of hBDs and CVL levels of total bacterial 16S rDNA. We found that in controls CVL levels of bacterial 16S rDNA positively correlated with CVL levels of hBD-3 ($r = 0.44$, $P = 0.007$) but not with CVL levels of hBD-2 ($r = 0.11$, $P = 0.50$) (Fig. 5A, B). In HIV+ donors, there was no association between levels of bacterial 16S rDNA and levels of either hBD-2 or -3 in CVL (Fig. 4C, D), suggesting that if expression of mucosal antibacterial defenses in the female genital tract is induced in response to prevalent bacteria, this response may be dysregulated in HIV infection or the microbes more effective at induction of defensin expression are relatively under-represented in HIV infection.

CVL Levels of Bacterial 16S rDNA Inversely Correlate with CD4+ T Cell Counts in HIV Infection and Inversely Correlate with Age in Both HIV+ Subjects and Controls

Levels of bacterial 16S rDNA, but not levels of *Lactobacillus* DNA, exhibited a weak, inverse correlation with circulating CD4+ T cell counts but not with plasma levels of HIV RNA in HIV+ subjects ($r = -0.35$, $P = 0.056$ and $r = 0.16$, $P = 0.41$, respectively) (Fig. 6A, B), suggesting that immune status may determine regulation of innate defenses and perhaps control of bacterial replication in the female genital tract.

In both controls and HIV+ women, younger age was associated with higher levels of bacterial DNA in CVL (Fig. 6C, D). After adjustment for circulating CD4+ T cell counts, we estimated that each additional year of age was associated with a mean decrease of 1.8×10^7 copies/ μ l in CVL levels of bacterial DNA in HIV+ donors ($P = 0.01$). Age was not associated with CVL levels of hBDs (data not shown).

DISCUSSION

In this cross-sectional study, we investigated hBD levels, and DNA levels of total bacteria, BV related bacteria, *Lactobacilli*, and three *Lactobacillus spp.* in CVLs from HIV infected women and control women. By measuring the presence of conserved sequences of 16S ribosomal DNA common to most bacteria, we found that viremic HIV+ women have higher CVL levels of total bacteria than do control subjects, and this may be related to the dysregulation of mucosal innate antimicrobial defenses in HIV infection.

Lactobacilli are generally considered beneficial in the female genital tract because they produce lactic acid and bacteriocins [24, 25]. Loss of *Lactobacillus* colonization in the female genital tract is recognized in HIV infection [3, 26], and vaginal levels of lactobacilli have been inversely associated both with vaginal levels of HIV and with the presence of BV [4, 5, 27]. We found that CVL levels of total *Lactobacillus* DNA tended to be decreased in HIV+ subjects compared to levels controls, and CVL levels of BV-associated bacterial DNA tended to be increased in HIV+ subjects compared to levels controls but for most comparisons, these differences were not significant. Stronger positive correlations were seen between CVL levels of lactobacilli and levels of hBD-2 and hBD-3 in controls than in HIV+ subjects, and a positive association was seen between CVL levels of bacterial 16S rDNA and hBD-3 in controls but not in HIV+ subjects. This may reflect a function of lactobacilli and other bacteria in contributing to mucosal levels of beta defensins, as these organisms have been shown to induce hBD-2 [28]. Previous studies have shown that lactobacilli induce enterocyte hBD-2 *in vitro*, which suggests a role of lactobacilli in maintaining mucosal antimicrobial levels in healthy women [28, 29]. Weak, non-significant correlations were seen with levels of *lactobacillus* species and hBD-3 in healthy controls that were not apparent in HIV infection. Loss of these correlations in HIV+ subjects suggests that there is an impaired antimicrobial peptide response at mucosal sites in HIV infection. The positive correlation between CVL levels of bacterial 16S rDNA and hBD-3 in healthy controls but not in HIV+ women also is consistent with an impaired cervicovaginal antimicrobial peptide response in HIV infection. This is supported further by the negative correlation between bacterial DNA levels in CVL and CD4 T cell counts in the HIV infected women.

We also found that in both controls and HIV+ subjects, age inversely correlated with bacterial DNA levels in CVLs. The reason for this novel finding is not clear but may be related to frequency of sexual activity that we did not capture in our study, and/or to differences in sex hormone levels and the resultant differences in the mucosal environment of younger and older subjects.

In summary, we found elevated levels of total bacterial 16S rDNA in CVLs from subjects with chronic HIV infection, especially in those with uncontrolled viremia. In HIV+ women, total bacterial DNA levels inversely correlated with CD4 T cell counts. We also found that CVL levels of three *Lactobacillus spp.* correlated with CVL levels of hBD-2 and hBD-3 in controls better than they did in HIV+ subjects, and that CVL levels of total bacterial DNA were positively correlated to CVL levels of hBD-3 in healthy controls but not in HIV+ women. These findings are consistent with a model wherein HIV infection results in impairment of innate mucosal defenses and increased bacterial colonization of the female genital tract.

Acknowledgments

This work was supported by funds from the National Institutes of Health: DE016110-01, AI076981, AI091526, P01-DE019759, and the Center for AIDS Research at Case Western Reserve University, AI-36219.

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Fig. 1. Increased levels of bacterial 16S rDNA in CVL of viremic HIV+ subjects

CVL levels of total bacterial 16S rDNA and BV-related bacterial DNA were measured by qPCR. (A) Increased CVL levels of bacterial 16S rDNA in viremic HIV+ subjects. CVL levels of BVAB1 DNA (B), BVAB2 DNA (C), and *G. vaginalis* DNA (D). Data were compared using nonparametric Mann-Whitney U test.

Fig. 2. CVL DNA levels of total *Lactobacillus* and three *Lactobacillus* spp. in healthy and HIV+ subjects

CVL levels of total *Lactobacillus* DNA and three *Lactobacillus* spp. DNA were measured by qPCR. For each cohort, CVL levels of total *Lactobacillus* DNA (**A**), *L. crispatus* (**B**), *L. iners* (**C**) and *L. jensenii* (**D**) are shown. Data were compared using nonparametric Mann-Whitney U test.

Fig. 3. CVL levels of hBD-2 correlate with CVL levels of hBD-3 in healthy control and HIV+ women

Levels of hBD-2 and hBD-3 were measured by ELISA in CVL samples. (A) CVL levels of hBD-2. (B) CVL levels of hBD-3. Data were compared using the nonparametric Mann-Whitney U test. (C) CVL levels of hBD-2 correlate with CVL levels of hBD-3 in healthy control women. (D) CVL levels of hBD-2 correlate with CVL levels of hBD-3 in HIV+ women. The data were analyzed using Spearman's rank correlation test.

Fig. 4. Correlations between CVL defensin levels and *Lactobacillus* DNA levels in healthy controls and HIV+ women

(A–F). The relationship between CVL DNA levels of *Lactobacillus* species *L. crispatus*, *L. iners*, or *L. jensenii*, and CVL levels of hBD-2/ hBD-3 among healthy controls. (G–L) The relationship between CVL DNA levels of *Lactobacillus* species *L. jens*, *L. iners* or *L. cris* and CVL levels of hBD-2/ hBD-3 among HIV+ women. Correlations were determined by Spearman's rank correlation test.

Fig. 5. Correlations between CVL defensin levels and total bacterial DNA levels in healthy controls and HIV+ women

(A, B). The relationship between CVL levels of bacterial 16S rDNA and CVL levels of hBD-2/hBD-3 among healthy controls. (C, D). The relationship between CVL levels of bacteria 16S rDNA and CVL levels of hBD-2/hBD-3 among HIV+ women. Correlations were determined by Spearman's rank correlation test.

Fig. 6. CVL bacterial 16S rDNA levels are inversely related to age and inversely related to peripheral CD4+ T cell counts

(A) The relationship between CVL levels of 16S rDNA and peripheral CD4+ T cell number in HIV+ women. (B) The relationship between CVL levels of 16S rDNA and plasma levels of HIV RNA. (C) Correlation between CVL levels of bacteria 16S rDNA and age in healthy control women. (D) Correlation between CVL levels of bacteria 16S rDNA and age in HIV+ women. Correlations were determined by Spearman's rank correlation test.

Clinical Characteristics of Study Participants

Table 1

	Controls				HIV-Infected Subjects							
					HIV on HAART				HIV, HAART-naïve,			
	N	Median	25 th	75 th	N	Median	25 th	75 th				
Age	36	39	34.5	45	15	39	36	45	16	39	33	48
CD4						685	434	784		432	285	598
VL							50	50	50	7660	3350	28650
Log ₁₀ VL							1.7	1.7	1.7	3.88	3.53	4.46

25th: percentile 25.

75th: percentile 75.

N: Valid sample size.

CD4: CD4⁺ T cell counts, cells/ul.

VL: plasma HIV RNA, copies/ml.

Table 2

Sequences of PCR Primers

Name	Sequence (5'-3')	Target	References
Bac-8F	AGTTTGATCCTGGCTCAG	16S rDNA	[17]
Bac-515R	GWATTACCGCGGCKGCTG	16S rDNA	[17]
BVAB1F	GTATATTTTCTACGGAACACAGG	BVAB1	[20]
BVAB1R	TTTGCTCCGATCGCTCCTT	BVAB1	[20]
G. vagF	GGGCGGGCTAGAGTGCA	<i>G. vaginalis</i>	[20]
G. vagR	GAACCCGTGGAATGGGCC	<i>G. vaginalis</i>	[20]
LactoF3	TGGAAACAGRTGCTAATACCG	<i>Lactobacillus</i>	[19]
LactoR3	GTCCATTGTGGAAGATTCCC	<i>Lactobacillus</i>	[19]
LactoF2	ATGGAAGAACACCAAGTGGCG	<i>Lactobacillus</i>	[4]
LactoR2	CAGCACTGAGAGGCGGAAAC	<i>Lactobacillus</i>	[4]
L. crispF	AGCGAGCGGAACAAACAGATTTAC	<i>L. crispatus</i>	[19]
L. crispR	AGCTGATCATGCGATCTGCTT	<i>L. crispatus</i>	[19]
L. jensF	AAGTCGAGCGAGCTTGCCATAGA	<i>L. jensenii</i>	[19]
L. jensR	CTTCTTTCATGCGAAAGTAGC	<i>L. jensenii</i>	[19]
L. inerF	CTCTGCCTTGAAGATCGGAGTGC	<i>L. iners</i>	[19]
L. inerR	ACAGTTGATAGGCATCATCTG	<i>L. iners</i>	[19]