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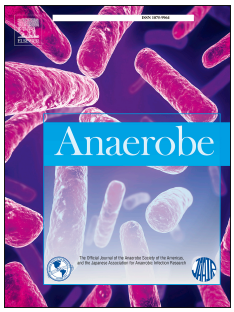

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Quantification, isolation and characterization of *Bifidobacterium* from the vaginal microbiomes of reproductive aged women

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CANADA

Abstract

The vaginal microbiome plays an important role in women's reproductive health. Imbalances in this microbiota, such as the poorly defined condition of bacterial vaginosis, are associated with increased susceptibility to sexually transmitted infections and negative reproductive outcomes. Currently, a "healthy" vaginal microbiota in reproductive aged women is understood to be dominated by *Lactobacillus*, although "atypical" microbiomes, such as *Bifidobacterium*-dominated profiles, have been described. Despite these observations, vaginal bifidobacteria remain relatively poorly characterized, and questions remain regarding their actual abundance in the microbiome. In this study, we used quantitative PCR to confirm the relative abundance of *Bifidobacterium* in the vaginal microbiomes of healthy reproductive aged women (n=42), previously determined by deep sequencing. We also isolated and phenotypically characterized vaginal bifidobacteria (n=40) in the context of features thought to promote reproductive health. Most isolates were identified as *B. breve* or *B. longum* based on *cpn*60 barcode sequencing. Fermentation patterns of vaginal bifidobacteria did not differ substantially from corresponding type strains of gut or oral origin. Lactic acid was produced by all vaginal isolates, with *B. longum* strains producing the highest levels, but only 32% of isolates produced hydrogen peroxide. Most vaginal bifidobacteria were also able to tolerate high levels of lactic acid (100 mM) and low pH (4.5 or 3.9), conditions typical of vaginal fluid of healthy women. Most isolates were resistant to metronidazole but susceptible to clindamycin, the two most common antibiotics used to treat vaginal dysbiosis. These findings demonstrate that *Bifidobacterium* is the dominant member of some vaginal microbiomes and suggest that bifidobacteria have the potential to be as

protective as lactobacilli according to the current understanding of a healthy vaginal microbiome.

Keywords: *Bifidobacterium*, vaginal microbiome, fermentation pattern, hydrogen peroxide, lactic acid, antibiotic

1. Introduction

Bifidobacteria were first described by Tissier in 1899, who isolated a bacterium from breast-fed infant feces and named it *Bacillus bifidus* [1]. In 1924, Orla-Jensen proposed the genus *Bifidobacterium* as a separate taxon for these organisms [2], which currently includes more than 30 species [3]. Bifidobacteria are Gram-positive, anaerobic, non-motile, non-spore forming rod-shaped bacteria, with varied branching. They belong to the *Bifidobacteriaceae* family and have high genomic G+C content (55-67 mol%) [3]. Bifidobacteria are known to colonize the human vagina, oral cavity and, more abundantly, the gastrointestinal tract (GIT) [4]. Several studies have shown their influence on human physiology and nutrition [5–9]. In newborns, bifidobacteria play an important role as one of the primary colonizers of the GIT, representing 60 to 91% of fecal bacteria in breast-fed infants [10,11]. This proportion decreases with age and it may represent less than 10% of the adult fecal microbiota [12,13]. Bifidobacteria provide protection from pathogens in the GIT through the production of bacteriocins [7], inhibition of pathogen adhesion [5], and modulation of the immune system [14,15]. Due to these health-promoting effects, bifidobacteria have been extensively studied as probiotics [8,16–18].

Early microbial colonization is an essential process in the maturation of the immune system [19]. This initial colonization may be affected by many factors, such as the mode of delivery (vaginal or caesarean section), feeding type (breast-fed or formula-fed), exposure to antibiotics and hygiene [20]. However, the relative contributions of maternal microbiota (gut, breast milk, vaginal) and environmental sources to the bifidobacteria population of the neonatal gut remain unresolved.

While *Bifidobacterium* spp. present in the gut are well described, vaginal bifidobacteria remain relatively poorly characterized, and it is not known if vaginal adaptation has resulted in distinct phenotypic features that distinguish them from gut populations. Although a healthy vaginal microbiota is defined as *Lactobacillus*-dominated, several studies have identified vaginal *Bifidobacterium*-dominated profiles in 5-10% of healthy, reproductive aged women [21–24]. Furthermore, vaginal bifidobacteria are reported to produce lactic acid and hydrogen peroxide; attributes of vaginal lactobacilli credited with maintaining homeostasis in the vaginal microbiome [25].

Culture-independent techniques are useful tools in microbiome characterization, but methods based on amplification and sequencing of 16S rRNA genes, have been reported to underrepresent *Bifidobacterium* in microbial communities [26]. The abundance of *Bifidobacterium* in the vaginal microbiota may also be underestimated due to the similarity of their 16S rRNA sequences to those of *Gardnerella vaginalis. G. vaginalis* is also a member of the *Bifidobacteriaceae* family and is a commonly detected microorganism associated with bacterial vaginosis (BV) [27]. The use of the *cpn*60 "universal target" (UT) region as a barcode for microbiome profiling results in better resolution of closely related species, including those within *Bifidobacteriaceae* [28], and *cpn*60 based human fecal microbiome profiles have been shown to more accurately represent *Bifidobacterium* content than a 16S rRNA based approach [26]. Previous studies of the vaginal microbiome [21] or synthetic mixtures of vaginal organisms [29] have demonstrated a strong correlation between *cpn*60 sequence read abundance and organism abundance determined by quantitative PCR. However, regardless of the target used, relative abundance of specific organisms within complex communities may not be

represented accurately by methods that rely on polymerase chain reaction (PCR) amplification and its inherent biases.

Considering the lack of information about *Bifidobacterium* spp. of vaginal origin, their importance as a potential source for the neonatal gut microbiome, and their potential health-promoting effects in the vagina, a better understanding of the properties of vaginal bifidobacteria is needed. In this study, our main objectives were: 1) to apply species-specific quantitative PCR to confirm the relative abundance of *Bifidobacterium* in the vaginal microbiomes of reproductive aged women previously determined based on *cpn*60 barcode sequencing, and 2) to characterize vaginal *Bifidobacterium* isolates based on carbohydrate fermentation patterns, hydrogen peroxide production, lactic acid production, resistance to low pH and lactic acid, and susceptibility to antibiotics.

2. Material and Methods

2.1 Samples and microbiome profiles

Vaginal microbiome profiles from 492 healthy women were previously published by our research group [30,31]. Profiles were created by PCR amplification and deep sequencing of the *cpn*60 UT region. Total bacterial load in each sample was also estimated as part of these studies using a SYBR Green assay based on the amplification of the V3 region of the 16S rRNA gene. The remaining vaginal swabs and DNA extracts from these studies, archived at -80 ˚C, were available for use in the current study.

2.2 *Bifidobacterium* quantitative PCR assays

Sequences with similarity to *Bifidobacterium breve, Bifidobacterium dentium* and *Alloscardovia omnicolens* (Bifidobacteriaceae family) that were detected at high frequency in the previously published studies [30,31] were selected as targets for quantitative PCR. Signature regions within the *cpn*60 UT unique to each target were determined using Signature Oligo software (LifeIntel Inc., Port Moody, BC, Canada) and primers were designed using Primer-blast software [32] and Primer3 [33] (Table 1).

To create plasmids for use in standard curves, target sequences were amplified from vaginal swab DNA extracts. The resulting PCR products were purified and ligated into cloning vector pGEM-T-Easy (Promega, Madison, WI) and used to transform competent *E. coli* DH5α. Insertion of the intended target sequence was confirmed by DNA sequencing. Optimal annealing temperature for each assay was determined using an annealing temperature gradient, and specificity of each primer set was confirmed by using plasmids containing *cpn*60 UT sequences from closely related species as template.

All qPCR reactions were performed in duplicate and each batch of reactions included a no template control and a standard curve consisting of serial dilutions of plasmids containing targets. Each reaction consisted of 2 µL of template DNA, 1× iQ SYBR Green Supermix (BioRad, Mississauga, ON, Canada) and 400 nM each primer, in a final volume of 25 µL. A MyiQ thermocycler (BioRad) was used for all reactions with the following protocol: 95 °C for 3 m, followed by 40 cycles of 95 °C for 15 s, 65 °C for 15 s, 72 °C for 15 s. A dissociation curve was subsequently performed for 81 cycles at 0.5 °C increments from 55 °C to 95 °C to confirm the purity of PCR products.

2.3 Calculation of proportional abundance of *Bifidobacterium* in vaginal samples

Vaginal microbiome profiles (n=492) were ranked according to the proportional abundance of *Bifidobacterium* (all *Bifidobacterium* species and *Alloscardovia* combined), based on the previously determined *cpn*60 sequence read counts [30,31].

B. breve, B. dentium and *A. omnicolens* DNA was quantified in vaginal swab DNA extracts from selected samples using the SYBR Green qPCR assays described in the previous section. Previously determined total 16S rRNA copy number per sample [30,31] was used an as estimate of total bacterial population. The ratio between $\log_{10}$ copy number of *Bifidobacterium* and 16S rRNA $\log_{10}$ copy number was used as an estimate of the proportional abundance of each target species in the selected vaginal microbiome samples.

Proportional abundance determined from deep sequencing of *cpn*60 UT amplicons (percent of sequence reads) and by quantitative PCR for each of the three targets evaluated (*B. breve, B. dentium* and *A. omnicolens)* were compared using Spearman rank correlation in IBM SPSS (Statistical Package for the Social Sciences, version 21).

2.4 Isolation of vaginal *Bifidobacterium*

A complete list of isolates used in the study and their sources is provided in Supplemental Table 1. Bifidobacteria were isolated from vaginal swabs, which were collected in previous studies and had been stored at -80 °C. For one group of samples (healthy pregnant and non-pregnant Canadian women from Vancouver, BC and Toronto, ON), 12 *Bifidobacterium*-dominated samples were selected based on the previous

159 microbial profiling data [30,31]. Eluted material from these 12 vaginal swabs was plated
160 on Columbia agar containing 5% sheep blood (CSB, BD Canada, Mississauga, ON). For
161 the second group of samples (Adolescent women, Winnipeg, MB) [25], material from 27
162 vaginal swabs was plated on a Bifidus selective medium agar (BSM agar, Sigma-Aldrich,
163 Oakville, ON); no sequence data from these microbiome samples was available. Frozen
164 swabs were thawed and sample was eluted from each swab by vortexing in phosphate-
165 buffered saline (PBS). Dilutions were prepared from the eluted sample and spread onto
166 CSB agar or BSM agar followed by incubation using the GasPak EZ anaerobic system
167 (BD Canada, Mississauga, ON) at 37 °C for 72 h. After isolating pure colonies, a freezing
168 buffer (4% (v/v) skim milk, 1% (w/v) glucose, 20% (v/v) glycerol) was added to the
169 isolates for long-term storage at -80 °C. DNA preparations from isolates were made using
170 Chelex 100 (Bio-Rad Laboratories, Inc., Mississauga, ON).

171 Bifidobacteria (n=16) previously isolated in a study of commercial sex workers in
172 Nairobi, Kenya [25], were also included in the study. *Bifidobacterium* spp. and
173 *Lactobacillus crispatus* type strains (*B. breve* ATCC 15700, *B. longum* subsp. *infantis*
174 ATCC 15697, *B. dentium* ATCC 27534 and *L. crispatus* ATCC 33820) were acquired
175 from the American Type Culture Collection (Manassas, VA). *Lactobacillus crispatus*
176 vaginal isolates 67-1, B12-1 and N4D05 were from our lab culture collection.

177 **2.5 *cpn*60 PCR, sequencing and phylogenetic analysis**

178 The *cpn*60 UT region was used for species identification of isolates. The target
179 region was amplified from genomic DNA using modified versions of the *cpn*60
180 "universal" primers, optimized for high G+C templates: H1594 (5'-CGC CAG GGT TTT

CCC AGT CAC GAC GAC GTC GCC GGT GAC GGC ACC ACC AC-3') and H1595 (5'-AGC GGA TAA CAA TTT CAC ACA GGA CGA CGG TCG CCG AAG CCC GGG GCC TT-3'). The M13 (-40)F and M13 (-48)R sequencing primer landing sites are underlined.

PCR was carried out with 2 µL template DNA in a reaction mixture containing 1× PCR buffer (0.2 M Tris-HCl at pH 8.4, 0.5 M KCl), 2.5 mM $MgCl_2$, 200 µM dNTP mixture, 400 nM each primer, 2 U AccuStart Taq DNA polymerase and water to a final reaction volume of 50 µL. Cycling conditions were as follows: 94 °C for 3 m, 40 cycles of 94 °C for 30 s, 57 °C for 60 s, 72 °C for 60 s, and a final extension at 72 °C for 10 m. Amplification was confirmed by resolving the PCR products in 1% agarose gel (expected *cpn*60 UT amplicon is ~652 bp). Purified PCR products were sequenced with primers M13(-40)F or M13(-48)R. Following assembly of the forward and reverse sequences and removal of amplification primer sequences, the resulting 552 bp sequences were compared to the cpnDB reference database (www.cpndb.ca) [34] for identification.

Phylogenetic analysis of isolates was done using PHYLIP (Phylogeny Inference Package) version 3.5 [35]. The *cpn*60 sequences of the isolates and reference strains from the cpnDB reference database were aligned with ClustalW [36]. Alignments were bootstrapped with seqboot; distances were calculated with the maximum likelihood option of dnadist. Dendrograms were constructed from distance data by neighbor-joining with neighbor. Consensus trees were calculated with consense, and branch lengths were superimposed on consensus trees using dnaml.

202 **2.6 Carbohydrate fermentation patterns**

203 The ability of vaginal *Bifidobacterium* isolates (n=39) to metabolize 49 different
204 carbohydrates was tested using the API 50 CH kit and API 50 CHL broth (bioMérieux,
205 France) according to manufacturer's instructions. Assay strips were incubated
206 anaerobically at 37 °C for 8 days. Results for each isolate were recorded at two times (2
207 and 8 days) as positive or negative for acid production from each carbon source based on
208 colour reaction. If acid production (colour change) was observed at 8 days of incubation,
209 it was recorded as a delayed reaction.

210 Type strains *B. breve* ATCC 15700, *B. longum* subsp. *infantis* ATCC 15697 and
211 *B. dentium* ATCC 27534 were included in the assay for comparison with vaginal isolates.
212 Previously published descriptions of carbon source utilization by other type strains were
213 also used for comparison (*B. longum* subsp. *longum* [37], *B. bifidum* [38], *B. adolescentis*
214 [37], *B. kashiwanohense* [39], *B. catenulatum* [40], *B. pseudocatenulatum* [41] and *A.*
215 *omnicolens* [42]). A Jaccard index was used to measure pairwise distances between
216 isolates based on their fermentation patterns by using the *vegan* package (function
217 'vegdist') in R [43]. This distance matrix was used for hierarchical clustering using the
218 'hclust' function in R, with UPGMA (Unweighted Pair Group Method with Arithmetic
219 Mean).

220 **2.7 Hydrogen peroxide assay**

221 A modified Brucella agar media (mB) containing 0.25% (w/v)
222 tetramethylbenzidine (TMB, Sigma-Aldrich, Oakville, ON) and 0.01% (w/v) horseradish
223 peroxidase (HRP, Sigma-Aldrich, Oakville, ON) was used for chromogenic detection of

hydrogen peroxide (H_20_2) production [44]. Isolates (n=40) grown on CSB agar were streaked onto mB agar plates. Alternatively, 1 mL of sterile PBS was used to wash colonies off the CSB plate and the resulting suspension was adjusted to 1 McFarland and spotted on to mB plates using a sterile replicator. mB plates were incubated anaerobically at 37 °C for 72 h, removed from the incubator and held at room temperature in air for 30 minutes for colour development. Hydrogen peroxide production was reported as positive or negative based on the colour of the colonies (no colour/pale beige = negative, green/blue = positive). Type strains *B. breve*, *B. longum* subsp. *infantis* and *B. dentium* were also included in the assay. Two independent cultures (biological replicates) were tested for each isolate.

2.8 Lactic acid assay

Isolates were grown overnight in Modified Reinforced Clostridial broth (for *Bifidobacterium* and *Alloscardovia*, n=40) or deMan, Rogosa and Sharpe broth (MRS) (for *Lactobacillus*, n=4). All cultures were adjusted to an initial optical density (OD_{600}) ≅ 0.1. After incubation, broth cultures were pelleted and supernatants were heat-treated at 80 °C for 15 minutes to stop enzymatic reactions. Lactic acid quantification was conducted using the D- and L-lactic acid Enzymatic BioAnalysis/Food Analysis UV method kit (R-Biopharm, Darmstadt, Germany). Results were reported as concentration of lactic acid per OD_{600}. Type strains *B. breve*, *B. longum* subsp. *infantis* and *B. dentium* were also included in the assay. Three biological replicates were performed for each isolate. Concentration of lactic acid produced by different species was compared by Kruskal-Wallis test using IBM SPSS (Statistical Package for the Social Sciences, version

21).

2.9 Tolerance of bifidobacteria to low pH and lactic acid

Experiments to test tolerance of bifidobacteria to lactic acid and low pH were based on O'Hanlon, Moench and Cone (2011) [45] with minor modifications. A subset of *Bifidobacterium* spp. (n=15) and *Gardnerella vaginalis* (n=3) isolates were grown overnight in Modified Reinforced Clostridial broth (pH 6.8) or NYC III broth (pH 7.3), respectively. For species where only one or two isolate(s) were available, all were included. Where more than two isolates were available (*B. breve, B. longum, B. dentium* and *B. bifidum*), two isolates were selected randomly to be included in the assay.

An aliquot (50 μL) of the overnight inoculum was added to each control or experimental medium (final volume 5 mL) and then incubated anaerobically at 37 °C for 2 hours. Control media was prepared at pH 6.8. Experimental media was prepared at pH 4.5 with the following concentrations of lactic acid: 0 mM, 1 mM, 10 mM, 100 mM and 1000 mM, and at pH 3.9 with (100 mM) and without lactic acid (0 mM). After 2 hours exposure, each sample was serially diluted with the appropriate medium containing 200 mM HEPES and track-plated [46]. The pH of each experimental or control medium was re-measured after the 2 hours incubation to confirm it had remained within 0.2 pH units of the starting pH. Agar plates were incubated anaerobically at 37 °C for 48 h and colony forming units (cfu/mL) were counted. Type strains *B. breve*, *B. longum* subsp. *infantis* and *B. dentium* were also included in the assay. The assay for each isolate was performed in triplicate. The percent bacterial survival was calculated based on the ratio of colony counts ($\log_{10}$ cfu/mL) of bacteria that had been incubated in experimental media (treatment) compared to colony counts ($\log_{10}$ cfu/mL) of bacteria that had been incubated

269 in control media (control), according to the following formula:

$$Percent\ survival = \frac{log_{10}{}^{cfu}/_{mL}\ treatment}{log_{10}{}^{cfu}/_{mL}\ control} \times 100$$

270

271 **2.10 Susceptibility to antibiotics**

272 Susceptibility of bifidobacteria to clindamycin and metronidazole was evaluated
273 using Etest strips (bioMérieux, France) in a subset of bifidobacteria isolates (n=22). The
274 subset selection was based on the following criteria: for species where ≤4 isolate(s) were
275 available, all were included. Where more than four isolates were available (*B. breve, B.*
276 *longum, B. dentium* and *B. bifidum*), four isolates were selected randomly to be included
277 in the assay. Overnight cultures grown in Modified Reinforced Clostridial broth were
278 adjusted to 1 McFarland. The suspension was spread evenly on Columbia sheep blood
279 agar plates and Etest strips were placed on the agar surface. Plates were incubated
280 anaerobically at 37 °C for 48 h. The minimum inhibitory concentration (MIC) for each
281 antibiotic was read as the lowest antibiotic concentration at which growth was inhibited.
282 Two biological replicates were performed for each isolate, and the average result was
283 reported.

284

285 **3. Results**

286 **3.1 Confirmation of *Bifidobacterium*-dominated vaginal microbial profiles**

287 We identified 21/492 (4.2%) of the previously published vaginal microbiome

288 profiles that were dominated (>50% of sequence reads) by *Bifidobacterium*-like
289 sequences, of which eight were dominated by *B. breve*, five by *B. longum*, three by *B.*
290 *dentium* and five by *A. omnicolens.* An additional 6% (29/492) of microbiome profiles
291 had intermediate (1-50%) levels of *Bifidobacterium*-like sequences and 59.5% (293/492)
292 of profiles had low (<1%) levels. *Bifidobacterium*-like sequences were undetected in
293 30.3% (149/492) of samples.

294 All samples (n=492) were ranked based on the total *Bifidobacterium*-like relative
295 abundance, previously determined by *cpn*60 amplicon sequencing. A total of forty-two
296 samples with high (>50%, n=11), medium (1-50%, n=11), low levels (<1%, n=10) and
297 undetected *Bifidobacterium*-like sequences (n=10) (i.e., samples with ≤ 2 reads) were
298 randomly selected from the 492 samples for qPCR analysis. This sample set (n=42) was
299 assayed for each of the three targets (*B. breve, B. dentium* and *A. omnicolens*) by species-
300 specific qPCR. The copy number for each target determined by qPCR was expressed as a
301 proportion of the total bacterial load, estimated by 16S rRNA gene copy number [30,31].
302 Sequence read numbers (% abundance), qPCR results for *B. breve, B. dentium* and *A.*
303 *omnicolens*, and previously determined total 16S rRNA copy numbers are provided in
304 Supplemental Table 2, and results are summarized in Figure 1.

305 For samples in the high category, the three targets analyzed (*B. breve, B. dentium*
306 and *A. omnicolens*) comprised approximately 100% of the estimated total bacterial load
307 (Figure 1, left panel). In two of the *B. breve* dominated samples where *A. omnicolens* was
308 also detected in the microbiome profiles, both targets were detected by qPCR. *B. breve*
309 and *B. dentium* were also detected by qPCR in two of the three high category samples
310 where the microbiome profiles were dominated by other *Bifidobacterium* species not

311 targeted by any of the qPCR assays. All five samples with medium levels (1-50% of
312 sequence reads) of *B. breve* based on *cpn*60 amplicon sequencing, had detectable *B.*
313 *breve* sequences by qPCR (Figure 1, middle panel). Estimates of proportional abundance
314 of *B. breve* in these samples based on qPCR (77-113%), however, were much higher than
315 the proportional abundance of corresponding sequence reads in the *cpn*60 sequence-based
316 microbiome profiles (all <10%). Two of the three samples with medium levels of *B.*
317 *dentium* were qPCR positive for this target, but again the qPCR based estimate of
318 proportional abundance (82-102%) was much higher than that predicted by sequence read
319 numbers (both <25%). Samples with medium levels of *A. omnicolens* were negative by
320 species-specific qPCR, but *A. omnicolens* was detected by qPCR in other samples in the
321 medium category that were dominated by *B. breve*, *B. dentium* or other bifidobacteria.
322 Samples in the low and undetected categories were negative for all three qPCR assays
323 (Figure 1, right panel).

324 When all samples (n=42) were considered, regardless of abundance category,
325 there was a positive correlation between *cpn*60 amplicon sequencing data (% of sequence
326 reads) and qPCR values ($\log_{10}$ copies per swab) for all three targets (*B. breve* ρ=0.671,
327 p<0.0001; *B. dentium* ρ=0.502, p=0.001; *A. omnicolens* ρ=0.784, p<0.0001). The
328 correlations remained significant when samples with zero values were removed from
329 analysis.

330 **3.2 Vaginal *Bifidobacterium* isolates**

331 A total of 40 isolates from the vaginal swabs of 26 women from Canada and
332 Kenya were included in the study (Supplementary Table 1). Based on the *cpn*60 gene
333 PCR amplification, sequences of the 40 isolates were compared to cpnDB and the nearest

type strain sequence was identified as *B. breve* n=15 (98.9-99.8% sequence identity over 552 bp), *B. longum* n=11 (99.1-100%), *B. dentium* n=4 (99.1-99.5%), *B. bifidum* n=3 (100%), *B. adolescentis* n=2 (94.4-95.1%), *B. kashiwanohense* n=2 (97.6-97.8%), *B. catenulatum* n=1 (99.1%), *B. pseudocatenulatum* n=1 (99.3%) and *Alloscardovia omnicolens* n=1 (99.5%). A phylogenetic tree based on the *cpn*60 UT sequences of the 40 vaginal isolates and 12 reference sequences from type strains of human origin is shown in Figure 2. Isolates whose nearest reference species were *B. adolescentis* (N1D05, N5F04) or *B. kashiwanohense* (N4G05, N5G01) clustered separately from the type strains with good bootstrap support.

3.3 Carbohydrate fermentation patterns

Vaginal bifidobacteria isolates were tested for their ability to metabolize 49 different carbon sources. The relatedness of the fermentation patterns of vaginal bifidobacteria (n=39) and three available type strains (n=3) was visualized by UPGMA clustering (Figure 3). As expected, most strains clustered based on species identity, except for the two *B. kashiwanohense*-like isolates (N4G05 and N5G01) and three *B. longum* isolates ((W)35-1, (I)239-2 and (IV)239). (I)239-2 and (IV)239 were the only *B. longum* isolates that metabolized glycogen, gentiobiose and L-fucose, and (W)35-1 was the only *B. longum* strain not able to metabolize D-ribose. Also, most *B. longum* isolates (7/11) did not cluster with the type strain (*B. longum* subsp. *infantis* ATCC 15697) based on their fermentation patterns. The use of trehalose by *B. breve* differed between the type strain and most vaginal isolates (type strain was negative and 13/15 vaginal isolates were positive). Vaginal *B. dentium* isolates mainly differed from the type strain due to their ability to use D-cellobiose (4/4). Overall, for *B. breve* isolates (n=15), there was a

357 complete agreement between vaginal isolates and the type strain ATCC 15700 (gut
358 origin) for utilization of 71% (35/49) of carbon sources tested. For *B. longum* (n=10),
359 vaginal isolates and type strain ATCC 15697 (gut origin) had a complete agreement for
360 51% (25/49) of carbon sources. For *B. dentium* (n=4), a complete agreement of 84%
361 (41/49) was observed between vaginal isolates and type strain ATCC 27534 (oral cavity
362 origin).

363 A second analysis was performed considering the percentage of vaginal isolates
364 that were positive for each carbon source in comparison to the literature description of the
365 type strains (Supplemental Table 3). This analysis enabled the comparison of isolates for
366 which the type strain was not available for inclusion in our fermentation assays. For *B.*
367 *adolescentis*, vaginal isolates (2/2) did not utilize D-sorbitol and D-melezitose, unlike the
368 type strain [38]. *B. kashiwanohense* isolates (2/2) differed from their type strain due to
369 the ability to metabolize D-trehalose and D-melezitose [39]. Regarding *B. bifidum*, the
370 main difference was the fermentation of D-raffinose by all vaginal isolates (3/3), which
371 differs from the type strain and other gut *B. bifidum* strains [3]. Also, vaginal *B. bifidum*
372 did not ferment D-cellobiose, diverging from its type strain [3]. Despite these few
373 differences, overall fermentation patterns of vaginal isolates did not differ considerably
374 from literature descriptions of the type strains.

375 **3.4 Production of hydrogen peroxide**

376 Hydrogen peroxide production was detected in 32.5% (13/40) of the
377 bifidobacteria. All *B. dentium* (n=4) strains were positive and all *B. kashiwanohense*
378 (n=2), *B. catenulatum* (n=1), *B. pseudocatenulatum* (n=1) and *A. omnicolens* (n=1) were
379 negative. Production of hydrogen peroxide by *B. breve* (3/15), *B. longum* (4/11), *B.*

bifidum (1/3) and *B. adolescentis* (1/2) was variable (Figure 4).

3.5 Production of lactic acid

Lactic acid production was measured for all vaginal bifidobacteria isolates using a commercial assay. Four vaginal isolates of *L. crispatus*, a species associated with high lactic acid production in the vagina, were included for comparison. All vaginal bifidobacteria produced lactic acid (n=40) (Figure 5). While bifidobacteria produced L-lactic acid only, *L. crispatus* produced both D and L lactic acid isomers. As expected, there was significant correlation between the absolute values of total lactic acid concentration (mM) and supernatant pH ($p < 0.01$, Pearson = 1), and culture OD_{600} ($p < 0.0001$, Pearson = 1) (data not shown). A Kruskal-Wallis test was conducted to evaluate differences in the production of lactic acid among species (only species with two or more isolates were included in the statistical analysis). The overall test was significant (χ^2 (6, n=30) = 24.7, $p \leq 0.0001$), hence pairwise comparisons among the groups were conducted with Mann-Whitney test. The highest concentration of lactic acid among the bifidobacteria was produced by *B. longum* isolates, which did not differ from the *L. crispatus* strains (Mann-Whitney, p=0.240) (Figure 5).

3.6 Tolerance to low pH and lactic acid

A subset of vaginal bifidobacteria isolates (15/40) and three type strains were selected for testing tolerance of low pH and high lactic acid concentrations and the percent survival values for each isolate under different conditions are shown in Figure 6.

All bifidobacteria tested were able to survive at pH 4.5 with up to 100 mM lactic acid; conditions typical of vaginal fluid of healthy women with a *Lactobacillus* dominated microbiome and that have been shown to be lethal to many vaginosis

403 associated bacteria [45]. Survival of a few isolates was affected by 1000 mM lactic acid,
404 which is ten times more concentrated than physiologic levels. Surprisingly, pH 4.5 and
405 100 mM lactic acid did not affect *G. vaginalis*. The experiment was repeated using media
406 at pH 3.9 (pK_a of lactic acid), which resulted in drastic reduction in *G. vaginalis* survival,
407 regardless of the inclusion of lactic acid. Most bifidobacteria were tolerant to this more
408 acidic condition, with or without lactic acid (Figure 6). *A. omnicolens* and *B.*
409 *kashiwanohense* (n=2) were the isolates most affected by lactic acid, with survival
410 declining considerably when incubated with 100 mM lactic acid (pH 3.9). Two
411 bifidobacteria type strains, *B. breve* ATCC 15700 and *B. longum* subsp. *infantis* ATCC
412 15697, were also affected by this condition, with only 45% survival compared to the
413 culture incubated at neutral pH.

414 **3.7 Susceptibility to antibiotics**

415 Selected representative isolates were tested for susceptibility to clindamycin and
416 metronidazole, the two antibiotics most widely used to treat vaginal dysbiosis (Table 2).
417 Only one isolate (*B. breve* (I)30-1) presented a MIC higher than 8 μg/mL for
418 clindamycin, which is the breakpoint of resistance for anaerobic bacteria according to
419 The National Committee for Clinical Laboratory Standards [47]. For metronidazole,
420 15/22 isolates had a MIC higher than ≥ 32 μg/mL, the breakpoint for metronidazole
421 resistance [47]. All fifteen of these isolates had a MIC ≥ 256 μg/mL, the maximum
422 antibiotic concentration in the Etest strip.

423

424 **4. Discussion**

As a result of microbiome characterization by culture-independent, DNA sequence based methods there is a growing appreciation of "atypical" vaginal microbiomes in healthy women, such as the *Bifidobacterium*-dominated profiles. However, it is known that DNA extraction methods and PCR amplification biases affect the sequencing outcome, resulting in a view of the abundances of species within the community that is inevitably distorted to some degree. Thus, for a careful investigation of the microbial composition, further evaluation using alternative techniques is recommended. Bifidobacteria present a particularly interesting and important subject for this type of investigation due to their recognized importance in the neonatal gut, their probiotic potential, and their frequent underrepresentation in PCR based microbiome profiles. Furthermore, the current diagnostic definition of a healthy vaginal microbiome is limited to a *Lactobacillus*-dominated community, and the available gold standard diagnostic method (Gram stain and Nugent scoring [48]) is interpreted based on this restrictive definition.

In this study, we used species-specific qPCR to quantify bifidobacteria DNA in vaginal samples showing a wide range of proportional abundances of bifidobacteria based on *cpn*60 amplicon sequencing. Our results showed a strong positive correlation between sequence read abundance and qPCR values for the three species tested (Supplemental Table 2). However, when we expressed bifidobacteria abundance as a proportion of the estimated total bacterial population, the values calculated for the medium abundance category (1-50% bifidobacteria sequences in the *cpn*60 microbiome profile), estimates were much higher than expected. One possible explanation for this is that the 16S rRNA based estimates were biased (underestimated) due to the composition

448 of the samples, resulting in a corresponding overestimation of proportional abundance of
449 bifidobacteria DNA. The biases of "universal" 16S rRNA gene primers are well
450 recognized [49,50], making estimates of total bacterial population size challenging using
451 this approach. Better approaches include flow cytometry [51], which counts bacterial
452 cells rather than relying on detection of sequences. However, fresh samples are preferred
453 for this approach, which were not available for our study. While it is also possible that the
454 *cpn*60 amplicon sequencing method underestimated the abundance of bifidobacteria due
455 to preferential amplification of other sequences present in the samples, this seems
456 unlikely given the abundance of *Bifidobacterium* sequence reads in other libraries within
457 the same studies (i.e. the high category) and previous demonstrations of the efficient
458 amplification of *Bifidobacterium cpn*60 sequences from complex samples [26]. A few
459 samples with medium levels of bifidobacteria sequence reads were qPCR negative for
460 targets expected to be present. The medium category included samples containing a wide
461 range (1-50%) of bifidobacteria abundance, and the lack of qPCR detection in samples
462 with bifidobacteria levels near the 1% cut off may be due to the detection limit of the
463 assays. Taken together, our results confirm that *cpn*60 amplicon sequencing reflects
464 relative abundance of bifidobacteria and that *Bifidobacterium* spp. can be the dominant
465 component of the vaginal microbiome. Future studies using more accurate methods for
466 total bacterial population quantification will be needed to better describe this relationship
467 in the context of overall community size.

468 To learn more about the characteristics of vaginal bifidobacteria detected in
469 sequence-based microbiome studies, we collected 40 isolates from vaginal swabs of
470 clinically healthy women, with most isolates being identified as *B. breve* or *B. longum*

(Figure 2). A few differences were observed in carbon source utilization patterns between vaginal isolates and type strains, however, most of those differences have also been described among other bifidobacteria of gut or oral cavity origin. For example, unlike the type strain, vaginal *B. bifidum* isolates did not ferment D-cellobiose, but this characteristic is also absent in some gut *B. bifidum* [3]. Similarly, our vaginal *B. dentium* isolates and other oral cavity isolates utilize D-cellobiose, which is not done by the type strain [3]. Thus, this lack of agreement in carbon source utilization between vaginal isolates and type strains does not necessarily indicate that there is a distinctive fermentation profile of vaginal bifidobacteria. Our results support the idea that different body sites, such as gut and vagina, host similar strains of bifidobacteria. However, a more detailed examination of the utilization of nutrients that differentiate these environments and the genome contents of vaginal and gut isolates of *Bifidobacterium* spp. may yet reveal signs of niche specialization that are not apparent in examinations of housekeeping functions.

Hydrogen peroxide and lactic acid production have been associated for decades with the protective role of vaginal *Lactobacillus*, but more recent findings have called into question the significance of the physiological concentrations of H_2O_2 produced by *Lactobacillus*. Vaginal H_2O_2-producing lactobacilli are claimed to inhibit growth of opportunist bacteria, which share the same niche with lactobacilli and may not have protective mechanisms like production of catalase or peroxidase [52]. However, it is unclear whether *Lactobacillus* is able to produce H_2O_2 under the hypoxic condition of the vagina [53], since oxygen is required for its production. Additionally, cervicovaginal fluid and semen contain proteins, glycoproteins, polysaccharides, and lipids that react

494 with and inactivate H_2O_2 [54]. O'Hanlon, Moench, & Cone (2011) also reported that
495 physiological concentrations of H_2O_2 had no microbicidal activity, while a
496 supraphysiologic concentration of H_2O_2 that was sufficient to inactivate bacterial
497 vaginosis associated bacteria, also inactivated vaginal lactobacilli. We decided to test our
498 bifidobacteria isolates for H_2O_2 production considering the wealth of previous studies and
499 descriptions of H_2O_2 production for *Lactobacillus*, the presumed indicator of a healthy
500 vaginal microbiome. Our results indicate that *in vitro* H_2O_2 production is not widespread
501 in vaginal bifidobacteria (Figure 4).

502 Lactic acid is a key element in promoting a healthy vaginal environment by
503 preventing the overgrowth of bacterial vaginosis-associated microorganisms [45]. One of
504 the protective mechanisms attributed to lactic acid is lowering the vaginal pH [55,56]. It
505 has also been demonstrated that lactic acid is able to disrupt the outer membrane of Gram
506 negative bacteria [57]. Bacteria are known as the primary source of lactic acid in the
507 vagina, with some species being able to produce both lactic acid enantiomers (D and L),
508 as opposed to human cells that only make the L form [55]. Here, we confirmed that
509 vaginal *Bifidobacterium* produced only L-lactic acid, as previously described for other
510 bifidobacteria [58]. While *L. crispatus* and *L. gasseri* are both able to produce a mixture
511 of DL-lactic acid, other *Lactobacillus* spp. have been shown to produce only L- (*L. iners*)
512 or D-lactic acid (*L. jensenii*) [59]. At physiological concentrations (~56 to 111 mM), both
513 forms of lactic acid are effective in decreasing HIV infectivity *in vitro*; at lower
514 concentrations, L-lactic acid has greater virucidal activity than D-lactic acid [60]. L-lactic
515 acid has also been shown to have greater antibacterial effect against *E. coli* (O157 and
516 non-O157) than D-lactic acid [61]. Although the biological significance of producing

different amounts of D and L forms remains unclear, the results of these studies suggest that the microbicidal effect of lactic acid involves more than acidity alone. Since bifidobacteria in general are known lactic acid producing bacteria, our observation of its production is far from surprising. However, what is noteworthy is that the levels of lactic acid produced by vaginal bifidobacteria, especially *B. longum*, are comparable to those of *L. crispatus*, the organism most often associated with a healthy vaginal microbiome [62].

Considering that lactic acid is a hallmark of a healthy vagina, knowing the levels of acid produced by different bifidobacteria is of interest when evaluating whether a vaginal microbiome dominated by *Bifidobacterium* should be considered as protective as a microbiome dominated by *Lactobacillus*. Moreover, assessing the ability of bifidobacteria to persist in high levels of lactic acid and low pH, conditions typical of the vaginal fluid of healthy women, was an important step in improving knowledge of bifidobacteria ecology. Other studies have shown that protonated lactic acid rather than lactate anion is the microbicidal form of lactic acid [45,60], which emphasizes the role of pH in mediating lactic acid activity. We initially conducted our experiments at pH 4.5, recreating conditions used in previous investigations of microbicidal activity of lactic acid against vaginosis-associated bacteria [45]. However, these conditions did not kill any of the three *G. vaginalis* isolates included in our study. We repeated the experiments at pH 3.9, a value of which is now understood to be within the range of expected pH for the cervicovaginal fluid of clinically healthy women (~3.5 - 4.5) [63] and observed differential survival of *G. vaginalis* and the bifidobacteria. Bifidobacteria resistance to low pH and high concentrations of lactic acid may be species specific, as isolates from the same species behaved similarly in most cases (Figure 6). *B. kashiwakonhense*-like

isolates, for example, were clearly less tolerant to these acidic conditions than *B. breve*, *B. dentium, B. bifidum* or *B. adolescentis* isolates. Further studies of larger numbers of isolates will be required to test this observation.

The normal microbiota plays an important role in the recovery of the microbiome after antibiotic treatment. While most vaginal bifidobacteria were susceptible to clindamycin, we observed high rates of metronidazole resistance. Metronidazole and clindamycin are the two antibiotics recommended by CDC to treat bacterial vaginosis, and can be administrated either orally or intravaginally [64]. Although metronidazole is the first-line treatment against bacterial vaginosis [65], many non-spore-forming, Gram-positive anaerobic rods are resistant to it, including *Propionibacterium, Atopobium, Mobiluncus, Bifidobacterium* and *Lactobacillus* [66]. *Gardnerella vaginalis*, a hallmark microorganism in BV, and *Prevotella* have also been demonstrated to be resistant to metronidazole [67,68]. Since *G. vaginalis*, *Atopobium*, *Mobiluncus* and *Prevotella* are considered vaginosis associated bacteria, this might explain the high rates of BV recurrence after metronidazole treatment [65]. Although antimicrobial resistance is a concern in BV treatment, bifidobacteria resistance might be a beneficial factor by facilitating the microbiota recovery after antibiotic administration.

5. Conclusion

In this study we confirmed that a subset of healthy, reproductive aged women have vaginal microbiomes dominated by *Bifidobacterium* spp.. We also demonstrated that vaginal bifidobacteria have the potential to be as protective as lactobacilli according to the current understanding of a “healthy” vaginal microbiome. These results have significant implications for women’s health diagnostics since current protocols based on

Gram staining and Nugent score would likely result in a diagnosis of “intermediate” or “consistent with BV” if a vaginal smear was dominated by *Bifidobacterium* rather than *Lactobacillus*. We expect our findings will help to guide clinicians and researchers to better assess a healthy vaginal microbiome, and avoid unnecessary interventions.

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Conflict of interest

The authors declared that they have no conflict of interests.

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812 **Figure Captions**

813 **Figure 1.** Detection of *B. breve*, *B. dentium* and *A. omnicolens* by sequencing and qPCR,
814 in 42 vaginal microbiomes. Results are shown for samples in the high (n=11, left panel),
815 medium (n=11, middle panel) and low/undetected (n=20, right panel with *)
816 bifidobacteria abundance categories as determined by *cpn*60 amplicon sequencing. All
817 samples in the "low" (n=10) and "undetected" (n=10) categories had identical results so
818 only one example is shown. The bar charts illustrate the proportion of sequences assigned
819 to each species detected, indicated by colour according to the legend. The lower table
820 shows the percentage of *Bifidobacterium* (*B. breve*, *B. dentium* or *A. omnicolens* $\log_{10}$
821 copies) out of the total bacterial load (estimated by 16S rRNA gene $\log_{10}$ copies)
822 (%Bif/16S (qPCR)).
823
824 **Figure 2.** Phylogenetic tree based on *cpn*60 UT sequences of vaginal bifidobacteria
825 (n=40) and reference strains^T. The tree was rooted with *Gardnerella vaginalis* ATCC
826 14018^T and constructed using the Dnaml method with bootstrap values calculated from
827 100 trees. The number at each node represents the percentage bootstrap support.
828
829 **Figure 3.** Carbohydrate fermentation patterns. A. UPGMA dendrogram derived from
830 Jaccard's similarity coefficients calculated among isolates based on their fermentation
831 patterns (isolates n=39, type strains^T n=3). B. Heatmap representing the fermentation of
832 49 carbon sources. Black = positive reaction; Grey = weak reaction; White = no
833 growth/fermentation; * = Delayed reaction.
834

Figure 4. Hydrogen peroxide production of bifidobacteria (n=39) indicated by blue colour on TMB medium (no image was available for isolate N3E01-2). Species designation of each isolate is indicated by coloured dot according to the legend. Positive controls (+). Negative control (-).

Figure 5. Total lactic acid concentration in culture supernatant of vaginal isolates and type strains. Grey boxes below the chart indicate species identification for each isolate. Numbers at the top are the average lactic acid concentrations (mM/OD_{600} ± standard deviation) produced by each species group (excluding type strains^{T}). Results shown are the average of at least three replicate experiments.

Figure 6. Survival (% log cfu/mL) of vaginal bifidobacteria (n=15), bifidobacteria type strains (n=3) and *G. vaginalis* (n=3) after incubation at low pH and high lactic acid concentrations in comparison with bacteria cultured at pH 6.8. Results shown are the average of at least three replicate experiments.

Supplementary Material

Table S1. Complete list of vaginal isolates used in the study.

Table S2. Sequence read numbers (% relative abundance), qPCR results for *B. breve, B. dentium* and *A. omnicolens*, and previously determined total 16S rRNA copy numbers of 42 samples used in the screening analysis.

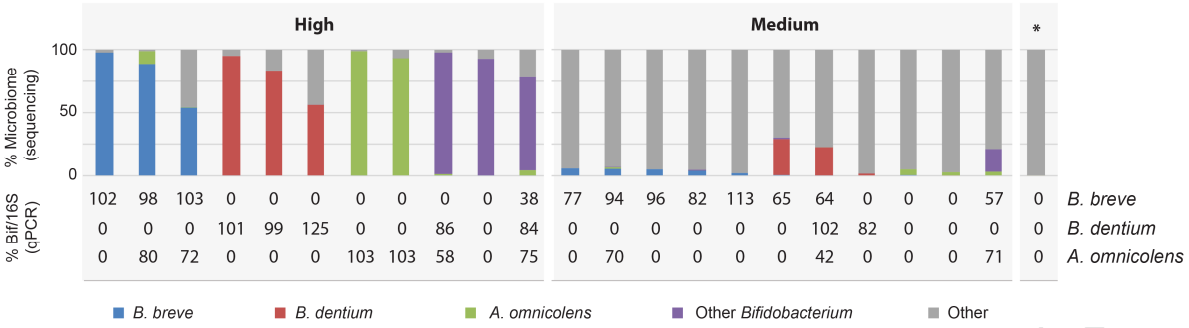
Table S3. Carbohydrate fermentation patterns of vaginal bifidobacteria isolates and of their type strains. Numbers represent the percentage of vaginal isolates that had positive reaction for each carbon source.

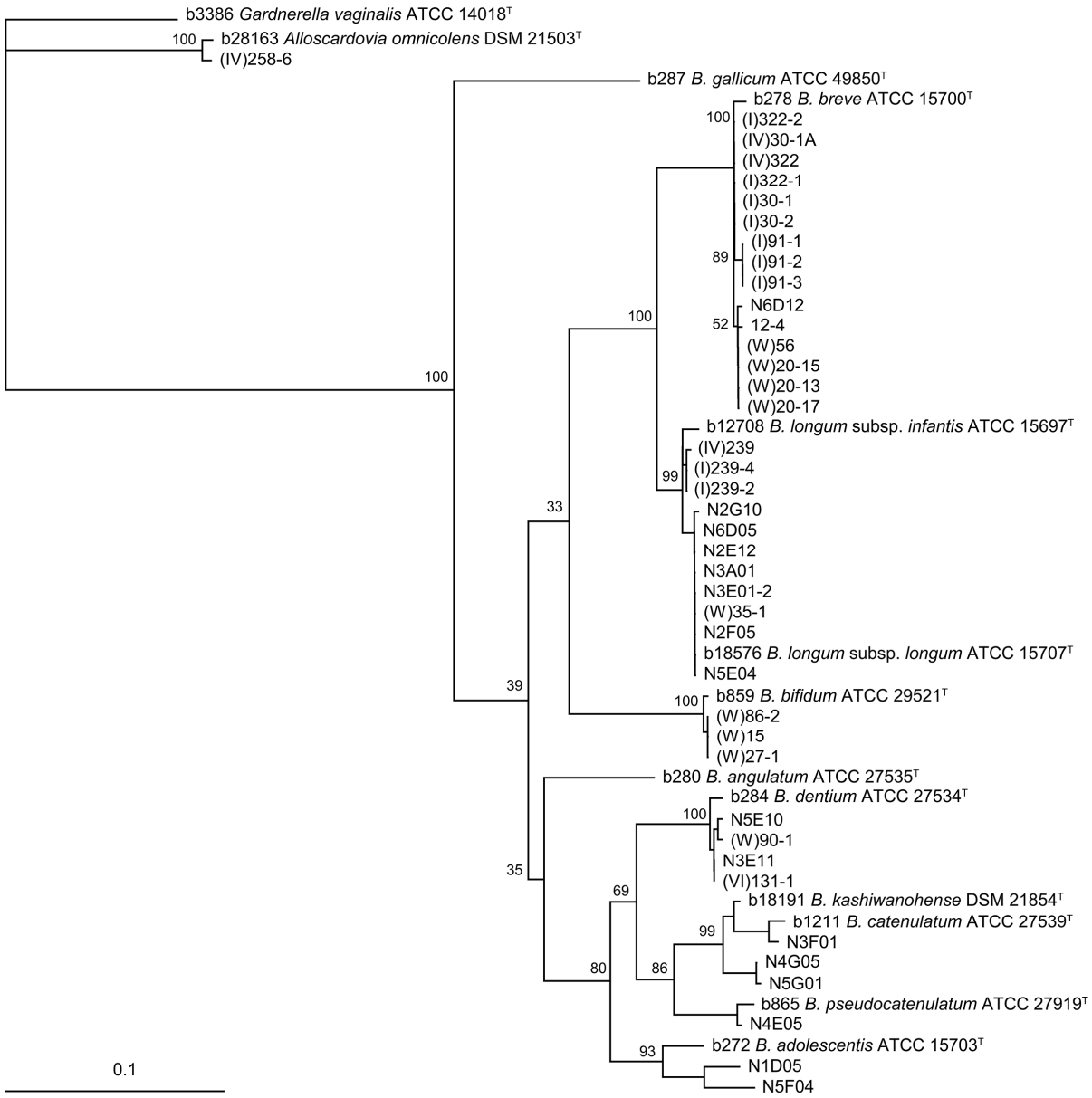
Table 1. Primers used in the bifidobacteria qPCR assays.

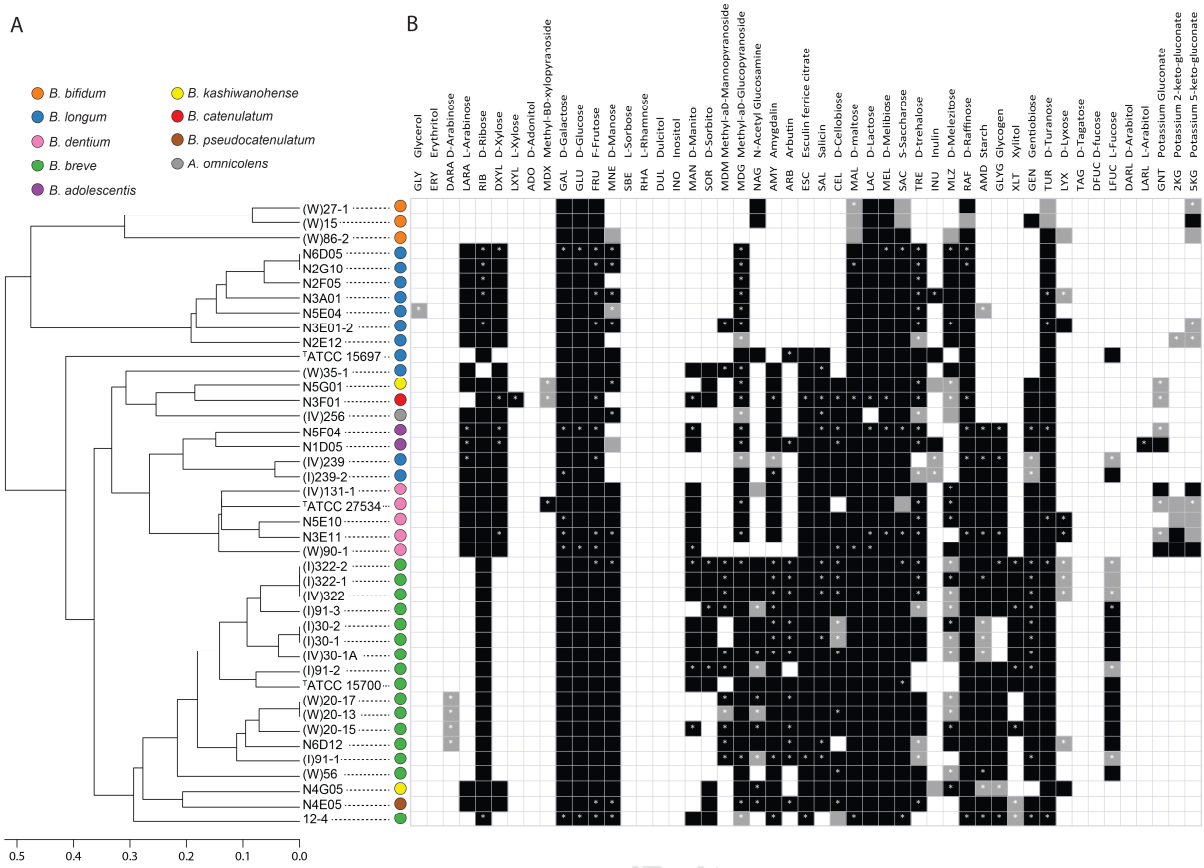
Target	Primer name	Sequence (5'->3')	Product size (bp)	Annealing Temperature (˚C)
B. breve	JH0472 (F)	AACCGTGCTCGCCCAGTC	150	65
	JH0473 (R)	TCCTTGGTCTCAACGTCCTT		
B. dentium	JH0474 (F)	GTGCTCGAAGACCCGTACAT	163	65
	JH0475 (R)	GGATGGTGTTCAGGATCAGG		
A. omnicolens	JH0470 (F)	GCACGAAGGCTTGAAGAACG	197	65
	JH0471 (R)	CCAAAGCCTCAGCAATACGC		

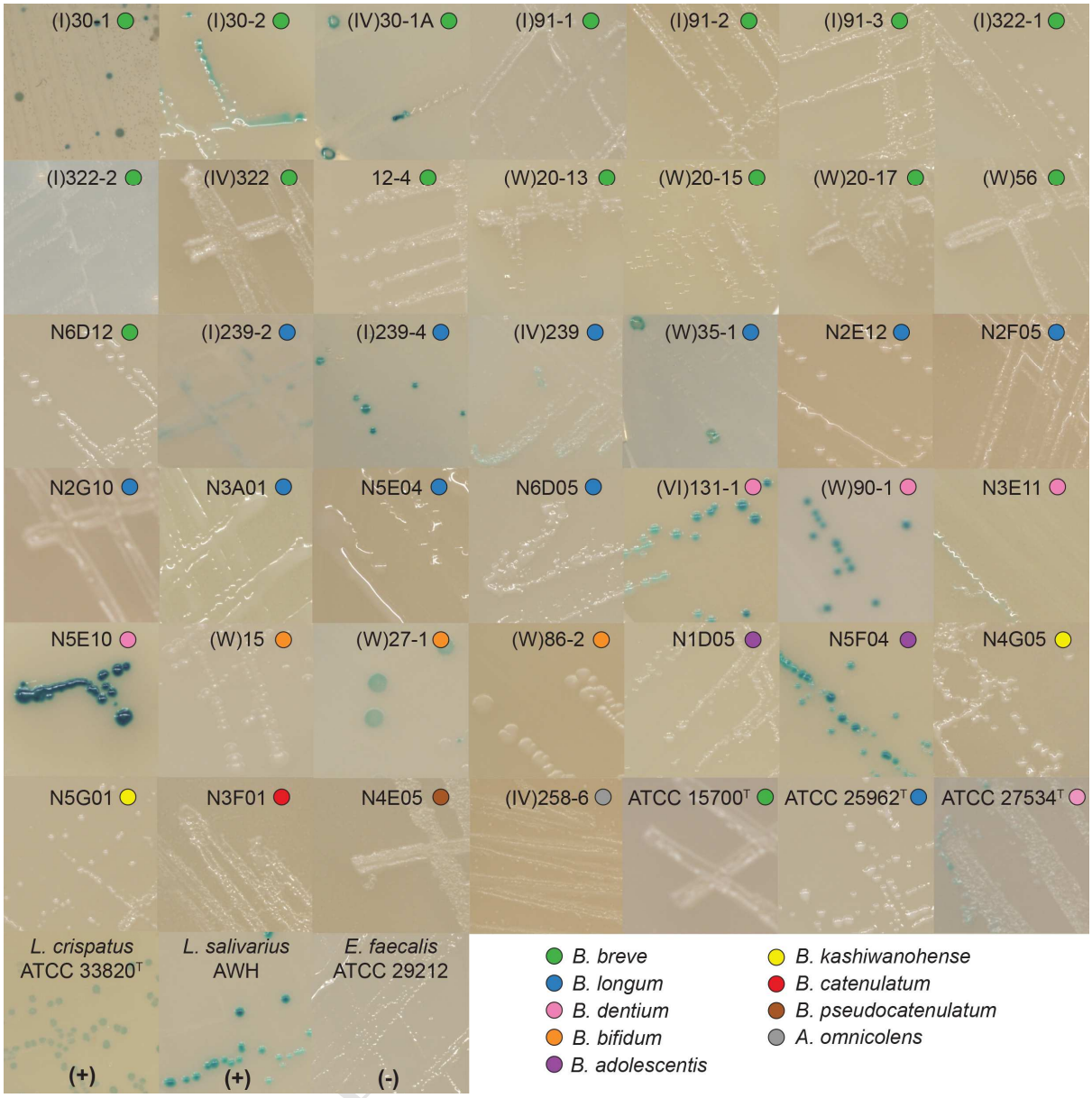
Table 2. Susceptibility of bacteria (n=22) to metronidazole and clindamycin. Results shown are derived from two replicate assays.

		Minimum inhibitory concentration (MIC µg/mL)	
Species	**Isolate**	**Metronidazole**	**Clindamycin**
B. breve	(I)30-1	8.0	32.0
	(I)91-1	>256	≤0.016
	(I)322-1	>256	0.035
	(W)56	>256	0.032
B. longum	(I)239-2	>256	≤0.016
	N2E12	>256	0.056
	(W)35-1	2.0	0.047
	N2F05	>256	0.125
B. dentium	(VI)131-1	4.0	≤0.016
	N3E11	>256	0.250
	N5E10	4.0	≤0.016
	(W)90-1	>256	≤0.016
B. bifidum	(W)15	2.0	0.032
	(W)27-1	2.0	0.032
	(W)86-2	4.0	0.056
B. adolescentis	N1D05	>256	0.158
	N5F04	>256	0.028
B. kashiwanohense	N4G05	>256	0.047
	N5G01	>256	0.028
B. catenulatum	N3F01	>256	0.040
B. pseudocatenulatum	N4E05	>256	0.040
A. omnicolens	(VI)258-6	>256	0.035





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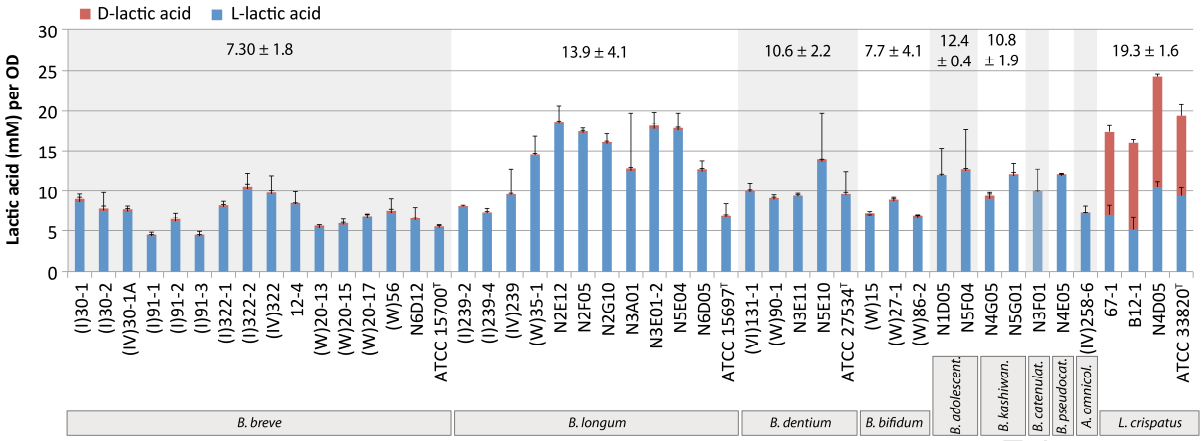
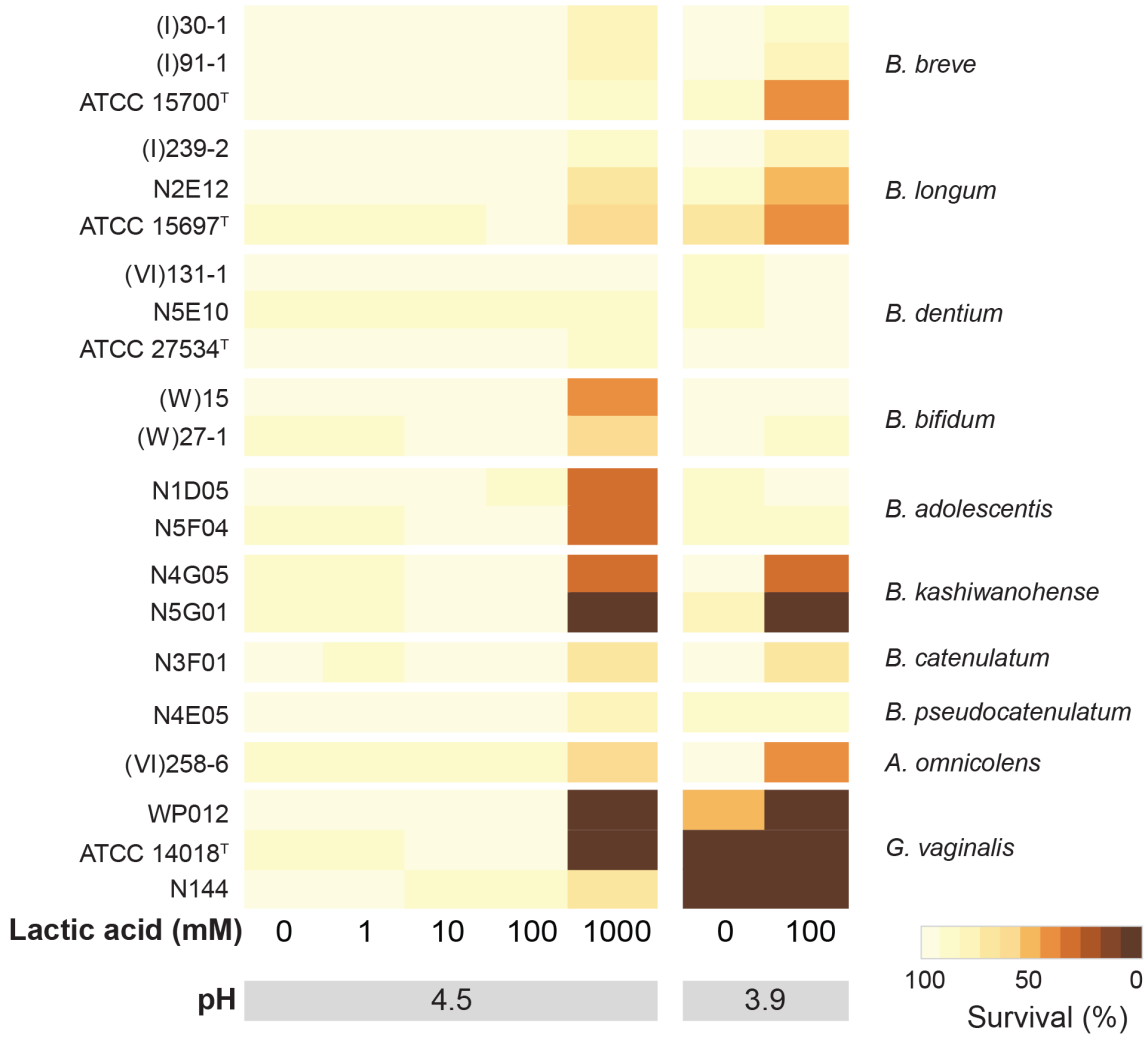
(I)91-2
(I)91-3
(I)322-1
(I)322-2
(IV)322
12-4
(W)20-13
(W)20-15
(W)20-17
(W)56
N6D12
(I)239-2
(I)239-4
(IV)239
(W)35-1
N2E12
N2F05
N2G10
N3A01
N5E04
N6D05
(VI)131-1
(W)90-1
N3E11
N5E10
(W)15
(W)27-1
(W)86-2
N1D05
N5F04
N4G05
N5G01
N3F01
N4E05
(IV)258-6
ATCC 15700T
ATCC 25962T
ATCC 27534T
L. crispatus ATCC 33820T
(+)
L. salivarius AWH
(+)
E. faecalis ATCC 29212
(-)
B. breve
B. longum
B. dentium
B. bifidum
B. adolescentis
B. kashiwanohense
B. catenulatum
B. pseudocatenulatum
A. omnicolens

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N5E10
ATCC 27534T
(W)15
(W)27-1
N1D05
N5F04
N4G05
N5G01
N3F01
N4E05
(VI)258-6
WP012
ATCC 14018T
N144
B. breve
B. longum
B. dentium
B. bifidum
B. adolescentis
B. kashiwanohense
B. catenulatum
B. pseudocatenulatum
A. omnicolens
G. vaginalis
Lactic acid (mM)
0
1
10
100
1000
0
100
pH
4.5
3.9
100
50
0
Survival (%)

Highlights

- Healthy vaginal microbiomes can be dominated by *Bifidobacterium*
- Phenotypic features of bifidobacteria related to reproductive health were analyzed
- Fermentation patterns of vaginal bifidobacteria were similar to those from the gut
- Vaginal bifidobacteria produced and tolerated lactic acid and low pH