Established *Gardnerella* biofilms can survive metronidazole treatment by reducing metabolic activity

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Abstract

Gardnerella spp., a hallmark of bacterial vaginosis, can form biofilm and it has been suggested that failure of antibiotic treatment of bacterial vaginosis and recurrent vaginosis are linked to its ability to form biofilm. Here, we tested the hypothesis that biofilm formation provides protection from the effects of metronidazole. We performed a broth microdilution assay to measure the minimum inhibitory concentration (MIC) of metronidazole for thirty-five *Gardnerella* isolates in two different growth media: one medium in which *Gardnerella* spp. grow primarily as biofilm and the other medium in which *Gardnerella* spp. grow primarily in planktonic form. The MIC of *Gardnerella* isolates observed in the two conditions were highly correlated (R^2= 0.69, $p < 0.001$) and 27/35 isolates had no difference in MIC between the two growth modes. When established biofilms were treated with metronidazole, live *Gardnerella* could be recovered following treatment in most cases (7/9 isolates tested). Metabolic activity of established biofilms of thirty-one isolates with and without metronidazole treatment was measured using a resazurin assay. Most (27/31) isolates showed reduced metabolic activity following treatment with 128 μg/ml of metronidazole relative to untreated controls. The amount of biofilm produced by *Gardnerella* isolates was not enhanced by sub-inhibitory concentrations of metronidazole and scanning electron microscopy revealed no architectural differences between treated and untreated biofilms. Our results suggest that *Gardnerella* spp. growing in established biofilms reduce metabolic activity as a mechanism of protection from the bactericidal effects of metronidazole.

42 **Introduction**

43 *Gardnerella* spp. are considered a hallmark of bacterial vaginosis: a vaginal dysbiosis defined by
44 the shift from a *Lactobacillus* spp. dominated microbiome to a more diverse microbiome,
45 comprising many aerobic and anaerobic bacteria, including *Gardnerella* spp.. The genus
46 *Gardnerella* includes at least four cpn60-defined subgroups corresponding to four whole-genome
47 sequence defined clades, which were more recently amended to thirteen genome species (1–3).
48 One major diagnostic feature of bacterial vaginosis is the presence of clue cells – epithelial cells
49 coated with multispecies biofilm (4, 5). It has been observed that *Gardnerella* accounts for a
50 significant proportion of this multispecies biofilm *in vivo* (6), and several studies have shown
51 that *Gardnerella* spp. can also form biofilm *in vitro* (7–10). Since the recognition of multiple
52 *Gardnerella* spp., it has also been demonstrated *in vitro* that *Gardnerella* can form multispecies
53 biofilm (8). Biofilm formation is often considered a stress response that protects bacterial cells
54 from environmental stresses such as antimicrobial substances, immune cells, and predators (11–
55 15). Alternatively, biofilm may be a natural and preferred mode of growth for some bacteria in
56 particular environments (12).

57 Growth in biofilm form can provide protection from antibiotics in a variety of ways including
58 enhanced production of extracellular matrix, reduction of metabolic activity, generation of
59 environmental heterogeneity, and induction of phenotypic diversity (16, 17). Slow diffusion of
60 antimicrobials within a biofilm may allow the bacteria living within to produce more
61 extracellular matrix to prevent penetration of antibiotics (18). In fact, for some well characterized
62 bacterial species, sublethal concentration of antibiotics have been demonstrated to lead to
63 increased production of biofilm (19–22).

64 Metronidazole, a bactericidal drug that causes DNA breakage by formation of reactive oxygen
65 species (23), is widely prescribed for the treatment of bacterial vaginosis (9) and there is
66 increasing concern about treatment failure (24, 25). Resistance to metronidazole can occur
67 through reduced uptake of the prodrug, increased export and nitroimidazole reductase activity
68 (26, 27). The ability of *Gardnerella* spp. to form biofilm is often suggested to be correlated with
69 metronidazole treatment failure and recurrent BV (9, 24, 25, 28). Although metronidazole
70 resistance in *Gardnerella* spp. has been reported, no mechanisms other than their capacity to
71 form biofilm has yet been suggested (5, 24, 25, 29). The objectives of our current study,
72 therefore, were to determine if biofilm formation by *Gardnerella* spp. provides protection against
73 metronidazole, if sub-inhibitory concentrations of metronidazole stimulate biofilm production,
74 and to identify possible mechanisms of protection against metronidazole treatment of established
75 *Gardnerella* biofilm.
76
77 **Methods**
78 **Bacterial Isolates**
79 Thirty-five *Gardnerella* isolates representing the four subgroups defined by cpn60 barcode
80 sequence (n= 9, subgroup A (including *G. swidsinskii* and *G. leopoldii*); *n*= 10, subgroup B (*G.*
81 *piotii* and genome sp. 3); *n* = 9 subgroup C (*G. vaginalis*); and *n*= 7, subgroup D (genome sp. 8,
82 9 and 10) were revived from freezer stocks on Columbia agar plates supplemented with 5% (v/v)
83 sheep blood by incubating them anaerobically for 48 h at 37 ºC (BD GasPak EZ Anaerobe Gas
84 Generating Pouch System, NJ, USA). To prepare inoculum for the broth microdilution assay,
85 approximately ten well-isolated colonies from each blood agar plate were transferred to 5 ml

86 brain heart infusion (BHI) medium supplemented with 0.25% (w/v) maltose and 10% (v/v) heat
87 inactivated horse serum and incubated anaerobically for 18 h at 37 ºC.
88
89 **Broth microdilution assay**
90 A stock solution, of metronidazole (102.4 mg/ml, M3761-25G, Sigma-Aldrich, ON, Canada) in
91 DMSO was prepared and stored at -20 ºC. Immediately before each experiment, the stock
92 solution was diluted 1:100 in DMSO to make a 1024 µg/ml solution. A broth microdilution assay
93 was performed to determine the minimum inhibitory concentration (MIC) of metronidazole (30).
94 Briefly, 100 µl of media were aliquoted into each well of a flat bottom 96 well plate (Corning
95 Costar, NY, USA) using a multichannel pipettor. To make two-fold serial dilutions, 100 µl of
96 1024 µg/ml metronidazole was added to each well of the first column of a 96-well plate. After
97 mixing by pipetting up and down 4-6 times, 100 µl was transferred to the second column, and the
98 process was repeated to column 10. After pipetting up and down, 100 µl from column 10, instead
99 of transferring to column 11, was discarded. Column 11 was used as growth control (no
100 antibiotic). Column 12 was used negative control (sterile media). A freshly grown broth culture
101 was adjusted to an OD_{595} of 0.5, corresponding to 10^6-10^7 cfu/ml, and 5 µl of adjusted broth
102 culture was added to each well of the 96 well plate, up to column 11. The plates were then
103 incubated anaerobically at 37 °C for 72 h. The process was repeated for all 35 isolates, and each
104 isolate was tested in two growth media: BHI + 0.25% maltose (v/v) and BHI + 0.25% maltose
105 (v/v) + 10% heat inactivated horse serum.
106
107 **Co-culture microbroth dilution assay**

108 Four representative isolates of the four subgroups were selected for a co-culture assay (Subgroup
109 A, VN003; Subgroup B, N170; Subgroup C, N165, and Subgroup D, NR002). All four isolates
110 were revived on blood agar plates. Colonies were harvested and resuspended in BHI + 0.25%
111 maltose (v/v) + 10% inactivated horse serum. The resuspended isolates were then incubated for
112 18 h anaerobically at 37 °C. The OD_{595} of fresh cultures were adjusted to 0.5. Isolates were
113 mixed in equal proportions in snap cap tubes (15 ml) in six combinations: AB, AC, AD, BC, BD,
114 and CD. Broth microdilution assays of co-cultures were performed as described above.
115
116 **Quantification of total growth, planktonic and biofilm growth**
117 The total growth in each well at 72 h was determined by measuring optical density at 595 nm
118 using a microplate reader (VarioSkan LUX Multimode plate reader). After measuring the total
119 growth, which includes both the planktonic bacteria and any biofilm formed at the bottom of the
120 well, the supernatant portion (planktonic growth) was transferred to a fresh flat-bottom 96 well
121 plate scanned at 595 nm. Biofilm quantification was performed using a CV assay as described
122 previously (8). Briefly, the 96-well plates were thoroughly washed twice with water. The wells
123 were then stained with 1% (w/v) crystal violet for 20 minutes. Then the plates were washed
124 twice with water and were dried before the addition of 33% (v/v) glacial acetic for biofilm
125 solubilization. The plates were then read at 595 nm to quantify biofilm.
126
127 **Metabolic activity and viability measurement**
128 Bacterial colonies harvested from blood agar plates were transferred to BHI + 0.25% maltose +
129 10% heat-inactivated horse blood serum and were incubated anaerobically for 18h. Inoculum
130 was prepared using 18 h old culture by adjusting the OD to 0.5 (corresponding to 10^6-10^7 cfu/ml)

131 in BHI + 0.25% maltose and 100 µl was pipetted into each well of duplicate 96-well plates (one
132 plate for viability assay and one for metabolic activity measurement). One column of each plate
133 was maintained containing sterile media only as negative control. After 48h of anaerobic
134 incubation at 37 °C, each plate was divided into two sections: rows A through D as control and E
135 through H as treatment. Overall OD_{595} was recorded, and the planktonic portion was transferred
136 to a new plate to measure planktonic OD_{595} only. Fresh BHI + 0.25% maltose was pipetted into
137 the control wells. For the treatment wells, fresh BHI + 0.25% maltose was supplemented with
138 metronidazole at a final concentration of 128 µg/ml. Media supplemented with metronidazole
139 were pipetted into treatment wells (Rows E-H) and the 96-well plates were incubated
140 anaerobically at 37 °C for another 24h. Following 24h incubation, overall OD_{595} was measured.
141 The planktonic fraction (supernatant) was transferred to a new plate and OD_{595} was recorded.

142 To determine viability of the cells growing in the biofilm mode post-treatment, biofilm formed at
143 the bottom of wells were scraped into 100 µl of PBS added to each well, pipetted up and down to
144 completely resuspend the cells and then 10 µl was transferred into new 96-well plate containing
145 90 µl of PBS using a multichannel pipettor. A serial dilution was made in the fresh 96-well plate
146 and 10^{-3} through 10^{-6} dilutions were spotted (2.5 µl each spot) onto blood agar plates. The blood
147 agar plates were incubated for 48 h and the colonies were counted on each spot. Each experiment
148 contained four technical replicates.

149 To measure metabolic activity, 100 µl of PBS (pH 7) was added to each well of the original plate
150 after removal of the planktonic growth. CellTitre Blue® reagent (20 µl)(Promega G8080) was
151 pipetted into both plates: biofilm growth plate and planktonic growth plate, avoiding direct
152 exposure of light. The incubation period for planktonic and biofilm fractions varied: visual
153 colour change was observed in planktonic fractions containing plates sooner (at 20 min) than the

154 biofilm cells containing plates. Plates were monitored every 20 min for up to 2 hours of
155 incubation at 37 °C. Fluorescence for the biofilm forming cells was recorded after 2 h.
156 Fluorescence was measured using VarioSkan LUX Multimode plate reader at 560 nm
157 (excitation) and 590 nm (emission). Metabolic activity was measured by subtracting RFU values
158 of the sterile control wells from the RFU values of the test wells.
159

160 **Scanning Electron Microscopy**

161 One *Gardnerella* isolate (N170, subgroup B, genome sp. 3) was grown anaerobically for 48 h on
162 blood agar. Ten well isolated colonies were harvested and transferred to BHI + 0.25% maltose +
163 10% horse blood serum. After 18h of growth, inoculum was prepared by adjusting the OD_{600} to
164 0.5 in BHI + 0.25%. An aliquot of 500 µl of inoculum was pipetted into 24-well plates
165 containing autoclaved round glass coverslips. The 24-well plate was incubated anaerobically for
166 48 h before replacing the media with fresh BHI + 0.25% maltose (control) and BHI + 0.25%
167 maltose supplemented with 128 µg/ml of metronidazole (treatment). The planktonic fraction was
168 removed 24 h post-treatment and the coverslips were flooded with PBS. Biofilm cells were fixed
169 by carefully adding 500 µl of 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) and by
170 incubating for 4 hours at 4 °C. Cells were further fixed by adding 1% osmium tetroxide (OsO_4)
171 for 1 h followed by dehydration by consecutive washes in 30%, 50%, 70%, 80%, 90%, 95%, and
172 100% (3×) ethanol. After fixation, the coverslips were carefully washed twice with 0.1 M
173 sodium cacodylate (pH 7.2), and then 500 µl of fresh wash buffer was added to each well and the
174 24-well plate was sealed with parafilm. Fixed cells were dried and substituted using
175 hexamethyldislazane (HMDS) by first adding 1:2 HMDS and EtOH solution for 10 min followed
176 by treatment with 2:1 HMDS and EtOH solution for an additional 10 min. Finally, cells were

177 treated with 100% HMDS twice for 10 min. Excess HMDS was removed, and coverslips were
178 air dried overnight. Prior to microscopic observation, samples were treated with 10 nm gold. The
179 biofilm cells were then observed at different magnifications (400X, 6000X, 20,000X, 45,000X)
180 using a scanning electron microscope (Hitachi UHR FE-SEM SU8000 Series). All captured
181 images were evaluated by an observer blinded to the treatment to avoid observation bias.
182
183 **Statistical analysis**
184 To test if biofilm growth and planktonic growth were significantly different in the two different
185 media (with or without serum), a Mann-Whitney test was performed. To determine the
186 relationship between the MIC values for *Gardnerella* isolates in the two different culture
187 conditions, a Pearson-coefficient test was performed. To determine if the cell counts were
188 significantly different between controls and treatments, a Mann-Whitney U test and Holm-Šídák
189 multiple comparisons were performed. To test if metabolic activity is significantly different
190 between treated and control biofilms, a multiple unpaired t-test and Holm-Šídák multiple
191 comparisons were performed. All statistical analyses were performed using GraphPad Prism
192 (v.9.2.0).
193
194 **Results**
195 **Impact of serum on biofilm formation**
196 To investigate if the presence of serum affects biofilm formation, all isolates were grown in BHI
197 + 0.25% (w/v) maltose with or without addition of 10% (v/v) heat inactivated horse serum.
198 Thirty-five isolates were grown in four technical replicates. Planktonic growth was significantly
199 higher in media containing serum compared to serum-free media while biofilm growth was

200 significantly higher in serum-free media than in media with serum (p <0.0001, Mann-Whitney U

201 test, Fig 1).

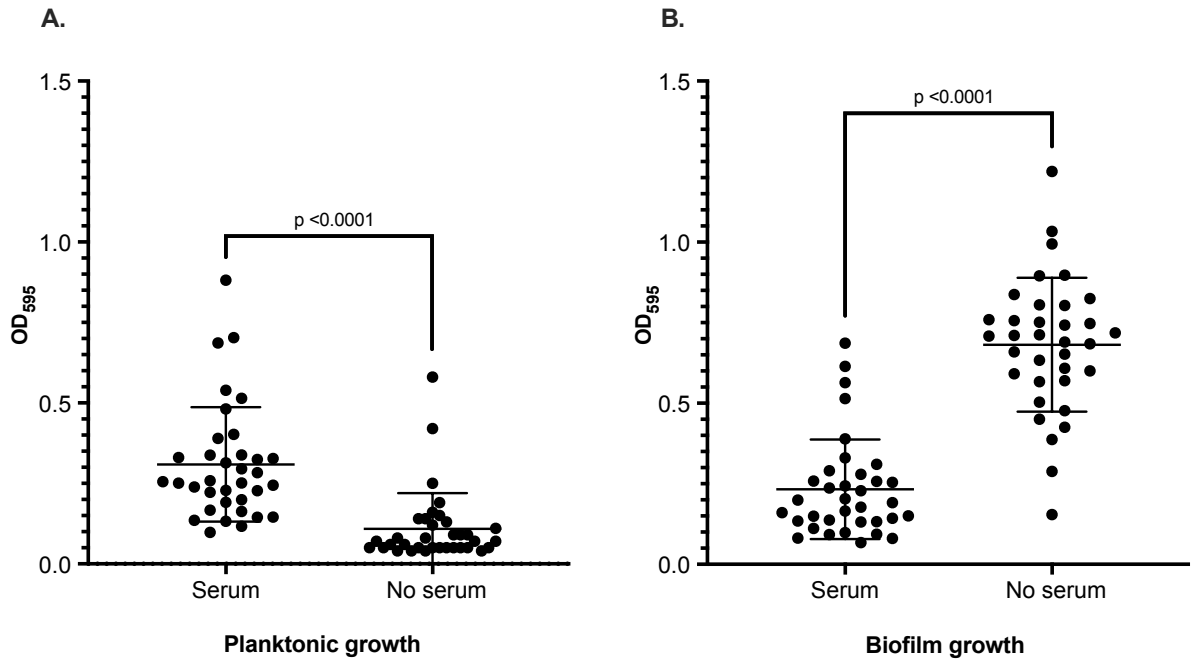

Fig 1: Growth mode is affected by serum (A) Planktonic growth in media with serum (BHI+ 0.25% maltose (w/v) + 10% (v/v) heat inactivated horse serum) and without serum (BHI+ 0.25% (w/v) maltose) measured by absorbance of broth culture. (B) Biofilm formation in media with serum (BHI+ 0.25% (w/v) maltose + 10% (v/v) heat inactivated horse serum) and without serum (BHI+ 0.25% (w/v) maltose) measured by crystal violet stain. Each data point is the average of four technical replicates of each of 35 isolates. Mann-Whitney test was performed to test significance and p values are indicated.

202

203 **Impact of growth mode on susceptibility to metronidazole**

204 Since serum significantly reduces biofilm formation and encourages planktonic growth, we used

205 presence or absence of serum to control mode of growth of the tested isolates in subsequent broth

206 microdilution assays. If growth in biofilm reduces susceptibility to metronidazole, we would

207 expect to see higher MIC values for isolates grown in serum-free media than in media with

208 serum. Thirty-five *Gardnerella* isolates were grown in metronidazole concentrations ranging

209 from 1 µg/ml to 512 µg/ml (two-fold serial dilution) in BHI + 0.25% (w/v) maltose with or

210 without 10% (v/v) heat inactivated horse serum. The MIC for most of the tested isolates (19/35,

211 54.28%) was 64 µg/ml (range 4-128 µg/ml). Overall, MIC values determined in the two media
212 were highly correlated (Pearson correlation co-efficient, R^2= 0.69, p <0.001) and 27/35 isolates
213 had the same MIC in both media. Of the 35 isolates tested, the observed MIC differed between
214 the two conditions for eight (8/35, 23%) isolates: NR010 of subgroup A; GH007, GH019 &
215 GH022 of subgroup B; and N165, GH015, GH021, and VN001 of subgroup C. Except N165, the
216 seven other isolates (GH007, GH015, GH019, GH021, GH022, NR010, and VN001) had a lower
217 MIC value in serum free media than in media with serum. There was no apparent relationship
218 between subgroup and MIC (Table 1).

219

220 **Table 1**. MIC of thirty-five *Gardnerella* isolates in
221 BHI+ 0.25% (w/v) maltose with or without 10%
222 (v/v) heat inactivated horse serum.

		MIC (µg/ml)	
Subgroup	**Isolate**	**+ serum**	**No serum**
A	GH005	32	32
	NR010	1	32
	NR015	32	32
	NR016	64	64
	NR019	64	64
	NR020	64	64
	VN003	32	32
	WP021	64	64
	WP022	16	16
B	GH007	16	8
	GH019	4	4
	GH022	32	2
	N95	64	64
	N101	64	64
	N144	64	64
	N170	64	64
	NR026	64	64
	VN002	64	64
	W11	4	4
C	ATCC14018	32	8
	GH015	32	32
	GH021	16	8
	N165	64	128
	NR001	8	4
	NR038	64	64
	NR039	64	64
	VN001	32	4
	WP023	16	16
D	NR002	64	64
	NR003	64	64
	NR043	64	64
	NR044	64	64
	NR047	64	64
	N160	64	64
	WP012	64	64

223

224 **Viability of treated biofilm vs. control biofilm**

225 In the broth microdilution assay, a suspension of cells is exposed to antibiotic simultaneously
226 with inoculation of assay plates. To investigate if established biofilms formed by *Gardnerella*
227 provide protection against subsequent metronidazole treatment, preformed biofilms (48 h old) of

228 nine *Gardnerella* isolates were treated for 24 h with 128 μg/ml metronidazole, which is double
229 the highest recorded MIC value for any of the tested isolates. Total viable counts of isolates
230 treated with metronidazole and untreated controls were compared. Viable cells were recovered
231 from all controls and all treated biofilms except VN003 and ATCC 14018, which had no viable
232 cells after treatment. In cases where viable cells were recovered, there were no significant
233 differences in cfu/ml between treated and untreated (Fig 2, Mann-Whitney test, Holm-Šídák
234 multiple comparisons).

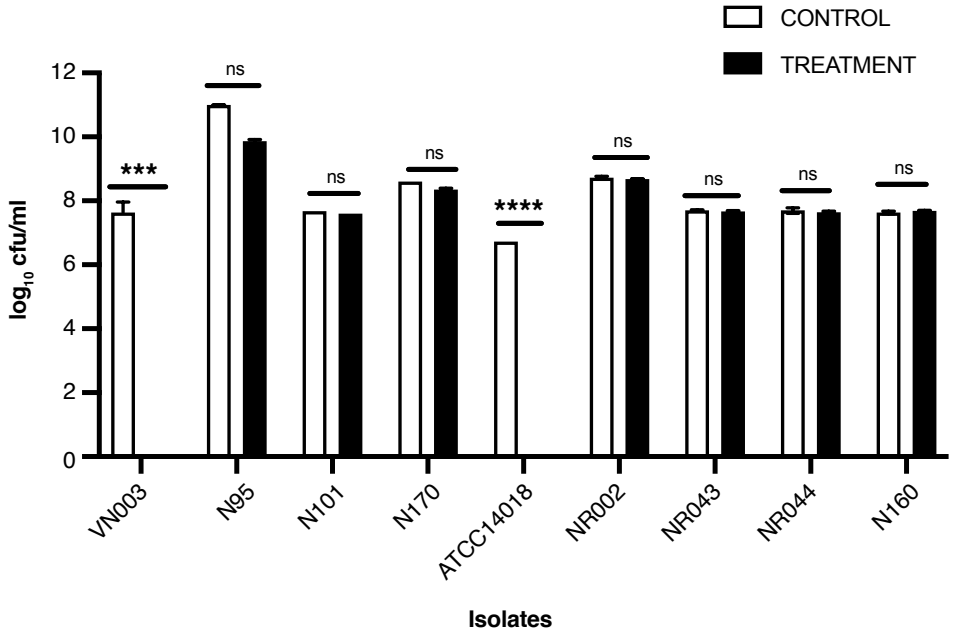

Fig 2: *Gardnerella* isolates in established biofilms can survive metronidazole treatment. 48 h old established biofilms of nine *Gardnerella* isolates were either treated with 128 μg/ml of metronidazole or not treated (controls). Biofilm cells were scraped, resuspended and total viable counts were performed on blood agar plates. Error bars show standard deviation of four technical replicates. Mann-Whitney U test was performed to test statistical significance (**** = p<0.0001, *** = p <0.001, ns = not significant).

235

236 **Metabolic activity of treated biofilm vs. control biofilm**

237 To determine if treated and untreated biofilms differ in metabolic activity, a resazurin assay was
238 performed. If cells are metabolically active, resazurin is reduced to resosurfin – a highly

239 fluorescence substance. We measured fluorescence (560/590nm) for thirty-one *Gardnerella*
240 isolates growing in biofilm mode, which were untreated or treated with 128 μg/ml metronidazole
241 for 24 h. Metabolic activity, measured as RFU values, was appreciably higher in untreated
242 biofilms: in 87% (27/31) of isolates metabolic activity was lower in the metronidazole treated
243 biofilm than in the corresponding untreated control (Fig 3). Of the 27 isolates which showed
244 reduced metabolic activity in preformed biofilms treated with metronidazole, the reduction was
245 significant in seventeen isolates (Fig 3, unpaired t-test, Holm-Šídák multiple comparisons).
246 Although three isolates (NR021, N95, NR026) showed higher metabolic activity in treatment
247 than in controls, these differences were not significant (Fig 3).

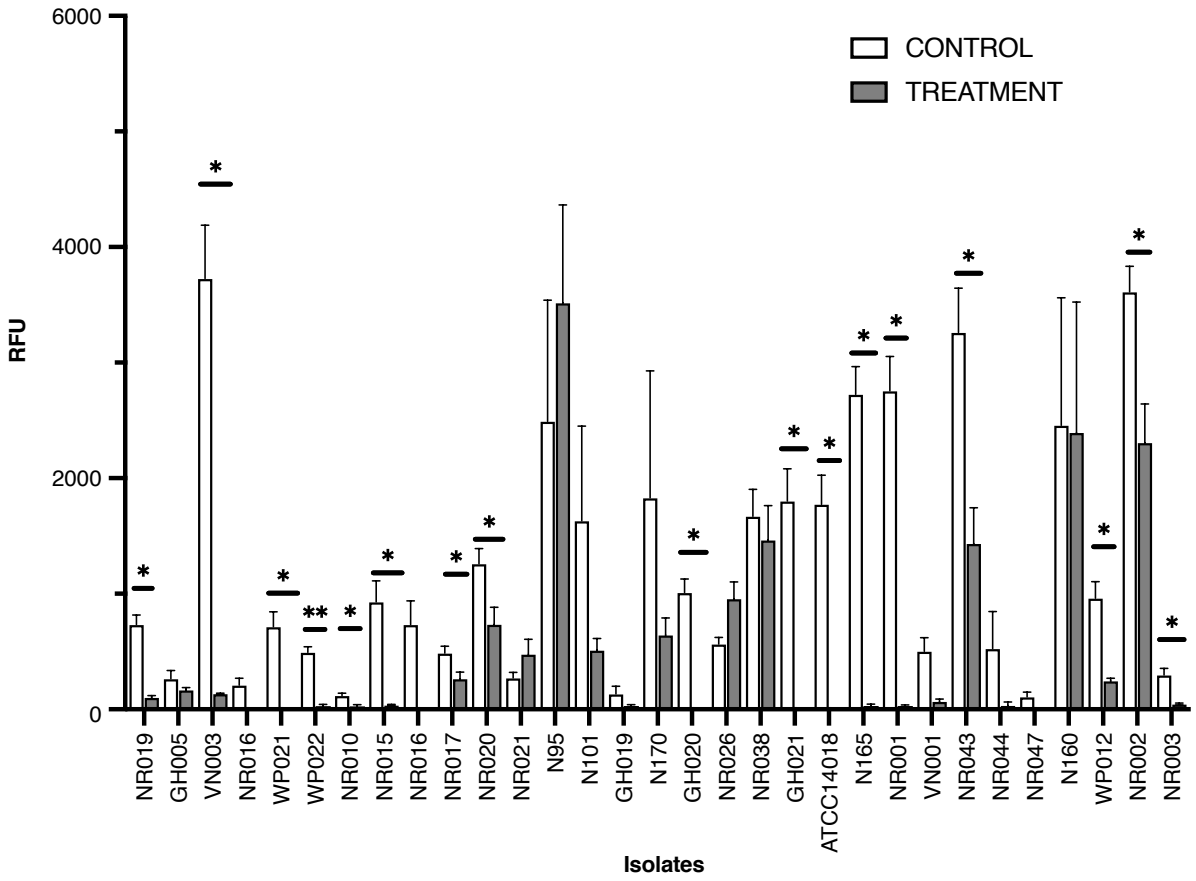

Fig 3: Metabolic activity of *Gardnerella* isolates growing in biofilms is reduced after metronidazole treatment. Thirty-three *Gardnerella* isolates were grown in BHI + 0.25% maltose for 48 h to form biofilms. Biofilms were treated with 128 μg/ml of metronidazole for 24 h, with four replicates per isolate. Metabolic activity was measured as baseline-subtracted relative fluorescence units (RFU) at 560/590 nm. An unpaired t-test and Holm-Šídák multiple comparisons were performed to test if the differences were statistically significant (* = $p < 0.005$, ** = $p < 0.05$).

248

249 **Impact of sub-inhibitory metronidazole on biofilm formation**

250 To investigate if sub-MIC concentrations of metronidazole stimulate biofilm formation by
251 *Gardnerella*, we compared the amount of biofilm growth of each isolate at each concentration of
252 metronidazole in serum-free media. No enhancement of biofilm formation was observed in most
253 of the tested isolates in the presence of metronidazole compared to biofilm formation in the
254 absence of metronidazole, except for NR026 (Subgroup B), VN001 (Subgroup C), and NR043
255 (Subgroup D) (Fig 4).

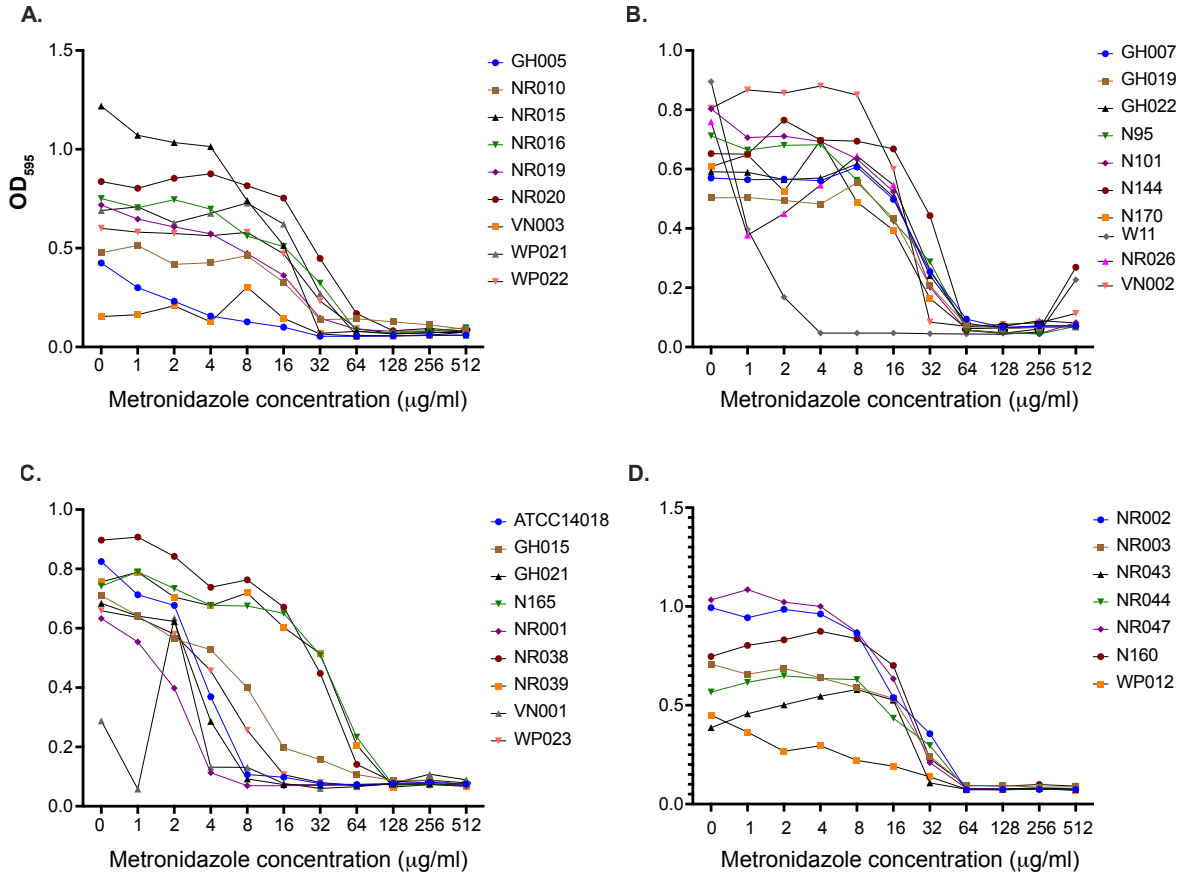

Fig 4: Biofilm formation at different concentrations of metronidazole. Thirty-five *Gardnerella* isolates were grown in media without serum. Each isolate was replicated four times in a 96-well plate. Crystal violet staining was performed to quantify biofilm biomass and OD_{595} measured. Each data point is the average of four technical replicates. Results are shown for isolates in cpn60-defined subgroups A, B, C and D (panels A-D).

256

257 **Architecture of metronidazole treated and untreated *Gardnerella* biofilms**

258 Next, we investigated if the structure of biofilm formed by isolate N170 and treated with
259 metronidazole differed from untreated biofilm. We chose N170 as a representative isolate
260 because it is a good biofilm former. There was no visible difference between treated and
261 untreated biofilms (Fig 5). Morphological heterogeneity of *Gardnerella* cells was observed in
262 both treated and untreated biofilms. Extracellular polymeric substances were observed in both
263 control and treatment; however, tightly packed cells were more common than extracellular

264 polymeric matrix in both biofilms (Fig 5). Dividing cells were observed in both treated and

265 untreated biofilms (Fig 5E-H).

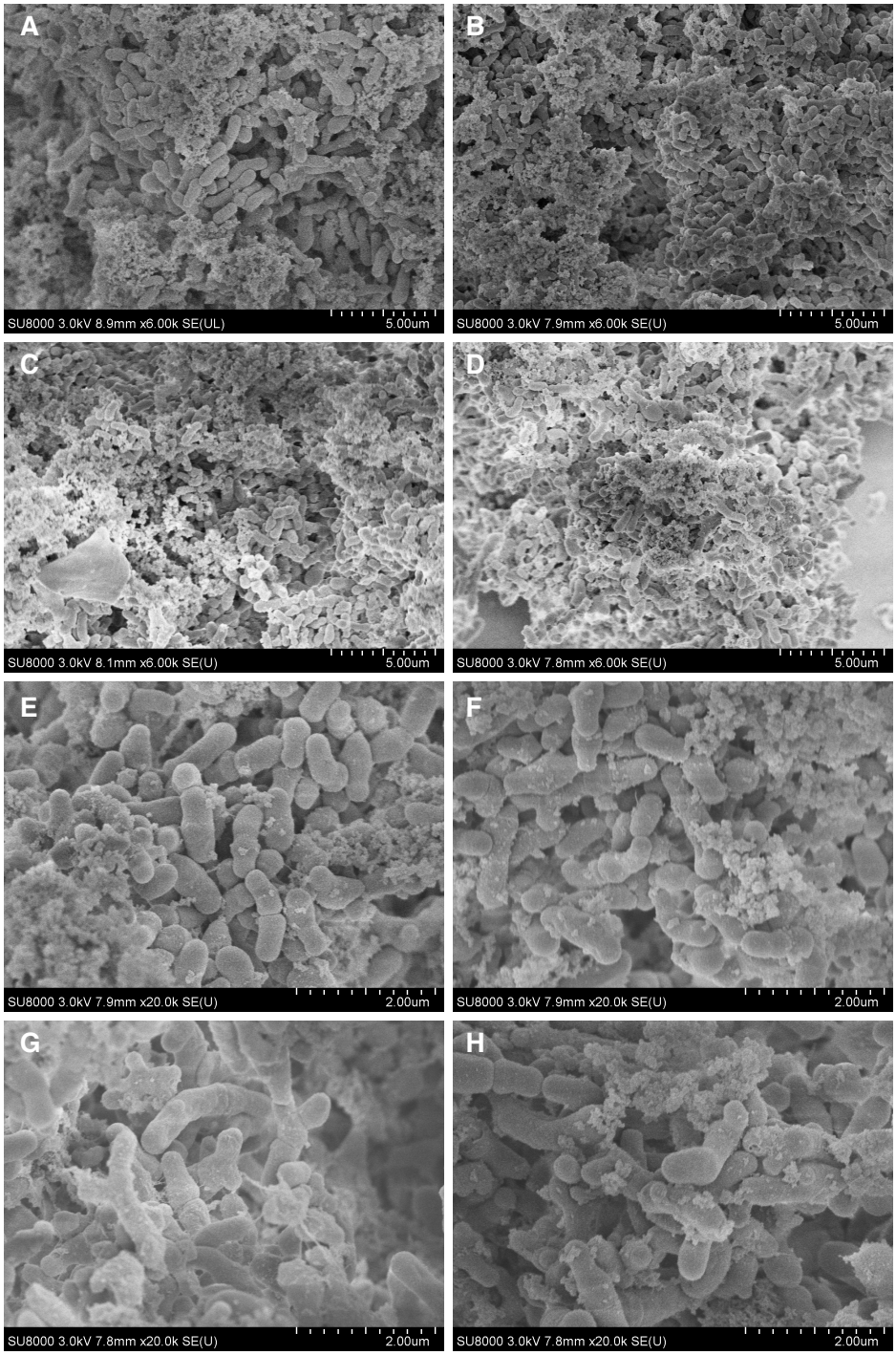

Fig 5: *Gardnerella* N170 biofilms. Established biofilms of isolate N170 were treated with 128 g/ml of metronidazole for 24 h. Un-treated controls were maintained. SEM reveals no architectural or morphological differences between treated (A, C, E, G) and control (B, D, F, H) biofilms. Panels A-D are representative images captured at 6,000× and E-H are images at 20,000×.

266

267

268 **Effect of co-culture on survival of *Gardnerella* treated with metronidazole**

269 Since stressful conditions can lead to cooperation between bacterial species, we tested if the
270 susceptibility of *Gardnerella* isolates grown in co-culture was different than when grown alone.
271 We also investigated if biofilm formation is enhanced when *Gardnerella* isolates are co-cultured
272 in the presence of metronidazole. No co-culture had a higher MIC than the highest MIC value of
273 either of the two co-cultured isolates (Table 2), and no co-culture produced more biofilm than
274 any of the individual isolates included in the co-culture (data not shown).

275 **Table 2**. MIC of co-cultures of *Gardnerella* isolates in BHI+ 0.25% (w/v) maltose with or without 10% (v/v)
276 heat inactivated horse serum.

		MIC (µg/ml)					
		+ serum			no serum		
Isolate 1 (Subgroup)	**Isolate 2 (Subgroup)**	**co-culture**	**Isolate 1**	**Isolate 2**	**co-culture**	**Isolate 1**	**Isolate 2**
VN003 (A)	N170 (B)	64	32	64	32	32	64
VN003 (A)	N165 (C)	64	32	64	64	32	128
VN003 (A)	NR002 (D)	32	64	64	32	64	64
N170 (B)	N165 (C)	64	64	64	64	64	128
N170 (B)	NR002 (D)	16	64	64	16	64	64
N165 (C)	NR002 (D)	64	64	64	64	128	64

277

278 **Discussion**

279 It is widely reported that biofilm formation by bacteria provides protection against antibiotics
280 (16, 18, 31, 32). It has also been suggested that bacterial vaginosis treatment failure and
281 recurrence is largely because of the capacity of *Gardnerella* and other vaginal bacteria to form
282 biofilm (9, 28), however, this phenomenon has not been demonstrated *in vitro,* and a mechanism
283 by which biofilm formation protects *Gardnerella* has not been identified.

284 To facilitate two different modes of growth in our study: biofilm and planktonic, we used media
285 with or without horse serum. Although the mechanisms are yet to be fully understood, there are
286 reports that inclusion of serum discourages biofilm formation (33, 34), and that low molecular
287 weight proteins present in the serum may inhibit the transcription of biofilm genes (33). Also, it
288 has been proposed that for motile species, serum may promote twitching motility, which may
289 encourage planktonic growth (34). In our study, planktonic growth was dominant in the presence
290 of serum while biofilm growth was dominant in media without serum (Fig. 1). Despite this
291 dramatic difference in growth habit, MIC values in the two conditions were highly correlated and
292 identical MIC values were observed for most isolates regardless of growth medium (Table 1).
293 The MIC of the majority isolates (54.2%) in both media was 64 µg/ml, which is in agreement
294 with a previous study which also reported that the MIC of 50% of tested *Gardnerella* isolates
295 was 64 µg/ml (29). The lack of difference in MIC between biofilm and planktonic cultures
296 suggests that resistance to metronidazole and differences in MIC among isolates are the result of
297 properties of the individual isolates and not solely a function of growth mode (planktonic or
298 biofilm).

299 Although it has been widely accepted that biofilm formation is a stress response that protects
300 bacteria from insults, including antibacterial compounds, it has also been suggested that biofilm
301 may simply be a default mode of growth for many bacterial species in particular environments
302 (12). Based on observations of clinical specimens, this certainly seems to be the case for
303 *Gardnerella* in the vaginal microbiome (4, 5, 35, 36). While the broth microdilution assay and
304 MIC determination provides some information about susceptibility of isolates to metronidazole,
305 it does not simulate the situation *in vivo* where BV associated biofilm is well established prior to
306 treatment. Biofilm formation can protect bacterial cells by the production of extracellular

307 polymeric substances, by release of antibiotic modifying enzymes and extracellular DNA, and
308 reduction of metabolic activity and growth rate (17, 37). Since metronidazole, an antibiotic
309 bactericidal to a broad range of anaerobes, is a common choice of treatment for BV, we
310 investigated if established *Gardnerella* biofilm can protect its inhabitants from being killed by
311 metronidazole.

312 To understand how and to what extent biofilm can protect *Gardnerella* cells from metronidazole,
313 48 h old *Gardnerella* biofilms were exposed to 128 μg/ml metronidazole for 24 h, a
314 concentration higher than the maximum recorded MIC values of isolates we tested. We measured
315 viability and metabolic activity by total viable count and resazurin assay. The resazurin assay is a
316 high-throughput assay to assess metabolic activity (38, 39) and has been used to assess metabolic
317 activity of bacteria residing in biofilms (40, 41). Our results reveal that metabolic activity is
318 reduced in most of the tested isolates after exposure to metronidazole for 24 h (Fig 3), however,
319 in most cases we tested viable *Gardnerella* cells can be recovered from treated biofilms (Fig 2).
320 Our findings suggest that biofilms can protect cells from the killing effect of metronidazole at the
321 cost of reduced metabolic activity. Metronidazole enters bacteria by passive diffusion as a
322 prodrug and has limited activity until it is reduced, which occurs within bacterial cells (42, 43),
323 and thus, metabolic activity is necessary for the bactericidal effects of the drug to occur.
324 Exposure to antibiotics can also cause the emergence of persister cells in a biofilm, which can
325 survive antibiotic treatment by reducing metabolic activity (13).

326 Reduction of metabolic activity at antibiotic concentrations exceeding the MIC has also been
327 demonstrated in *E. coli* and *S. aureus*; however, in this study metabolic activity was also
328 increased at sub-MIC concentrations (44). Subinhibitory concentrations of antibiotics can
329 increase the production of extracellular matrix, enhancing biofilm biomass, slowing the diffusion

330 of antibiotics, and reducing exposure of the bacteria within the biofilm (17, 18). We did not
331 observe any enhancement of biofilm formation in the vast majority of the tested *Gardnerella*
332 isolates at sub-MIC concentrations of metronidazole (Fig 4). Enhancement of biofilm formation
333 by sub-inhibitory concentrations of antibiotics likely depends on the mechanisms of actions of
334 antibiotics. Yu et al. demonstrated enhancement of biofilm formation in *Enterococcus faecalis* (a
335 host associated Gram-positive cocci often associated with nosocomial infection) in response to
336 cell wall synthesis inhibitors such as ampicillin, oxacillin, and Fosfomycin, but not in response to
337 protein synthesis, DNA synthesis, and RNA synthesis inhibitors, such as erythromycin,
338 ciprofloxacin, and rifampicin (22). It has been suggested that antimicrobial compounds, such as
339 pyocins, which kill bacteria by damaging the cell wall, can enhance cellular attachment at sub-
340 lethal concentrations leading to increased biofilm formation (45). Metronidazole, however, kills
341 primarily anaerobic and facultative anaerobic bacteria by formation of reactive oxygen species
342 (ROS), which damages bacterial DNA (23). Therefore, it is conceivable that due to its mode of
343 action, metronidazole would not be expected to stimulate biofilm formation.

344 Since metabolic activity was reduced in biofilms treated with metronidazole and no enhancement
345 of biofilm formation was observed at sub-MIC levels, we sought to determine if metronidazole
346 treatment affects the structure of *Gardnerella* biofilm. We performed an SEM to visualize any
347 morphological or structural differences between biofilms treated with metronidazole and
348 untreated control biofilms. Although no differences were observed between treated and untreated
349 biofilms formed by isolate N170, we observed that the biofilm was largely composed of bacterial
350 cells in compact masses, and extracellular polymeric substance was not a major component of
351 the structure. Although SEM alone cannot determine if any other changes were happening in the
352 biofilms treated with metronidazole (sensors, florescence microscopy, and nanoparticles can

353 provide a better understanding of what changes may occur in a biofilm treated with antibiotics),
354 the fact that biofilm formation was not enhanced by sub-inhibitory concentrations of
355 metronidazole and extracellular polymeric substance was not abundant in *Gardnerella* biofilm
356 suggest that extracellular polymeric substance does not play a major role in providing protection
357 against metronidazole (46, 47).

358 When exposed to antimicrobials, otherwise competing bacteria may cooperate for a greater
359 purpose: to survive environmental stress (48, 49). Targeting cooperative behaviours can be an
360 effective method to treat biofilm infection (50). The results of our co-culture experiments
361 showed no evidence of synergy between *Gardnerella* spp. when exposed to antibiotics. This
362 finding is contrary to some other observations of either environmental (48) or well-characterized
363 Gram-negative bacterial isolates (50) but the overall dearth of information available regarding
364 cooperation between isolates when exposed to antibiotics makes it impossible to know if these
365 cases are the exception or the rule. We nevertheless, reported previously that coculturing
366 *Gardnerella* isolates does not have any synergistic effects (8, 51).

367 **Conclusions**

368 Overall, we have observed that established *Gardnerella* biofilm can survive metronidazole
369 treatment at levels above the MIC and this survival is associated with a reduction in metabolic
370 activity during antibiotic exposure. Metronidazole does not affect the architecture of *Gardnerella*
371 biofilm *in vitro* as observed by SEM. By reducing metabolic activity in response to exposure to
372 metronidazole, *Gardnerella* growing in established biofilms in the vaginal microbiome may
373 avoid the bactericidal effects of the drug and resume growth once treatment ends. From a clinical
374 perspective, management of recurrent vaginosis may be improved by methods to mitigate the

375 ecological processes that lead to expansion of *Gardnerella* populations and establishment of
376 biofilm.
377

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386

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