bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Inhibitory Effect of *Bacillus velezensis* on Biofilm Formation by *Streptococcus mutans***

2

3 **Running title:** *B. velezensis* inhibits biofilm formation

4

5 Yesol Yoo,[a]* Dong-Ho Seo,[b]* Hyunjin Lee,[a,b] Young-Do Nam,[b] Myung-Ji Seo[a,c#]

6

7 [a]Department of Bioengineering and Nano-Bioengineering, Graduate School of Incheon

8 National University, Incheon 22012, Republic of Korea

9 [b]Research Group of Gut Microbiome, Korea Food Research Institute, Wanju 55365, Republic

10 of Korea

11 [c]Division of Bioengineering, Incheon National University, Incheon 22012, Republic of Korea

12

13 *These authors contributed equally to this work.

14

15 [#]Address correspondence to Myung-Ji Seo, mjseo@inu.ac.kr.

16

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

17 **ABSTRACT**

18 *Streptococcus mutans* plays a key role in the development of dental caries and
19 promotes the formation of oral biofilm produced by glucosyltransferases (GTFs). *Bacillus*
20 *velezensis* K68 was isolated from traditional fermented foods and inhibits biofilm formation
21 mediated by *S. mutans*. Gene amplification results demonstrated that *B. velezensis* K68
22 contained genes for the biosynthesis of 1-deoxynojirimycin (1-DNJ), a known GTF
23 expression inhibitor. The presence of the GabT1, Yktc1, and GutB1 genes required for 1-DNJ
24 synthesis in *B. velezensis* K68 was confirmed. Supernatant from *B. velezensis* K68 culture
25 medium inhibited biofilm formation by 84% when *S. mutans* was cultured for 48 h, and
26 inhibited it maximally when 1% glucose was added to the *S. mutans* culture medium as a
27 GTF substrate. In addition, supernatant from *B. velezensis* K68 medium containing 3 ppb 1-
28 DNJ decreased *S. mutans* cell surface hydrophobicity by 79.0 ± 0.8% compared with that of
29 untreated control. The supernatant containing 1-DNJ decreased *S. mutans* adherence by 99.97%
30 and 98.83% under sugar-dependent and sugar-independent conditions, respectively. *S. mutans*
31 treated with the supernatant exhibited significantly reduced expression of the essential GTF
32 genes *gtfB*, *gtfC,* and *gtfD* compared to that in the untreated group. Thus, *B. velezensis*
33 inhibits the biofilm formation, adhesion, and GTF gene expression of *S. mutans* through 1-
34 DNJ production.

35

36 **IMPORTANCE**

37 Dental caries is among the most common infectious diseases worldwide, and its
38 development is closely associated with physiological factors of bacteria, such as the biofilm
39 formation and glucosyltransferase production of *Streptococcus mutans.* Biofilms are difficult
40 to remove once they have formed due to the exopolysaccharide matrix produced by the

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

41 microorganisms residing in them; thus, inhibiting biofilm formation is a current focal point of
42 research into prevention of dental caries. This study describes the inhibitory properties of
43 *Bacillus velezensis* K68, an organism isolated from traditional Korean fermented foods,
44 against biofilm formation by *S. mutans*. Herein, we show that *B. velezensis* inhibits the
45 biofilm formation, adherence to surfaces, and glucosyltransferase production of *S. mutans*.

46

47

48 **KEYWORDS**

49 *Bacillus velezensis*, biofilm, 1-deoxynojirimycin, glucosyltransferase, *Streptococcus*
50 *mutans*

51

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

52 **INTRODUCTION**

53 Dental caries is among the world's major diseases, presenting in 60–90% of
54 schoolchildren and adults, and is caused by microorganisms using various dietary saccharides,
55 such as glucose, fructose, and sucrose (1). Especially, physiological properties such as acid
56 tolerance, biofilm formation, and expression of virulence genes, of Streptococci in the mouth
57 produced by the quorum-sensing system are known to affect oral health (2). *Streptococcus*
58 *mutans* is a gram-positive, facultative anaerobic bacterium and is the primary causative agent
59 of cariogenicity (3). *S. mutans* forms a biofilm and attaches to tooth surfaces, and the
60 hydrophobicity of the biofilm is related to surface proteins antigens such as antigen I/II
61 (SpaP), WapA, and SloC, on *S. mutans* (4). Oral biofilm produced by *S. mutans* is formed by
62 microbial communities attached to the enamel layer of tooth surfaces (5, 6).

63 Biofilm is not easily removed because it is enclosed in a matrix of polysaccharides;
64 thus, inhibition of the initial stages of biofilm formation is an important area of study (7).
65 Biofilm is synthesized by glucosyltransferases (GTFs), which produce extracellular
66 polysaccharides, in *S. mutans* (8). GtfB synthesizes α-1,3-linked insoluble glucan; GtfC
67 synthesizes both insoluble and soluble glucan; and GtfD synthesizes α-1,6-linked soluble
68 glucan (9-11). Various studies have been conducted on the anti-caries effects of
69 microorganisms that inhibit the growth of *S. mutans* using treated culture medium from lactic
70 acid bacteria and antibiotics or lytic enzymes from *Bacillus* spp. (12-17). Quercitrin and
71 deoxynojirimycin (DNJ) were recently shown to inhibit the GTF gene expression in *S.*
72 *mutans*, which led to biofilm formation (18).

73 This study focused on inhibition of *S. mutans* oral biofilm by a *Bacillus velezensis*
74 strain. *B. velezensis* K68 was isolated from traditional fermented foods, and its inhibitory

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

75 activity against *S. mutans* biofilm was analyzed. Additionally, the effects of treatment with *B.*
76 *velezensis* culture supernatant were determined on *S. mutans* cell surface hydrophobicity,
77 adherence, and regulation of virulence genes. We demonstrated that *B. velezensis* may
78 prevent dental caries through the inhibition of biofilm produced by *S. mutans.*

79

80 RESULTS AND DISCUSSION

81 *S. mutans* biofilm inhibition by *B. velezensis* K68 isolated from fermented food

82 Because metabolites from some *Bacillus* species are known to inhibit biofilm
83 formation by *S. mutans*, we isolated a *Bacillus* strain K68 exhibiting inhibitory effects on
84 biofilms from fermented food. The 16S rRNA gene sequence (GenBank accession no.
85 MG589484) of the isolated strain K68 was 99.64% similar to that of *Bacillus velezensis* CR-
86 502 (Fig. S1). To investigate the inhibition of biofilm formation by *S. mutans* KCTC 3065,
87 cells were treated with the supernatant from *B. velezensis* K68 culture medium containing 1%
88 glucose and incubated at 37°C for different time periods (Fig. 1). In general, biofilm
89 formation time for *S. mutans* in medium with 1% glucose is 24–48 h. The weak inhibition at
90 the initial time (6 h) is likely due to insufficient biofilm formation at the time. After 12 h, *B.*
91 *velezensis* K68 culture significantly inhibited biofilm formation compared to negative control
92 (*B. subtilis* 142). In addition, the inhibitory effect increased in a concentration-dependent
93 manner with *B. velezensis* K68 culture medium supernatant. Lytic enzymes and antibiotics
94 produced by *Bacillus* spp. inhibit the growth of *S. mutans*, preventing biofilm formation (16,
95 17). Interestingly, there was no significant effect on *S. mutans* growth after treatment with *B.*
96 *velezensis* K68 culture medium (data not shown). Previous studies showed that 1-
97 deoxynojirimycin (1-DNJ) from mulberry (*Morus alba*) prevents biofilm formation and

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

98 adhesion of *S. mutans* (7). Some *Streptomyces* and *Bacillus* spp. are known to produce 1-DNJ
99 (19, 20). Therefore, *B. velezensis* K68 presumably inhibits the biofilm formation of *S. mutans*
100 by producing 1-DNJ.

101

102 **Genetic and analytical confirmation of 1-DNJ production by *B. velezensis* K68**

103 Some *Bacillus* spp. produce 1-DNJ and a promising operon including GabT1
104 (putative aminotransferase), Yktc1 (putative phosphatase), and GutB1 (putative
105 oxidoreductase) could be essential to its biosynthesis (21). To determine whether *B.*
106 *velezensis* K68 produces 1-DNJ, the presence of these three genes was confirmed by PCR
107 (Fig. S2). A 1.2 kb fragment of *gabT1*, 0.9 kb of *yktc1*, and 1.0 kb of *gutB1* were successfully
108 amplified. The obtained genes showed sequence differences from that of known 1-DNJ
109 biosynthetic genes. The deduced amino acid sequence of each gene was analyzed, resulting
110 that the GabT1, Yktc1, and GutB1 of *B. velezensis* K68 showed the highest homology to each
111 protein from *B. velezensis* FZB42 (Table 1). The putative 1-DNJ biosynthetic gene cluster
112 sequence assembled with *gabT1*, *yktc1* and *gutB1* of *B. velezensis* K68 has been deposited in
113 GenBank under accession no. MH142722.

114 Triple quadrupole LC-mass spectrometry analysis showed that *B. velezensis* K68
115 produced 1-DNJ (data not shown). ESI in positive ion mode resulted in a peak on the
116 chromatogram at 164.16 *m/z* for $[M+H]^+$. This peak was confirmed to represent 1-DNJ
117 ($C_6H_{13}NO_4$, 163.17 g/mol) with a retention time of 9.19 min. The 1-DNJ in the supernatant of
118 the *B. velezensis* K68 culture medium was identical to a standard 1-DNJ solution, suggesting
119 that *B. velezensis* K68 produced 1-DNJ.

120

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

121 **Inhibitory effect of *B. velezensis* K68 on hydrophobicity and adherence of *S. mutans***

122 Cell surface hydrophobicity is involved in interactions between bacterial and
123 epithelial cells and is important to initial bacterial adherence to tooth surfaces (22, 23). After
124 treatment with 3 ppb 1-DNJ from the supernatant of *B. velezensis* K68 culture medium, *S.*
125 *mutans* cell-surface hydrophobicity dramatically decreased from 79.0 ± 0.8% (untreated) to
126 8.1 ± 2.1% (treated) (Fig. 2A). The cell-surface hydrophobicity of *S. mutans* is known to be
127 related to its cell-surface proteins. Hasan *et al* argued that quercitrin and 1-DNJ reduced *S.*
128 *mutans* hydrophobicity by binding proteins on its cell surface (Ag I / II) (18). Its
129 hydrophobicity was likely reduced by the 1-DNJ present in the supernatant of the *B.*
130 *velezensis* K68 culture medium.

131 Adhesion of *S. mutans* occurs by sugar-dependent and sugar-independent mechanisms.
132 Sugar-dependent attachment is mediated by glucan produced from sugars through
133 glucosyltransferases, whereas sugar-independent attachment is mediated by physicochemical
134 forces, such as electrostatic forces, hydrophobic interactions, and hydrogen bonding with the
135 constituents of saliva (24). In this study, the addition of glucose improved the adhesion of *S.*
136 *mutans* (Fig. 2B). However, *B. velezensis* K68 culture supernatant containing 3 ppb 1-DNJ
137 induced 99.97% and 98.83% decreases in adherence under sugar-dependent and sugar-
138 independent conditions, respectively, as compared to the adherence of untreated control.
139 Previous studies showed that 1-DNJ reduced the adherence of *S. mutans* in both sugar-
140 dependent and sugar-independent conditions and showed a greater effect in the presence of
141 sugar (18). *S. mutans* produces water-soluble glucans, such as dextran, and water-insoluble
142 glucans, such as mutan, from sugars using various glucosyltransferases (25, 26). 1-DNJ is
143 known to be an expression inhibitor of glucosyltransferases (18). Thus, our findings indicate

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

144 that *B. velezensis* K68 produces 1-DNJ, which binds to cell surface proteins on *S. mutans* and
145 acts as a competitive inhibitor of glucosyltransferases to reduce the hydrophobicity and
146 adhesion of *S. mutans*.

147

148 **Inhibition of exopolysaccharide synthesis by *B. velezensis* K68**

149 Scanning electron microscopy (SEM) revealed the formation of exopolysaccharides
150 synthesized by *S. mutans* (18). The scanning electron micrographs show the effects of *B.*
151 *velezensis* K68 culture medium supernatant on the ability of *S. mutans* to synthesize
152 extracellular polysaccharides. The sample treated with the supernatant (Fig. 3B) showed
153 significant dispersion of its cells, suggesting a reduction in exopolysaccharide synthesis. In
154 contrast, the untreated and negative control samples (Fig. 3A, 3C) showed clear aggregation
155 of cells immobilized in the exopolysaccharide pool. These results are consistent with 1-DNJ
156 treatment of *S. mutans* (18). The effects of 1-DNJ are thought to be due to inhibition of the
157 glucan synthesis, attachment, and biofilm formation of *S. mutans*.

158

159 **Inhibitory effect of *B. velezensis* K68 on glucosyltransferase expression in *S. mutans***

160 Three glucosyltransferase genes, *gtfB*, *gtfC*, and *gtfD*, are involved in water-soluble
161 or water-insoluble glucan synthesis in *S. mutans* (27). The mRNA expressions of these genes
162 were significantly decreased after treatment with *B. velezensis* K68 culture medium
163 supernatant compared to their levels in untreated and negative control groups (Fig. 4). The
164 *gtfB* and *gtfC* produce water-insoluble glucans, which act as adhesion molecules to
165 immobilize bacteria on tooth pellicles (9). Therefore, *gtfB* and *gtfC* are critical toxic factors

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

166 related to cariogenicity (8). Both genes were independently expressed, and there was a
167 difference between their promoters (28). Additionally, the expression of the two genes is
168 decreased by ions (Ca, K, and Mg) (29). Interestingly, *gtfB* mRNA levels decreased in the *B.*
169 *subtilis*-treated group, likely due to the presents of ions in the *B. subtilis* culture medium. In
170 the *B. velezensis* K68-treated group, the mRNA expression of all three *gtf* genes was
171 significantly reduced, and thus the biofilm formation and adherence of *S. mutans* were
172 decreased compared to those in the untreated group. These results were consistent with those
173 observed in treatments with *Lactobacillus salivarius* and 1-DNJ (14).

174 This study was conducted to determine the ability of *B. velezensis* K68, which was
175 isolated from traditional Korean fermented foods, to inhibit biofilm formation by *S. mutans*.
176 The 1-DNJ biosynthetic gene in *B. velezensis* K68 was successfully amplified through PCR,
177 and 1-DNJ production was confirmed by LC-Mass. The 1-DNJ produced by *B. velezensis*
178 K68 inhibited the expression of the GTF genes responsible for biofilm formation by *S.*
179 *mutans*. In addition, physicochemical analyses showed that culture medium containing 1-DNJ
180 inhibited biofilm formation by *S. mutans*. These results indicate that *B. velezensis* K68 can be
181 used to produce fermented food useful for dental health. The applicability of *B. velezensis*
182 K68 warrants further investigation.

183

184 **MATERIALS AND METHODS**

185 **Bacterial strains and culture conditions**

186 *Streptococcus mutans* KCTC 3065 was purchased from the Korean Collection for
187 Type Cultures (Daejeon, Republic of Korea). *Bacillus velezensis* K68 was isolated from
188 traditional Korean fermented foods. *S. mutans* KCTC 3065 was routinely cultured in brain-

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

189 heart infusion (BHI) broth (MB Cell, Los Angeles, CA, USA), and *B. velezensis* K68 was
190 successively cultured in tryptic soy broth (TSB; BD Biosciences, Franklin Lakes, NJ, USA)
191 at 37°C with continuous shaking for 3 d. After cultivation, these media were centrifuged at
192 10,000 rpm for 10 min, and the supernatant was filtered with a 0.2 μm filter to remove cell
193 debris.
194

195 **Isolation of *Bacillus* strains harboring 1-DNJ biosynthetic genes**

196 Extraction of genomic DNA from *B. velezensis* K68 was performed using phenol-
197 chloroform isoamylalcohol as previously described (30). The 16S rRNA and 1-DNJ
198 biosynthesis genes of *B. velezensis* K68 were amplified by polymerase chain reaction (PCR).
199 The PCR primer sets for the genes were as follows: 27F (5′-AGA GTT TGA TCM TGG CTC
200 AG-3′) and 1492R (5′-CGG TTA CCT TGT TAC GAC TT-3′) for the 16S RNA gene, MJS-
201 23 (5′-ATG GGA ACG AAG GAA ATC ACG AAT CCA-3′) and MJS-24 (5′-TCA CTT
202 GAT TTC CTC CAA TAG CTT GCG-3′) for *gabT1*, MJS-19 (5′-GTG AGA GAC TAT
203 ATC ATY GRG CTT GGA-3′) and MJS-20 (5′-TTA GGA GTC CAG ACC AAC GCC TTC
204 ATA-3′) for *yktc1*, and MJS-21 (5′- ATG AAG GCG TTG GTC TGG ACT CCT AAT-3′)
205 and MJS-22 (5′-TTA TAA AAG TTY CGG ATC AGA CAC RAG-3′) for *gutB1* (31). PCR
206 was performed at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C
207 for 1 min 30 s, and then a final extension at 72°C for 7 min. The PCR products were
208 sequenced and analyzed for the construction of a phylogenetic tree. The samples were
209 dissolved in LC/MS-grade methanol (Thermo Fisher Scientific, Waltham, MA, USA). Triple
210 quadrupole LC analysis was conducted with a Finnigan TSQ Quantum Ultra EMR, a TSK gel
211 amide-80 column (3 μm, 2.0 × 150 mm; Tosoh, Tokyo, Japan), and 10 μL of injected sample
212 volume. Chromatograms were obtained using a flow late of 0.2 mL/min with 5 mM

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

213 ammonium acetate as eluent A and acetonitrile as eluent B. Elution gradient conditions were
214 set at 0 min (90% B), 10 min (40% B), 12 min (40% B), 13 min (90% B), and 20 min (90%
215 B).
216
217 **Biofilm formation assay**
218 Biofilm formation was assessed using flat-bottomed, polystyrene, 96-well microtiter
219 plates. *S. mutans* KCTC 3065 was cultivated overnight in BHI broth, and the resulting cell
220 suspension was diluted to 0.8–1.0 optical density (OD) at 600 nm. A total of 250 μL of liquid,
221 composed of 50 μL of the cell suspension, 150 μL of BHI broth containing 1% glucose, and
222 50 μL of *B. velezensis* K68 culture supernatant, was injected into each well of the microtiter
223 plates. The liquid was mixed and incubated at 37°C for 6, 12, 24, and 48 h, and planktonic
224 cells were gently removed with sterile water. Biofilm formation was compared with that of
225 *Bacillus subtilis* as a negative control. Attached cells in the wells were fixed with formalin
226 (37%, diluted 1:10) containing 2% sodium acetate for 1 h. Each well was stained with 250 μL
227 of 0.1% Crystal violet for 15 min at 25°C and rinsed with sterile water. Bound dye was
228 removed with 150 μL of 95% ethanol. Biofilm formation was observed by measuring the
229 suspension at 595 nm with a microplate reader (Multiskan FC plate reader; Thermo Fisher
230 Scientific) (18). Inhibition percentage was calculated as follows: inhibition percentage =
231 (untreated sample OD_{595} – test sample OD_{595})/untreated sample $OD_{595} \times 100$.
232
233 **Triple quadrupole LC-mass spectrometry**
234 Triple quadrupole LC-mass spectrometry was used to confirm 1-DNJ production by
235 *B. velezensis* K68. Cultivated samples were centrifuged at 10,000 rpm for 10 min (1580R,
236 Labogene, Seoul, Korea), and the supernatant was filtered with a 0.2 μm filter and freeze-

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

237 dried (TFD8503; Ilshinbiobase, Seoul, Korea) for liquid chromatography-mass spectrometry
238 confirmation of 1-DNJ synthesis. The samples were dissolved in methanol (LC/MS Grade,
239 Thermo Fisher Scientific). Triple quadrupole LC analysis was conducted with a Finnigan
240 TSQ Quantum Ultra EMR system and TSK gel amide-80 column (3 μm, 2.0× 150 mm;
241 Tosoh) injected with 10 μL samples. The chromatogram was obtained using a flow late of 0.2
242 mL/min with 5mM ammonium acetate as eluent A and acetonitrile as eluent B. Different
243 elution gradient conditions were set for 0 min (90% B), 10 min (40% B), 12 min (40% B), 13
244 min (90% B), and 20 min (90% B).
245
246 **Scanning electron microscopy**
247 Mature *S. mutans* KCTC 3065 was grown at 37°C for 24 h in BHI containing 1%
248 (w/v) glucose and placed on glass coverslips in cell culture plates (60 mm × 15 mm). The
249 coverslips were gently washed twice with 1× phosphate-buffered saline (PBS). Adherent
250 cells were fixed with PBS containing 2.5% glutaraldehyde for 1 h and gradually dehydrated
251 with a series of 25, 50, 75, 90, and 100% ethanol treatments. The prepared cells were dried
252 completely in an oven for 1 h. The cells were then coated with gold, and their morphologies
253 observed using an JSM-7001F field emission scanning electron microscope (JEOL Korea,
254 Seoul, Republic of Korea) (32).
255
256 **Cell-surface hydrophobicity**
257 The hydrophobicity of the *S. mutans* KCTC 3065 cell surface was measured by
258 comparing percentages of binding affinity of the cells to toluene. Cells cultivated in BHI
259 broth were washed twice with 0.85% (w/v) NaCl solution. They were then resuspended to an
260 approximate OD_{600} of 0.3. To calculate hydrophobicity, 3 mL of cell suspension was added to

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

261 250 μL of toluene. This solution was mixed with a vortex for 2 min and incubated at 25°C to
262 allow phase separation. After separation of the toluene phase, the OD of the aqueous phase at
263 600 nm was recorded with a spectrophotometer (33). The hydrophobicity percentage was
264 calculated as follows: $(OD_{initial} - OD_{final}) / OD_{initial} \times 100$.
265
266 **Bacterial adherence assay**
267 The ability of *S. mutans* KCTC 3065 to adhere to glass surfaces was determined as
268 previously described (34). Cells were grown at 37°C for 24 h in glass test tubes at an angle of
269 30° with BHI broth containing 1% (w/v) glucose. After incubation, the tubes were gently
270 spun down, and then the planktonic cells were removed. The attached cells were suspended in
271 0.5 M NaOH by vortexing and quantified at OD_{600}. The adherent percentage was calculated
272 as follows: $(OD_{adherent\ cells}/OD_{total\ cells}) \times 100$.
273
274 **Quantitative real-time polymerase chain reaction**
275 Total RNA was extracted from *S. mutans* KCTC 3065 cultures treated with *B.*
276 *velezensis* K68 culture supernatant using RNeasy mini-columns (Qiagen, Hilden, Germany)
277 with proteinase K solution (Qiagen) (35). cDNA was synthesized with a DiaStar RT kit
278 (Solgent, Daejeon, Korea) using random primers. Quantitative real-time reverse transcription
279 polymerase chain reaction (qRT-PCR) was performed with 2× Real-Time PCR mix
280 (including SYBR green; Solgent) using a StepOnePlus Real-Time PCR System (Applied
281 Biosystems, Foster City, CA, USA) run at 95°C for 5 min, followed by 40 cycles at 95°C for
282 15 s, 60°C for 1 min, and then an annealing and extension step at 60°C for 5 min. Primer
283 sequences were as follows: 5′-AGC AAT GCA GCC ART CTA CAA AT-3′ and 5′-ACG AAC
284 TTT GCC GTT ATT GTC A-3′ for *gtfB*, 5′-YCT CAA CCA ACC GCC ACT GTT-3′ and 5′-

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

285 TTA ACG TCA AAA TTA CGA CAT AAT C-3′ for *gtfC*, 5′-CAC AGG CAA AAG CTG AAT
286 TAA CA-3′ and 5′-AAT GGC CGC TAA GTC AAC AG-3′ for *gtfD*, and 5′-CCT ACG GGA
287 GGC AGC AGT AG-3′ and 5′-CAA CAG AGC TTT ACG ATC CGA AA-3′ for the 16S
288 rRNA gene. Relative quantification was performed using the 2-ΔΔCt method with a reference
289 gene (16S rRNA gene) from *S. mutans* KCTC 3065 (36, 37).
290
291 **SUPPLEMENTAL MATERIAL**
292 Supplemental material for this article may be found.
293 **SUPPLEMENTAL FILE 1,** PDF file, 0.2 MB.
294
295 **ACKNOWLEDGMENTS**
296 This research was supported by the Basic Science Research Programs through the
297 National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT &
298 Future Planning (NRF-2014R1A1A1002980) and the Ministry of Education (NRF-
299 2016R1D1A1B03931582) for M.-J.S. This work was also supported by the Main Research
300 Program (E0170602-02) of the Korea Food Research Institute (KFRI) funded by the Ministry
301 of Science and ICT & Future Planning for Y.-D.N.

302

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

303 **REFERENCES**

304 1. Metwalli KH, Khan SA, Krom BP, Jabra-Rizk MA. 2013. *Streptococcus mutans*,
305 *Candida albicans*, and the human mouth: a sticky situation. PLOS Pathog 9:e1003616.
306 https://doi.org/10.1371/journal.ppat.1003616.
307 2. Al-Sohaibani S, Murugan K. 2012. Anti-biofilm activity of *Salvadora persica* on
308 cariogenic isolates of *Streptococcus mutans*: *in vitro* and molecular docking studies.
309 Biofouling 28:29–38. https://doi.org/10.1080/08927014.2011.647308.
310 3. Wang H, Ren D. 2017. Controlling *Streptococcus mutans* and *Staphylococcus aureus*
311 biofilms with direct current and chlorhexidine. AMB Express 7:204.
312 https://doi.org/10.1186/s13568-017-0505-z.
313 4. Koga T, Okahashi N, Takahashi I, Kanamoto T, Asakawa H, Iwaki M. 1990. Surface
314 hydrophobicity, adherence, and aggregation of cell surface protein antigen mutants of
315 *Streptococcus mutans* serotype c. Infect Immun 58:289–296.
316 5. Marsh PD. 2006. Dental plaque as a biofilm and a microbial community –
317 implications for health and disease. BMC Oral Health 6:S14–S14.
318 https://doi.org/10.1186/1472-6831-6-S1-S14.
319 6. Zijnge V, van Leeuwen MBM, Degener JE, Abbas F, Thurnheer T, Gmür R, M.
320 Harmsen HJ. 2010. Oral biofilm architecture on natural teeth. PLoS One 5:e9321.
321 https://doi.org/10.1371/journal.pone.0009321.
322 7. Islam B, Khan SN, Haque I, Alam M, Mushfiq M, Khan AU. 2008. Novel anti-
323 adherence activity of mulberry leaves: inhibition of *Streptococcus mutans* biofilm by
324 1-deoxynojirimycin isolated from *Morus alba*. J Antimicrob Chemother 62:751–757.
325 https://doi.org/10.1093/jac/dkn253.
326 8. Bowen WH, Koo H. 2011. Biology of *Streptococcus mutans*-derived

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

327 glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms.
328 Caries Res 45:69–86. https://doi.org/10.1159/000324598.
329 9. Wen ZT, Yates D, Ahn S-J, Burne RA. 2010. Biofilm formation and virulence
330 expression by *Streptococcus mutans* are altered when grown in dual-species model.
331 BMC Microbiol 10:111. https://doi.org/10.1186/1471-2180-10-111.
332 10. Yousefi B, Ghaderi S, Rezapoor-Lactooyi A, Amiri N, Verdi J, Shoae-Hassani A. 2012.
333 Hydroxy decenoic acid down regulates gtfB and *gtfC* expression and prevents
334 *Streptococcus mutans* adherence to the cell surfaces. Ann Clin Microbiol Antimicrob
335 11:21–21. https://doi.org/10.1186/1476-0711-11-21.
336 11. Argimón S, Alekseyenko AV, DeSalle R, Caufield PW**.** 2013. Phylogenetic analysis of
337 glucosyltransferases and implications for the coevolution of mutans Streptococci with
338 their mammalian hosts. PLoS One 8:e56305.
339 https://doi.org/10.1371/journal.pone.0056305.
340 12. Chuang L-C, Huang C-S, Ou-Yang L-W, Lin S-Y**.** 2011. Probiotic *Lactobacillus*
341 *paracasei* effect on cariogenic bacterial flora. Clin Oral Investig 15:471–476.
342 https://doi.org/10.1007/s00784-010-0423-9.
343 13. Baca-Castañón ML, De la Garza-Ramos MA, Alcázar-Pizaña AG, Grondin Y,
344 Coronado-Mendoza A, Sánchez-Najera RI, Cárdenas-Estrada E, Medina-De la Garza
345 CE, Escamilla-García E. 2015. Antimicrobial effect of *Lactobacillus reuteri* on
346 cariogenic bacteria *Streptococcus gordonii*, *Streptococcus mutans*, and periodontal
347 diseases *Actinomyces naeslundii* and *Tannerella forsythia*. Probiotics Antimicrob
348 Proteins 7:1–8. https://doi.org/10.1007/s12602-014-9178-y.
349 14. Wu CC, Lin CT, Wu CY, Peng WS, Lee MJ, Tsai YC. 2015. Inhibitory effect of
350 *Lactobacillus salivarius* on *Streptococcus mutans* biofilm formation. Mol Oral

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

351 Microbiol 30:16–26. https://doi.org/10.1111/omi.12063.

352 15. Cagetti MG, Mastroberardino S, Milia E, Cocco F, Lingström P, Campus G. 2013.
353 The use of probiotic strains in caries prevention: a systematic review. Nutrients
354 5:2530–2550. http://doi.org/10.3390/nu5072530.

355 16. Kim S-Y, Ohk S-H, Bai D-H, Yu J-H. 1999. Purification and properties of
356 bacteriolytic enzymes from *Bacillus licheniformis* YS-1005 against *Streptococcus*
357 *mutans*. Biosci Biotechnol Biochem 63:73–77. https://doi.org/10.1271/bbb.63.73.

358 17. Sumathi C, Nandhini A, Padmanaban J. 2017. Antagonistic activity of probiotic
359 *Bacillus Megaterium* against *Streptococcus Mutans*. Int J Pharm Bio Sci 8:270–274.
360 http://dx.doi.org/10.22376/ijpbs.2017.8.1.p270-274.

361 18. Hasan S, Singh K, Danisuddin M, Verma PK, Khan AU. 2014. Inhibition of major
362 virulence pathways of *Streptococcus mutans* by quercitrin and deoxynojirimycin: a
363 synergistic approach of infection control. PLoS One 9:e91736.
364 https://doi.org/10.1371/journal.pone.0091736.

365 19. Zhu Y-P, Yamaki K, Yoshihashi T, Ohnishi Kameyama M, Li X-T, Cheng Y-Q, Mori Y,
366 Li L-T. 2010. Purification and identification of 1-deoxynojirimycin (DNJ) in okara
367 fermented by *Bacillus subtilis* B2 from Chinese traditional food (Meitaoza). J Agric
368 Food Chem 58:4097–4103. https://doi.org/ 10.1021/jf9032377.

369 20. Hardick DJ, Hutchinson DW, Trew SJ, Wellington EMH. 1992. Glucose is a
370 Precursor of 1-deoxynojirimycin and 1-deoxymannonojirimycin in *Streptomyces*
371 *subrutilus*. Tetrahedron 48:6285–6296. https://doi.org/10.1016/S0040-
372 4020(01)88220-X.

373 21. Kang K-D, Cho YS, Song JH, Park YS, Lee JY, Hwang KY, Rhee SK, Chung JH,
374 Kwon O, Seong S-I. 2011. Identification of the genes involved in 1-deoxynojirimycin

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

375 synthesis in *Bacillus subtilis* MORI 3K-85. J Microbiol 49:431–440.
376 https://doi.org/10.1007/s12275-011-1238-3.

377 22. Westergren G, Olsson J. 1983. Hydrophobicity and adherence of oral streptococci
378 after repeated subculture *in vitro*. Infect Immun 40:432–435.

379 23. Tahmourespour A, Kasra Kermanshahi R, Salehi R, Nabinejad A. 2008. The
380 relationship between cell surface hydrophobicity and antibiotic resistance of
381 streptococcal strains isolated from dental plaque and caries. Iran J Basic Med Sci
382 10:251–255. https://doi.org/ 10.22038/ijbms.2008.5239.

383 24. Staat RH, Langley SD, Doyle RJ. 1980. *Streptococcus mutans* adherence:
384 presumptive evidence for protein-mediated attachment followed by glucan-dependent
385 cellular accumulation. Infect Immun 27:675–681.

386 25. Germaine GR, Harlander SK, Leung W-LS, Schachtele CF. 1977. *Streptococcus*
387 *mutans* dextransucrase: functioning of primer dextran and endogenous dextranase in
388 water-soluble and water-insoluble glucan synthesis. Infect Immun 16:637–648.

389 26. Wenham DG, Davies RM, Cole JA. 1981. Insoluble glucan synthesis by mutansucrase
390 as a determinant of the cariogenicity of *Streptococcus mutans*. Microbiology
391 127:407–415. https://doi.org/ 10.1099/00221287-127-2-407.

392 27. Lynch DJ, Fountain TL, Mazurkiewicz JE, Banas JA. 2007. Glucan-binding proteins
393 are essential for shaping *Streptococcus mutans* biofilm architecture. FEMS Microbiol
394 Lett 268:158–165. https://doi.org/10.1111/j.1574-6968.2006.00576.x.

395 28. Hoshino T, Fujiwara T, Kawabata S. 2012. Evolution of cariogenic character in
396 *Streptococcus mutans*: horizontal transmission of glycosyl hydrolase family 70 genes.
397 Sci Rep 2:518. https://doi.org/ 10.1038/srep00518.

398 29. Chen P-M, Chen J-Y, Chia J-S. 2006. Differential regulation of *Streptococcus mutans*

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

399 *gtfBCD* genes in response to copper ions. Arch Microbiol 185:127–135.
400 https://doi.org/10.1007/s00203-005-0076-2.
401 30. Olson ND, Morrow JB. 2012. DNA extract characterization process for microbial
402 detection methods development and validation. BMC Res Notes 5:668–668.
403 https://doi.org/10.1186/1756-0500-5-668.
404 31. Seo M-J, Nam Y-D, Lee S-Y, Park S-L, Yi S-H, Lim S-I. 2013. Isolation of the
405 Putative biosynthetic gene cluster of 1-deoxynojirimycin by *Bacillus*
406 *amyloliquefaciens* 140N, Its production and application to the fermentation of
407 soybean paste. Biosci Biotechnol Biochem 77:398–401.
408 https://doi.org/10.1271/bbb.120753.
409 32. Bedran TBL, Grignon L, Spolidorio DP, Grenier D. 2014. Subinhibitory
410 concentrations of triclosan promote *Streptococcus mutans* biofilm formation and
411 adherence to oral epithelial cells. PLoS One 9:e89059.
412 https://doi.org/10.1371/journal.pone.0089059.
413 33. Kim DH, Kwon T-Y. 2017. *In vitro* study of *Streptococcus mutans* adhesion on
414 composite resin coated with three surface sealants. Restor Dent Endod 42:39–47.
415 https://doi.org/10.5395/rde.2017.42.1.39.
416 34. Limsong J, Benjavongkulchai E, Kuvatanasuchati J. 2004. Inhibitory effect of some
417 herbal extracts on adherence of *Streptococcus mutans*. J Ethnopharmacol 92:281–289.
418 https://doi.org/10.1016/j.jep.2004.03.008.
419 35. Tahmourespour A, Salehi R, Kermanshahi RK, Eslami G. 2011. The anti-biofouling
420 effect of *Lactobacillus fermentum*-derived biosurfactant against *Streptococcus mutans*.
421 Biofouling 27:385–392. https://doi.org/10.1080/08927014.2011.575458.
422 36. Tahmourespour A, Salehi R, Kasra Kermanshahi R. 2011. *Lactobacillus acidophilus*-

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

423 derived biosurfactant effect on GTFB and GTFC expression level in *Streptococcus*
424 *Mutans* biofilm cells. Brazil J Microbiol 42:330–339.
425 http://dx.doi.org/10.1590/S1517-83822011000100042.
426 37. Kim Y-J, Lee S-H. 2016. Inhibitory effect of *Lactococcus lactis* HY 449 on cariogenic
427 biofilm. J Microbiol Biotechnol 26:1829–1835.
428 http://dx.doi.org/10.4014/jmb.1604.04008.
429

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

430 **Table 1** Comparison of the deduced amino acid sequences of the 1-DNJ biosynthetic genes

431 isolated from *B. velezensis* K68

***B. velezensis* K68 (Nucleotide accession no., nucleotides, aa[a])**	***B. velezensis* FZB42[b] (aa, % identity/similarity to K68)**	**Predicted function**
gabT1 (MH142719, 1269, 422)	ABS72608 (425, 99.1%/99.1%)	4-aminobutyrate aminotransferase
yktc1 (MH142720, 951, 316)	ABS72609 (316, 99.1%/99.4%)	fructose-1,6 biphosphate/inositol monophosphatase
gutB1 (MH142721, 1047, 348)	ABS72610 (348, 99.4%/99.7%)	zinc-binding dehydrogenase

432 [a]Deduced amino acid sequences for each gene of *B. velezensis* K68.

433 [b]Amino acid accession no. for each ORF of *B. velezensis* FZB42.

434

bioRxiv preprint doi: https://doi.org/10.1101/313965; this version posted May 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

435 **Figure legends**

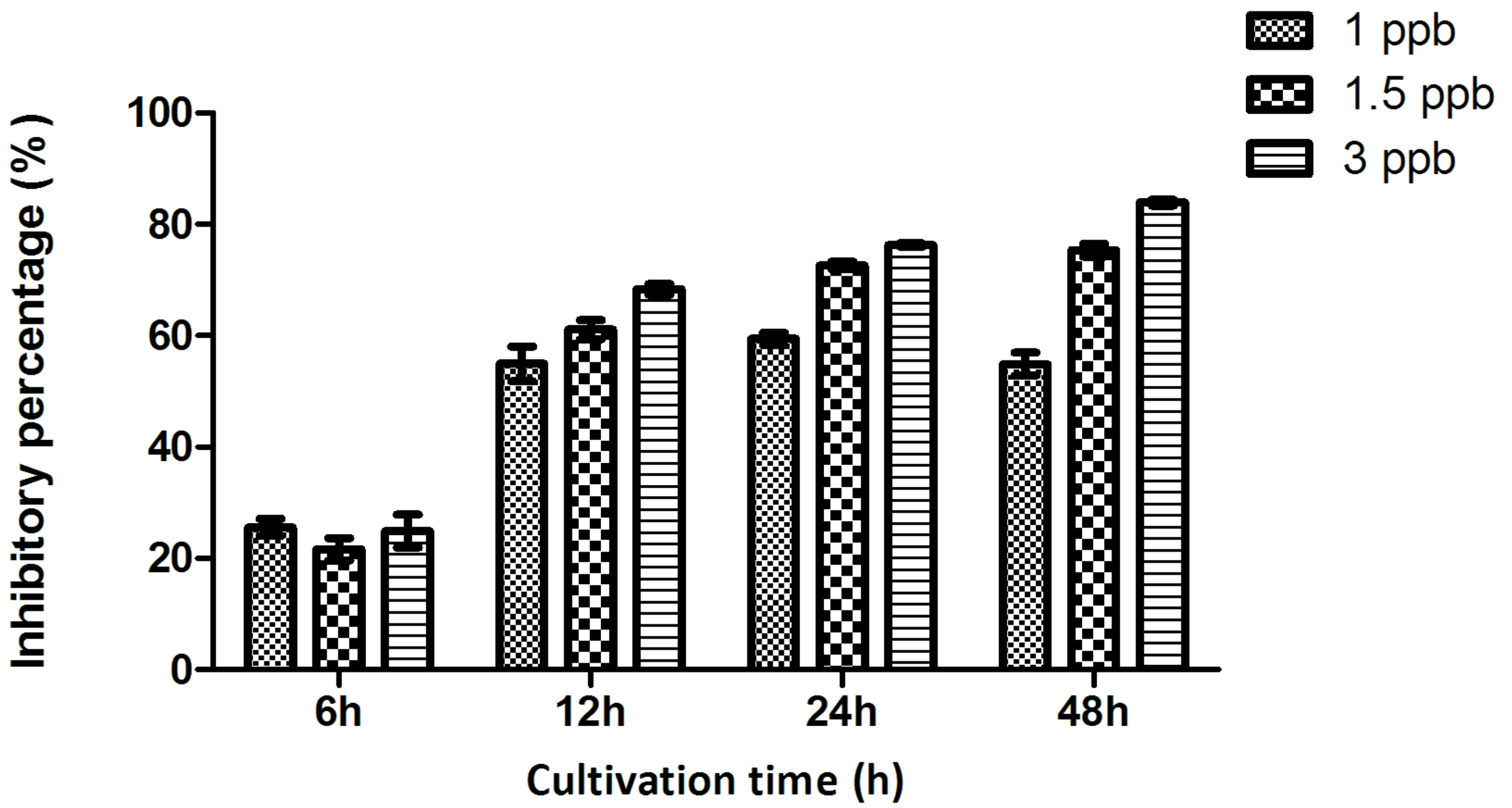
436

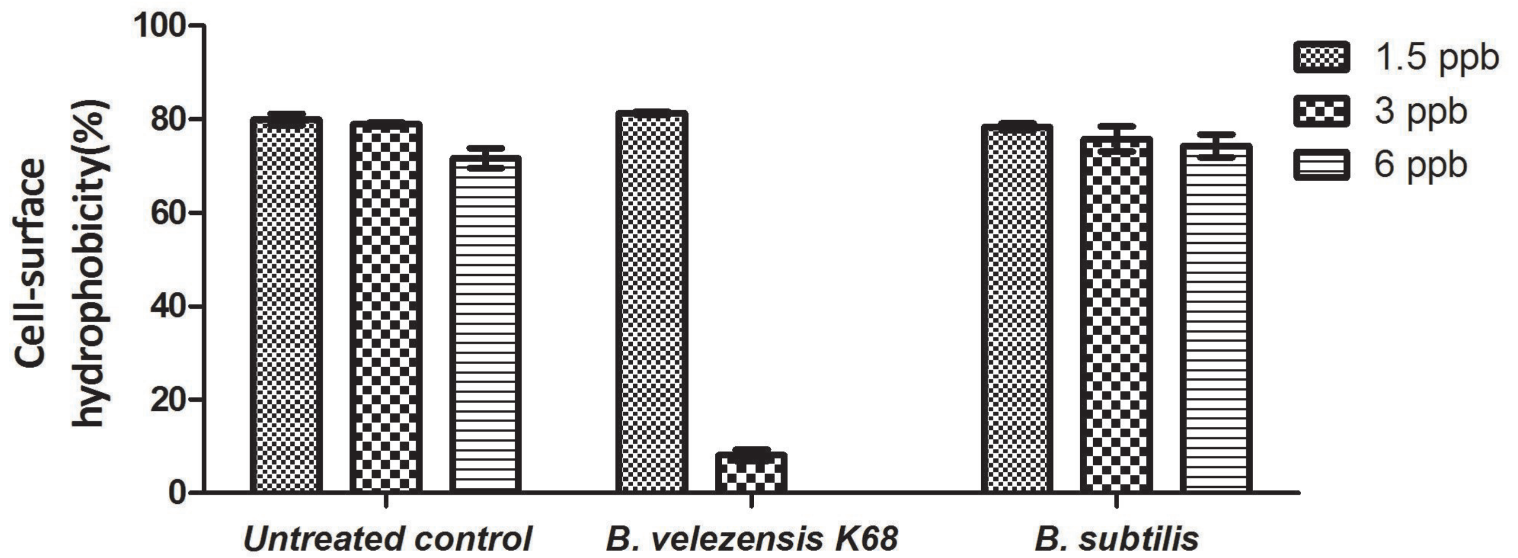
437 **FIG 1** Inhibition of biofilm formation with supernatant from *B. velezensis* K68 culture media.

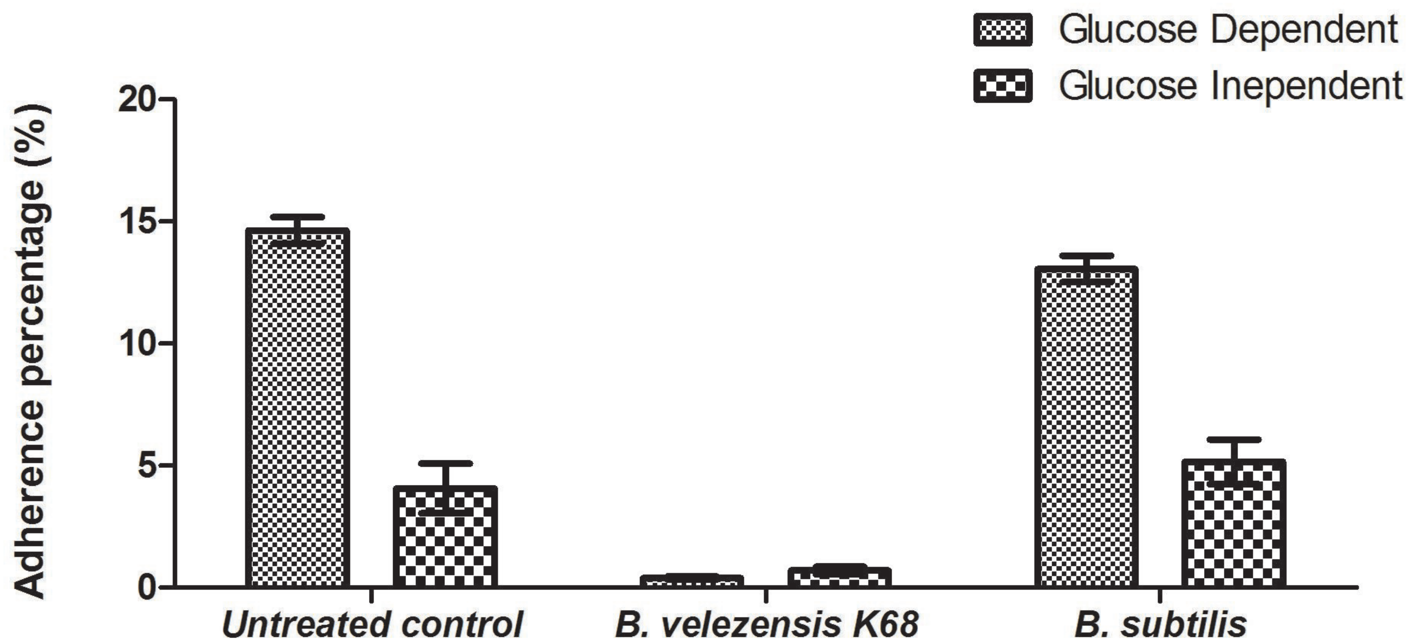
438 **FIG 2** Effects of the supernatant from *B. velezensis* K68 culture medium on the cell-surface
439 hydrophobicity (A) and adherence (B) of *S. mutans*.

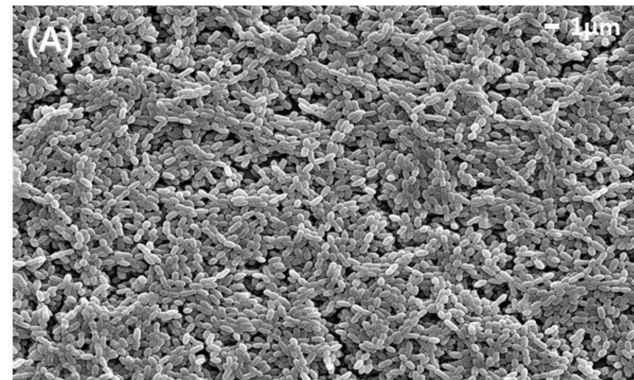
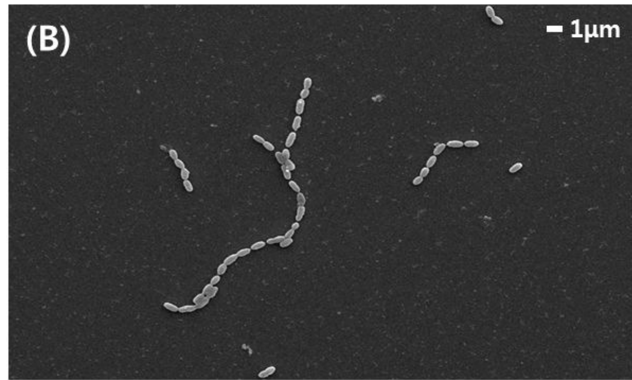
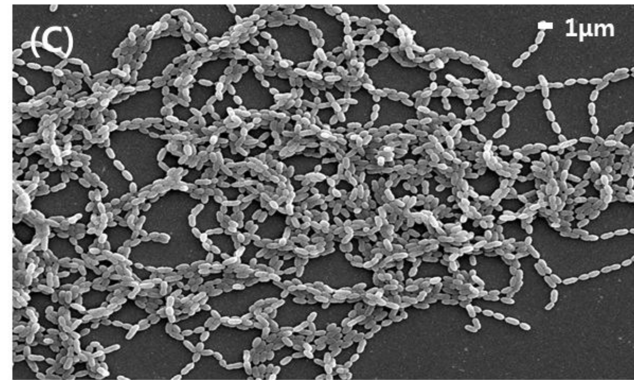
440 **FIG 3** Scanning electron micrographs of *S. mutans* biofilms. (a) *S. mutans* (untreated control),
441 (b) *S. mutans* treated with supernatant from *B. velezensis* K68 medium, (c) *S. mutans* treated
442 with supernatant from *B. subtilis* medium (negative control).

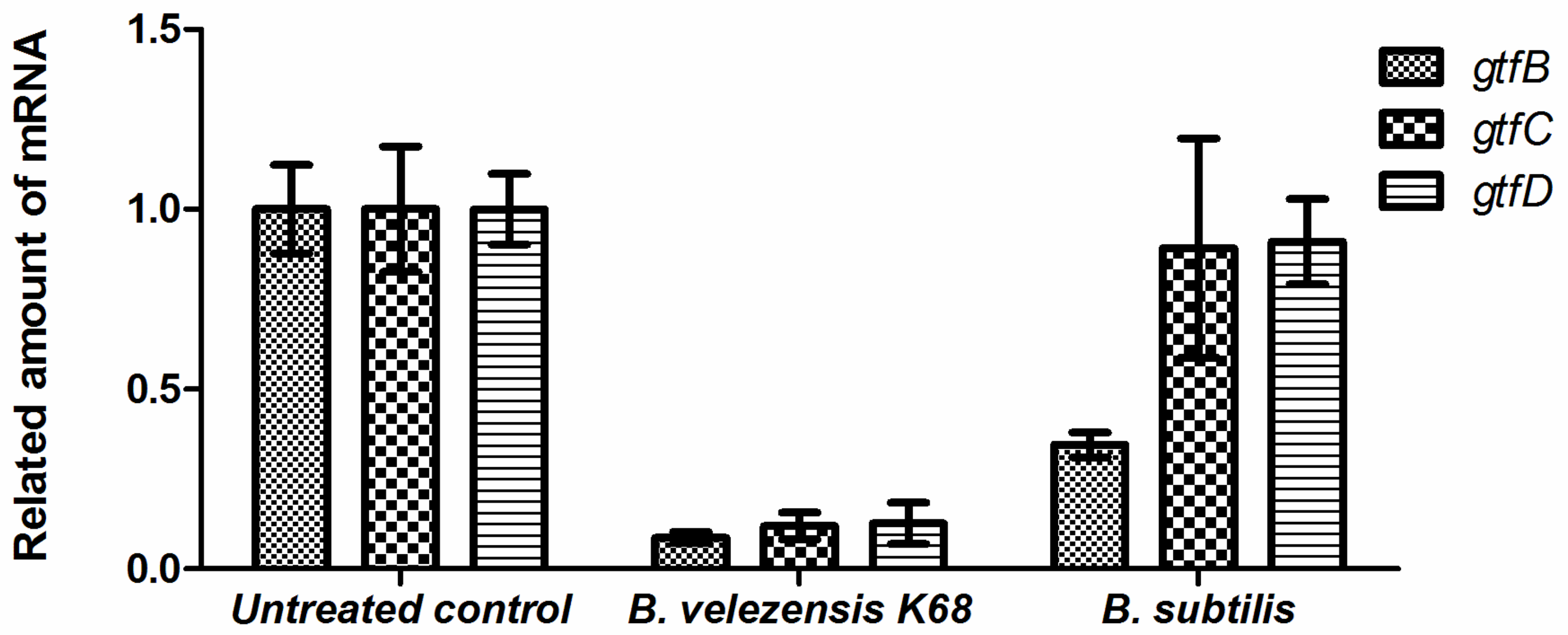
443 **FIG 4** *S. mutans* glucosyltransferase gene expression after treatment with supernatant from *B.*
444 *velezensis* K68 medium. *gtfB*, *gtfC*, and *gtfD*, which encode glucosyltransferases in *S. mutans*,
445 were analyzed by qPCR.


1 ppb
1.5 ppb
3 ppb
Inhibitory percentage (%)
100
80
60
40
20
0
6h
12h
24h
48h
Cultivation time (h)


(A)
Cell-surface hydrophobicity(%)
100
80
60
40
20
0
1.5 ppb
3 ppb
6 ppb
Untreated control
B. velezensis K68
B. subtilis


(B)
Adherence percentage (%)
20
15
10
5
0
Glucose Dependent
Glucose Inependent
Untreated control
B. velezensis K68
B. subtilis


(A)
1μm

(B)
1μm

(C)
1μm



Related amount of mRNA
1.5
1.0
0.5
0.0
gtfB
gtfC
gtfD
Untreated control
B. velezensis K68
B. subtilis