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1 **Method for screening antimicrobial gels against multi-species oral biofilms**

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6 Keywords: oral biofilm, antibacterial gels, *Porphyromonas gingivalis*, *Treponema denticola*,
7 *Streptococcus gordonii*, hyaluronic acid, oxygen, MBECTM assay.

8 Running headline: *In-vitro* assay to evaluate gels against oral biofilm.

9 10 **Abstract**

11 We described a microtiter plate-based method that was effectively tailored for testing gel
12 formulations against oral multispecies biofilms established on peg-lids. This method lifts the
13 limitations imposed mainly by the anaerobic nature of the targeted bacterial species and the viscous
14 properties of the targeted treatments.

15
16 Periodontitis is a polymicrobial chronic inflammatory disease of the periodontium caused
17 by the accumulation of dental plaque (Pihlstrom *et al.*, 2005). It is regarded as the second most
18 common disease worldwide, with severe forms affecting up to 10 to 15% of adults (Petersen and
19 Ogawa, 2012) resulting in tooth loss. Gel formulations of antibacterial agents are a promising
20 delivery system for the treatment of periodontitis *via* topical administration. Their advantages

21 include the ease of use, increased retention time at the site of application and controlled drug
22 release. Several authors have demonstrated the effectiveness of gel formulations in reducing
23 microbial content or plaque index (Gomes *et al.*, 2006; Noyan *et al.*, 1997; Paquette *et al.*, 1997;
24 Sauvêtre *et al.*, 1993) in human, animal-based or *in vitro* studies. Similar improvement in probing
25 depth and/or bleeding was reported (Esposito *et al.*, 1996; Graça *et al.*, 1997) on use of gel
26 formulations alone or in conjunction with other modes of treatment. Our objective is to develop a
27 process for high-throughput screening of the efficacy of gel-based formulations on a biofilm of oral
28 bacteria.

29 The protocol is based on the model biofilm we previously established (Martin *et al.*, 2018)
30 which includes three key periodontal biofilm species. *Streptococcus gordonii* is a representative
31 species of peroxygenic oral streptococci and produces hydrogen peroxide that can influence the
32 pathogenic switch of the oral subgingival biofilm (Chathoth *et al.*, 2020; Kreth *et al.*, 2008).
33 Whereas, *Porphyromonas gingivalis* and *Treponema denticola*, co-exist in deep periodontal
34 pockets (Kigure *et al.*, 1995; Kumawat *et al.*, 2016) display a coaggregation reaction (Grenier,
35 1992) and are associated with severe forms of periodontitis. A recent theory proposed that the
36 peroxygenic Streptococci (initial colonizers of dental plaque) and the sulfidogenic species such as
37 *P. gingivalis* and *T. denticola* (pathogenic late colonizers) may act as the key players in a dysbiosis
38 process (Chathoth *et al.*, 2020). We therefore choose to implement an assay and test the effect of
39 antimicrobial gels on this biofilm, considering the limitations imposed by both the anaerobic nature
40 of the targeted bacterial species which prevent the use of classical Live/Dead stain or viability-PCR
41 using PMA and the viscosity of the gels. The antimicrobial activity is evaluated by analysis of
42 biofilm development after treatment by qPCR. The conversion of genomic DNA quantity to cells
43 ml⁻¹ was made possible by considering specific genome weights (2.58x10⁻⁶ ng for *P. gingivalis*,

44 3.12x10⁻⁶ ng for *T. denticola* and 2.41x10⁻⁶ ng for *S. gordonii*) as described by Ammann *et al.*,
45 (2013). In this study, the primers used to distinguish between species were (5'→3'): 16S *S.*
46 *gordonii* F, AAG-CAA-CGC-GAA-GAA-CCT-TA; 16S *S. gordonii* R, GTC-TCG-CTA-GAG-
47 TGC-CCA-AC; 16S *P. gingivalis* F, TGG-GTT-TAA-AGG-GTG-CGT-AG; 16S *P. gingivalis* R,
48 CAA-TCG-GAG-TTC-CTC-GTG-AT; 16S *T. denticola* F, CGC-GTC-CCA-TTA-GCT-AGT-TG
49 and 16S *T. denticola* R, TTC-TTC-ATT-CAC-ACG-GCG-TC.

50 The mixed biofilm was grown for 6 or 24 hours on saliva-coated pegs of Minimum Biofilm
51 Elimination Concentration (MBEC™) Assay System (formerly the Calgary Biofilm Device) (Ceri
52 *et al.*, 1999) and challenged for 1 hour with two gels of similar viscosity already used for the
53 treatment of oral infections. The method is described in Figure 1. Hyalugel®-ADO (Ricerfarma,
54 Milan, Italy) mainly consists of hyaluronic acid (0.2 %, w/w); blue®m oral gel (blue®m Europe
55 B.V., Netherlands) has the oxygen donor sodium perborate (1.72 %, w/w) and the iron chelator
56 lactoferrin (0.2 %, w/w) as main active ingredients (Shibli *et al.*, 2021). A prior experiment using
57 the agar diffusion method indicated that both gel formulations exhibited antibacterial activity
58 against *S. gordonii* and *P. gingivalis*, with blue®m oral gel having better antibacterial activity than
59 Hyalugel®-ADO (Table S1).

60 In a preliminary assay to implement the method, the antimicrobial gels were tested on
61 mature biofilms, grown for 24 hours. However, no significant change was observed in the ability
62 of *P. gingivalis* and *T. denticola* to develop in the biofilm after the treatment for a further 24-hour
63 incubation in comparison to the control treated with the growth medium MMBC-3 (Martin *et al.*,
64 2018). Meanwhile, the biofilm growth of *S. gordonii* was severely affected by blue®m oral gel,
65 resulting in a dysbiosis with an increase in the ratio of anaerobic pathogens and a decreased total
66 biomass in comparison to the control (Table S2). This indicated that the treatment with these gels

67 could be effective as a preventive measure on a thinner biofilm. Therefore, we run the assays on 6-
68 hour biofilms.

69 The results corresponding to the treatment of a 6-hour biofilm are shown in Figure 2.
70 Confocal fluorescent microscopy imaging of biofilms was performed as a qualitative test to ensure
71 the presence of bacterial biofilm all over the pegs surface (Figure S1). The concentration of the
72 total bacteria (biofilm + planktonic) was significantly reduced from 4.11×10^9 cells ml⁻¹ in MMBC-
73 3 control to 5.15×10^6 cells ml⁻¹ and 1.5×10^7 cells ml⁻¹ for Hyalugel[®]-ADO and blue[®]m oral gel
74 respectively after treatment of the biofilms for one hour (Figure 2A). The samples treated with
75 Hyalugel[®]-ADO and blue[®]m oral gel displayed a biofilm reduction of 96.5 % and 97.6 %
76 respectively, in comparison to untreated control (taken as 0 % of reduction). This decrease in
77 biofilm concentration due to Hyalugel[®]-ADO and blue[®]m oral gel is also evident in the
78 microscopic images (Figure S1). Similarly, a decrease of over 99.5 % was observed in the
79 concentration of detached/planktonic cells on treatment with either of the gels in comparison to
80 control (MMBC-3-treated samples) (Figure 2A). The biofilms on the peg surface (Figure 2B) and
81 the corresponding planktonic (unattached or detached) (Figure 2C) cells contained a higher
82 proportion of *S. gordonii* in comparison to *P. gingivalis* and *T. denticola* cells. The concentration
83 of *S. gordonii* and *T. denticola* in the biofilm decreased significantly between the control samples
84 and the samples treated with Hyalugel[®]-ADO and blue[®]m oral gel, while the concentration of *P.*
85 *gingivalis* remained unchanged. Hyalugel[®]-ADO decreased the concentration of planktonic *S.*
86 *gordonii* and *P. gingivalis* compared to controls while no effect was observed on *T. denticola*.
87 blue[®]m oral gel induced a significant decrease in the concentration of planktonic *S. gordonii* cells
88 and an increase in planktonic forms of *P. gingivalis* and *T. denticola*. These results indicate that
89 both gels are effective in reducing biofilm development after a 1-hour treatment and that blue[®]m

90 oral gel is additionally effective in inducing detachment of anaerobic pathogenic species. The
91 method will then allow the number of product applications and the duration of the treatment to be
92 refined in order to optimise the beneficial effects.

93 In conclusion, this method appears suitable to evaluate the antimicrobial effects of gels
94 against a biofilm whose composition is relevant to predict the evolution of periodontitis. It allows
95 high-throughput testing and is easy to use since antimicrobial activity is assessed by the ability of
96 bacterial strains to grow after treatment. The use of qPCR allows monitoring of both the evolution
97 of the total biofilm and the behaviour of each partner in the biofilm. The modulation of biofilm
98 growth times and treatment exposure times will make it possible to refine the method on a case-
99 by-case basis.

100

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107

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200

201 **Legends of figures**

202 **Figure 1. The different steps of the method for screening the effectiveness of gels on a biofilm**
203 **of oral bacteria.** Strains of *Streptococcus gordonii* Challis DL1 (Chen *et al.*, 2004),
204 *Porphyromonas gingivalis* TDC60 (Watanabe *et al.*, 2011) and *Treponema denticola* ATCC35405
205 (Chan *et al.*, 1993) were used for the study. The lid with pegs (Nunc-TSP, polystyrene) was placed
206 over the microtiter plate and incubated in filter-sterilised saliva that was diluted twice in sterile
207 distilled water (Normal Saliva Human Fluid, Pool Human Donors, MyBioSource) for 30 minutes.
208 The saliva-coated pegs were immersed in 200 µl of inoculum consisting of *S. gordonii*
209 (OD_{600nm}:0.05), *P. gingivalis* (OD_{600nm}:0.1) and *T. denticola* (OD_{600nm}:0.1) and incubated in
210 anaerobic conditions for 6 or 24 hours at 37°C. The 6-hour or 24-hour three-species biofilms or
211 adherent cells present on the surface of the pegs were subjected to 150 µL of either MMBC-3 or
212 Hyalugel[®]-ADO or blue[®]m oral gel for 1 hour in anaerobic condition at 37°C. The pegs were then

213 incubated in a new microtiter well containing 200 μ l of MMBC-3 for 24 hours in anaerobic
214 conditions at 37°C. The detached/planktonic cells were collected from the microtiter plate and the
215 biofilm were collected from the surface of the pegs by sonication (30 min) and were quantified by
216 qPCR as previously described (Martin *et al.*, 2017). Six pegs (two per condition) were also kept
217 for microscopic visualisation.

218

219 **Figure 2. qPCR quantification of the number of bacteria (cells ml⁻¹) in the biofilm (collected**
220 **from the pegs) and in planktonic form (collected from the 96-well microtiter plate).** The 6-
221 hour three-species biofilm on the pegs were subjected to MMBC-3 or Hyalugel[®]-ADO or blue[®]m
222 oral gel for 1 hour and further incubated in MMBC-3 for 24 hours. The planktonic cells were
223 collected from the 96-well plate while the biofilm was collected from the pegs and quantified. (A)
224 Total concentration (cells ml⁻¹) of bacteria (planktonic/detached + biofilm), bacteria in biofilm and
225 bacteria detached or in planktonic form after each treatment. (B) Concentration of each species in
226 the biofilm after each treatment. (C) Concentration of each species in planktonic/ detached cells
227 for each treatment. White bars = MMBC-3, grey bars = Hyalugel[®]-ADO and black bars = blue[®]m
228 oral gel. p-value < 0.05 = *, p-value < 0.01 = **, p-value < 0.001 = ***

229

230 **Figure S1. Representative microscopic images of the 6-hour three-species oral biofilm on the**
231 **surface of the pegs.** Post 1-hour treatment (with MMBC-3, Hyalugel[®]-ADO or blue[®]m oral gel)
232 of the 6-hour biofilms on the surface of the pegs, the pegs were incubated in MMBC-3 for 24 hours.
233 The pegs were further broken from the lid using pliers and stained using Styo[®]9. The stained pegs
234 were visualized using the Leica TCS-SP5 confocal laser scanning microscope. To compare

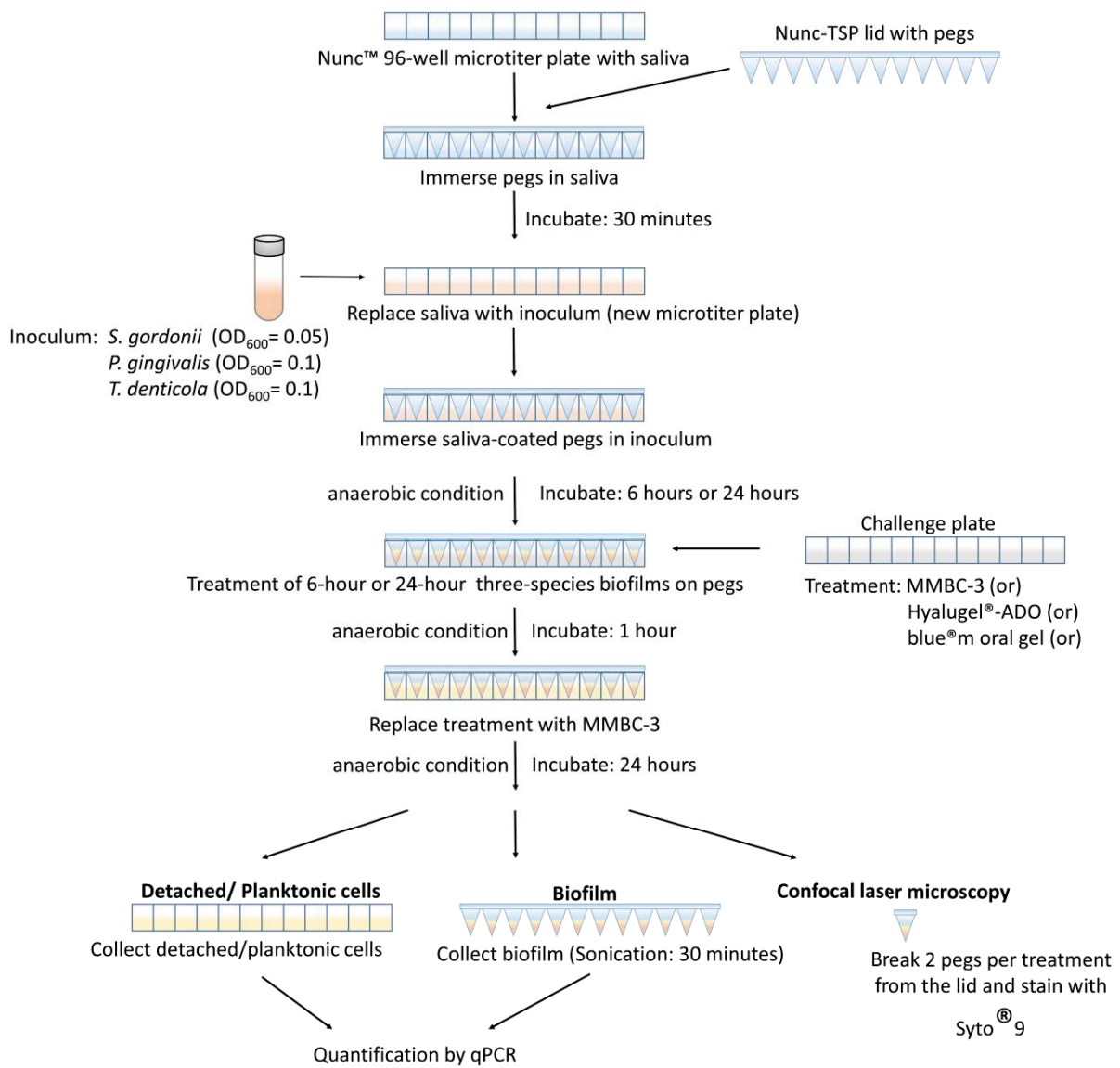
235 between the three treatments, maximum z-production of the Z stack were taken using 10X oil
236 immersion objective lens and numerical zoom of 2: (A), (B), (C). Magnified images were captured
237 using the 63X oil immersion objective lens, numerical zoom of 5.05: (D), (E), (F).

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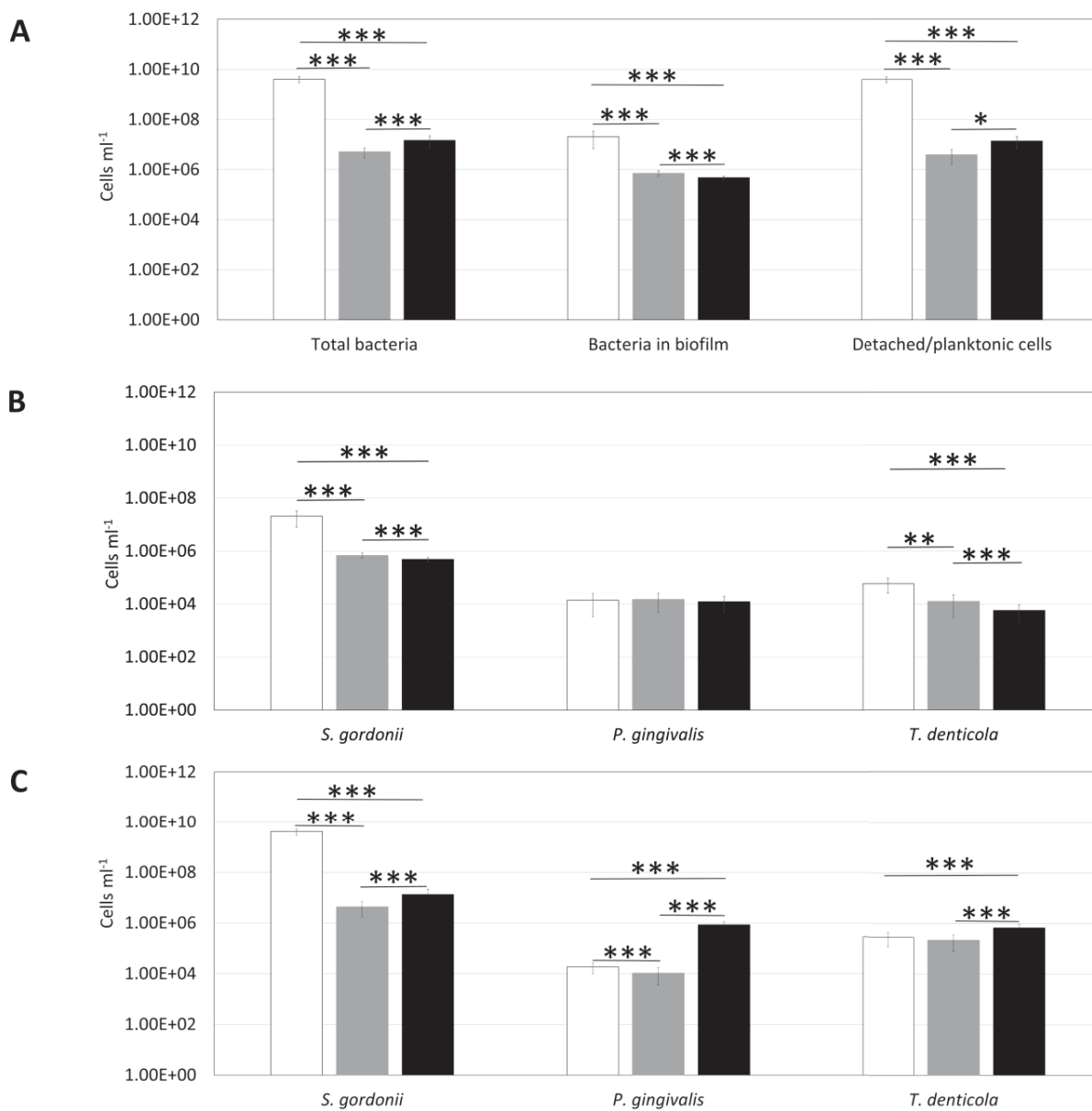


242

243 **Figure 1**

244

245



246

247

248 **Figure 2**