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1 Ecological approaches to oral biofilms: control without killing

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22 **Running title:** Biofilms: control without killing

23 **Key words:** Plaque control; biofilm; oral microbiome ; antimicrobial agents;

24 modelling

25 **Abstract**

26 Humans have co-evolved with micro-organisms, and have a symbiotic or mutualistic
27 relationship with their resident microbiome. As at other body surfaces, the mouth has
28 a diverse microbiota that grows on oral surfaces as structurally- and functionally-
29 organised biofilms. The oral microbiota is natural and provides important benefits to
30 the host, including immunological priming, down-regulation of excessive pro-
31 inflammatory responses, regulation of gastrointestinal and cardiovascular systems,
32 and colonisation by exogenous microbes. On occasions, this symbiotic relationship
33 breaks down, and previously minor components of the microbiota out-compete
34 beneficial bacteria, thereby increasing the risk of disease. Antimicrobial agents have
35 been formulated into many oral care products to augment mechanical plaque control.
36 A delicate balance is needed, however, to control the oral microbiota at levels
37 compatible with health, without killing beneficial bacteria and losing the key benefits
38 delivered by these resident microbes. These antimicrobial agents may achieve this
39 by virtue of their recommended twice daily topical use, which results in
40 pharmacokinetic profiles indicating that they are retained in the mouth for relatively
41 long periods at sub-lethal levels. At these concentrations they are still able to inhibit
42 bacterial traits implicated in disease (e.g. sugar transport/acid production; protease
43 activity) and retard growth without eliminating beneficial species. *In silico* modelling
44 studies have been performed which support the concept that either reducing the
45 frequency of acid challenge and/or the terminal pH, or by merely slowing bacterial
46 growth, results in maintaining a community of beneficial bacteria under conditions
47 that might otherwise lead to disease (controlling without killing).

48 **244/250 words**

49 From birth, the infant is exposed to and colonised by a wide range of micro-
50 organisms, derived mainly from the mother, although only a subset are able to
51 establish successfully [Sampaio-Maia and Monteiro-Silva, 2014]. These micro-
52 organisms increase in number and type over time, eventually resulting in the
53 presence of ten times more microbes than human cells. The biological properties of
54 each habitat determine which micro-organisms can colonise and grow, and dictate
55 which will be major or minor components of the resident microbiota of a site. This
56 results in different surfaces having distinct but characteristic microbiotas [Arrieta et
57 al., 2014; Chen and Jiang, 2014; Christensen and Bruggemann, 2014; Cogen et al.,
58 2008; Consortium, 2012; Crielaard et al., 2011; Faust et al., 2012; Sampaio-Maia
59 and Monteiro-Silva, 2014; Sanchez et al., 1990].

60 The human microbiome and the host have co-evolved to have a symbiotic or
61 mutualistic relationship [Chow et al., 2010]. The resident micro-organisms gain a
62 secure, warm, nutritious habitat from the host, and contribute to food digestion,
63 nutrition, regulation of human metabolism, differentiation of host mucosa, immune
64 development and function, and prevention of colonisation by exogenous and often
65 pathogenic microbes [Relman, 2012]. The microbiome thus helps to direct the
66 development of host immunity, and host immune responses in turn shape the
67 resident microbiome [Belkaid and Naik, 2013]. This relationship between the
68 resident microbiome and the host is dynamic and, whilst the composition of resident
69 populations in health is remarkably stable [Cho and Blaser, 2012], this can be
70 perturbed by changes in lifestyle, immune status or by broad spectrum antibiotic
71 therapy. Such perturbations have been associated with a number of clinical
72 disorders such as obesity, allergy and a variety of inflammatory diseases [Arrieta et
73 al., 2014; Costello et al., 2012]. For example, obesity in humans has been

74 associated with a reduction in bacterial diversity, although the differences in
75 individual species are not consistent among studies [Tagliabue and Elli, 2013]. It is
76 difficult to ascertain whether these changes in microbiota are causal or came as a
77 consequence of obesity. However, the transplantation into germ-free mice with a
78 caecal microbiota from obese animals led to a significantly greater increase in total
79 body fat than colonisation with a microbiota from lean mice [Turnbaugh et al., 2006].

80

81 **The composition and metabolism of the oral microbiota in health**

82 The mouth is similar to other habitats in the body in having a characteristic
83 microbiota, with different surfaces in the oral cavity supporting distinct microbial
84 communities [Faust et al., 2012], the composition and activity of which are dictated
85 by the local environmental conditions. The microbiota grows on oral surfaces as
86 structurally- and metabolically-organised communities of interacting species, termed
87 biofilms [Zijngel et al., 2010]. The properties of these biofilm communities are more
88 than the sum of the component organisms. These communities are in a dynamic
89 equilibrium with their environment, and there can be significant re-assortment and
90 rearrangement of the composition and metabolic activity of these microbial consortia
91 in response to changes in the biology of the mouth (e.g. eruption of teeth; flow of
92 saliva; integrity of the host defences) and in the lifestyle of the individual (e.g.
93 smoking, medication) [Crielaard et al., 2011; Sampaio-Maia and Monteiro-Silva,
94 2014]. The diet, in general, has little impact on the oral microbiota, except in the case
95 of frequency of intake of fermentable sugars and nitrate (in green vegetables) (see
96 later).

97 Traditionally, the oral microbiome has been characterised using sophisticated
98 but laborious culture techniques, involving dispersal of the biofilms followed by serial

99 dilution, plating and incubation on a range of selective and non-selective agar plates,
100 usually under strictly anaerobic conditions. However, comparisons of the number of
101 cells visualised by microscopy from a sample with the total viable counts
102 demonstrated that, at best, only about 50% of the oral microbiota could be cultured.
103 Recent advances in technology have resulted in the development of molecular and
104 culture-independent approaches that have enabled the detection of far more taxa
105 and a better description of the microbial richness of the oral microbiota. Although
106 there are inter-subject and inter-site variations in the microbiota, and only a limited
107 number of sites and subjects have been analysed, a core oral microbiome has been
108 proposed, which includes representatives of the following genera: *Streptococcus*,
109 *Veillonella*, *Granulicatella*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Rothia*,
110 *Actinomyces*, *Prevotella*, *Capnocytophaga*, *Porphyromonas* and *Fusobacterium*
111 [Zaura et al., 2009]. Most studies have focussed on the biofilms found on teeth;
112 these biofilms have the most diverse composition, and consistent differences are
113 found on distinct dental surfaces due to variations in key environmental properties.
114 The resident microbiome depends on complex host molecules, such as proteins and
115 glycoproteins, for nutrition. These are catabolised by oral bacterial communities in a
116 concerted and sequential manner resulting in the establishment of numerous
117 nutritional interdependencies (food webs) [Wright et al., 2013].

118 The bacteria found in occlusal fissures are mainly Gram positive (especially
119 streptococci), are facultatively anaerobic and metabolise host and dietary sugars,
120 and the site is influenced by the properties of saliva. In contrast, the biofilms from the
121 healthy gingival crevice contain many Gram negative and obligately anaerobic
122 species, that have a proteolytic style of metabolism, and the community is influenced
123 more by gingival crevicular fluid, GCF [Marsh and Martin, 2009]. This site distribution

124 is direct evidence that the composition and metabolism of the oral microbiota at a
125 site is sensitive to, and responsive to, the oral environment, and that there is a
126 dynamic relationship between them both.

127 The composition of the oral microbiota can remain stable over time (microbial
128 homeostasis) [Marsh, 1989]. This is not due to any biological indifference among the
129 members of the biofilm community; the relationship is not passive but highly
130 dynamic. As mentioned earlier, biofilm composition will shift in response to changes
131 in local environment and lifestyle. Such changes can perturb biofilm composition and
132 activity, and predispose a site to disease, but as these diverse microbial
133 communities confer important physiological benefits to the host, as will be discussed
134 next, oral care strategies should be focussed on maintaining the composition and
135 activity of these biofilms rather than trying to eliminate them.

136

137 **Benefits of the oral microbiota**

138 As in other habitats in the body, the general relationship between the oral
139 microbiota and the host is mutualistic. The micro-organisms are maintained in an
140 environment which is supplied with a diverse array of host molecules which serve as
141 nutrients, and the resultant microbiota provides benefits to the host. There is
142 evidence for active communication (“cross-talk”) between some of the resident
143 bacteria and host cells [Ivanov and Honda, 2012; Kamada and Nunez, 2014; Smith
144 and Garrett, 2011]. Some bacteria also regulate the activities, development and/or
145 deployment of host immune cells, while others promote mild inflammatory response
146 that help to “prime” the immune responses or down-regulate potentially damaging
147 pro-inflammatory host responses to the normal oral microbiota, while the host retains
148 the ability to respond to genuine microbial insults [Cosseau et al., 2008; Kaci et al.,

149 2014; Neish et al., 2000; Srinivasan, 2010]. The precise biological mechanisms
150 involved in this “cross-talk” are still being determined; pathogenic and non-
151 pathogenic bacteria may initiate different intracellular signalling pathways and innate
152 immune responses [Canny and McCormick, 2008; Milward et al., 2007; Neish, 2009],
153 but whether the bacteria have a Gram positive or a Gram negative cell wall structure
154 may be a stronger determinant than pathogenicity as to which pathways are
155 activated [Chino et al., 2009; Feezor et al., 2003].

156 The resident oral microbiota contributes to the host defences by preventing
157 the establishment of the many exogenous micro-organisms the host comes into
158 contact with on a regular basis. This ‘colonisation resistance’ is because the natural
159 oral microbiota is better adapted at attaching to oral surfaces, is more efficient at
160 metabolising the available nutrients for growth, and can produce inhibitory factors
161 and create hostile environments that restrict colonisation by potential microbial
162 invaders.

163 Resident oral bacteria also contribute to the general health of their host by
164 regulating gastrointestinal and cardiovascular systems via the metabolism of dietary
165 nitrate [Kapil et al., 2013]. Approximately 25% of ingested nitrate is secreted in
166 saliva, from where it is reduced to nitrite by oral bacteria. Nitrite regulates blood
167 flow, blood pressure, gastric integrity and tissue protection against ischemic injury.
168 Nitrite is converted to nitric oxide in the acidified stomach, and this has antimicrobial
169 properties, and contributes to defence against enteropathogens, and in the
170 regulation of gastric mucosal blood flow and mucus formation. The reduction of
171 nitrate to nitrite in saliva falls markedly in human volunteers [Dougall et al., 1995;
172 Govoni et al., 2008; Petersson et al., 2009] and laboratory animals [Petersson et al.,
173 2009] when the resident salivary microbiota is deliberately suppressed using

174 antimicrobial agents. The suppression of endogenous nitrate reduction in the animal
175 model resulted in a loss of the predicted biological benefits of nitrite, including
176 reduced gastric mucus thickness, while the expected fall in blood pressure following
177 a nitrate supplement was prevented [Petersson et al., 2009]. These findings confirm
178 that it is essential not to perturb or lose the beneficial functions of the resident oral
179 microbiota, which has implications for oral care professionals and the products
180 developed by oral care companies.

181

182 **The composition and metabolism of the oral microbiota in disease.**

183 There is a shift in the composition and metabolism of the oral microbiome in
184 disease. Numerous studies, using either traditional culture or contemporary
185 molecular approaches to compare the microbiota in biofilms from healthy surfaces to
186 that from sites with dental caries and periodontal diseases, have shown that there
187 are substantial differences in the composition of the microbiota in disease [Wade,
188 2013]. The application of sensitive molecular techniques has confirmed that many of
189 the bacteria associated with disease can be found in biofilms from healthy sites, but
190 they are present in clinically irrelevant numbers and at a far lower frequency [van
191 Winkelhoff and Boutaga, 2005]. Therefore, disease is due to shift in the composition
192 of the biofilm (dysbiosis) rather than as a result of exogenous 'infection', and is
193 associated with markedly higher proportions of certain species that, if present in
194 health, are normally only minor components in the biofilm.

195 An ecological hypothesis has been proposed to explain the relationship between
196 the resident oral microbiota and dental disease [Marsh, 2003]. Briefly, a substantial
197 change in local environmental conditions can alter the competitiveness of plaque
198 bacteria leading to the enrichment of organisms most suited to the new environment.

199 In other ecosystems, such dramatic shifts in microbiota are associated with a major
200 alteration to the habitat, such as to the nutrient status (e.g. the overgrowth of algae in
201 rivers following the wash-off of fertilisers from neighbouring farm land), pH (e.g. the
202 disruption of aquatic life in lakes by “acid rain”), atmosphere, or immune status (e.g.
203 reactivation of dormant *Mycobacterium tuberculosis* in the lungs of HIV-infected
204 patients). It has been argued that, in caries, an increased frequency of sugar intake,
205 or a reduction in saliva flow, results in plaque biofilms spending more time at low pH.
206 This selects for acid-producing and acid-tolerating species (most commonly mutans
207 streptococci, but not exclusively so) at the expense of health-associated bacteria that
208 prefer pH values around neutrality. Increases in the acidogenic bacterial populations
209 lead to even greater production of acid and raises the risk of demineralisation still
210 further. In periodontal disease, the inflammatory response to plaque accumulation
211 results in an increased flow of GCF which not only delivers components of the host
212 defences (immunoglobulins, complement, neutrophils, etc), but also introduces other
213 host molecules to the site, such as haemoglobin and transferrin, that act as essential
214 cofactors and nutrients for many anaerobic and proteolytic bacteria. The metabolism
215 of these bacteria makes the site more anaerobic and the local pH increases due to
216 proteolysis, and these environmental changes select for the diverse microbial
217 consortia that are implicated in periodontal diseases, including *Porphyromonas*
218 *gingivalis*, *Tannerella forsythia*, numerous spirochaetes and other currently
219 unculturable taxa [Marsh, 2003].

220

221 **Oral disease: control without killing?**

222 A key principle of the ‘Ecological Plaque Hypothesis’ is that disease can be
223 controlled not only by improving oral hygiene or targeting the putative pathogens

224 directly, but also by interfering with the environmental pressures that select for the
225 pathogenic micro-organisms, thereby driving dysbiosis. Unless there is an attempt to
226 interfere with the factor(s) driving the dysbiosis then the patient is likely to return to
227 the surgery suffering from further episodes of disease [Marsh, 2003]. In caries,
228 preventive strategies could include reducing the frequency and impact of the low pH
229 challenge, for example, by recommending snack foods containing non-fermentable
230 sweeteners, using fluoride products to promote remineralisation and and which
231 would also reduce acid production [Takahashi and Washio, 2011], or boosting saliva
232 flow, for example, with sugar-free gums. Similarly, the use of oxidising agents to
233 make sites less anaerobic, or novel anti-inflammatory compounds (such as lipoxins,
234 resolvins and protectins) [Hasturk et al., 2012; Freire and van Dyke, 2013] that
235 reduce GCF flow and promote healing, would help to restrict the growth of the
236 obligately anaerobic and proteolytic periodontal pathogens [Marsh, 2003]. Indeed,
237 studies in a rabbit model of experimental periodontitis using treatment with a resolvin
238 demonstrated a reduction in inflammation and tissue destruction. This was
239 accompanied by an alteration in the subgingival biofilm microbial community to one
240 with a less complex profile that was associated more with gingival health, including a
241 reduction in levels of *P. gingivalis*. This was despite the fact that the resolvin had no
242 inherent antibacterial activity [van Dyke, 2008]. The re-introduction of health-
243 associated subgingival bacterial communities to the treated periodontal pocket has
244 also been advocated [Teughels et al., 2007].

245 Strategies to treat or prevent infection by enhancing host defences have been
246 proposed [Hancock et al., 2012], which may find applications in periodontal
247 treatment. For example, vitamin D and some short chain fatty acids can increase
248 production of innate defence molecules, such as LL-37, by many cells including the

249 gingival epithelium [McMahon et al., 2011]. Host defences may not need to be
250 present at inhibitory levels but, like exogenously applied antimicrobials, may
251 influence the microbiota at sub-inhibitory levels; for example, low concentrations of
252 LL37 have been shown to have anti-biofilm activities, including inhibition of biofilm
253 formation, that are distinct from its bactericidal activities [Amer et al., 2010; Bishu et
254 al., 2014; Dean et al., 2011; Kai-Larsen et al., 2010; Overhage et al., 2008]. At sub-
255 inhibitory levels some host defences factors may also help to eliminate bacteria by
256 acting as adjuncts that increase the effectiveness of antibiotics and other
257 antimicrobials [Reffuveille et al., 2014; Yeung et al., 2011].

258 When taking a holistic view of the mutualistic relationship between the host and its
259 resident oral microbiota, there is a challenge to treat or prevent disease without
260 losing or disrupting the important benefits provided by this microbiota (see earlier).
261 Antimicrobial agents have been formulated into oral care products for many years
262 with claims made that they provide additional benefit at restricting or controlling
263 biofilm development. Such products are required to deliver two apparently
264 contradictory requirements in order to meet regulatory guidelines [American Dental
265 [Association, 2008; Marsh, 1992] – a potential microbiological paradox [Marsh,
266 1992]. These requirements are to deliver a relevant and measurable clinical and
267 microbiological benefit, while at the same time not disrupting the natural microbial
268 ecology of the mouth, for example, by permitting overgrowth by opportunistic
269 pathogens (e.g. yeasts) or exogenous micro-organisms [Marsh, 2010].

270 The pharmacokinetic (PK) profile of these agents, and the fact that their
271 targets are drug-tolerant biofilms, may provide an unexpected solution to this
272 apparent paradox. Most antimicrobial agents used in oral care products are
273 described as being broad spectrum on the basis of conventional MIC/MBC testing. In

274 these assays, the bacteria are cultured with a constant concentration of the agent for
275 prolonged periods (e.g. 48 hours). However, under their normal conditions of use in
276 the mouth (recommended: twice daily application for brief periods, <2 minutes) they
277 are present at MIC or MBC levels for a relatively short time, although some are
278 retained for many hours at sub-lethal concentrations (for example, metal salts,
279 Triclosan) (Figure 1) [Duckworth, 2013]. The MIC value of Triclosan did not predict
280 its spectrum of activity when assessed using a more realistic model in which the
281 inhibitor was pulsed into a mixed culture of oral bacteria [Bradshaw et al., 1993;
282 Marsh, 1992; ten Cate and Marsh, 1994]. Bacterial species with a similar MIC to
283 Triclosan showed markedly different sensitivities, while species with very different
284 MIC values could have equivalent responses during a transient pulse of the agent.
285 For example, *Streptococcus gordonii* and *S. mutans* had identical MIC values but the
286 former was barely affected by a pulse of Triclosan (<5%) whereas *S. mutans*
287 suffered a >95% reduction in its proportions [Bradshaw et al., 1993; Marsh, 1992]. In
288 this way, antimicrobial agents in oral care products may have a more selective mode
289 of action in which they mainly inhibit the growth and metabolism of organisms
290 implicated in disease while leaving those associated with oral health relatively
291 unaffected [Marsh, 2012].

292 This mode of action may be most appropriate for 'over-the-counter' products
293 that are used repeatedly and in an unsupervised manner, and one that is consistent
294 with the 'ecological approach' to control dental disease. At sub-lethal
295 concentrations, many of these agents could target key virulence traits of oral
296 bacteria, such as adhesion to surfaces, and sugar transport/acid production in
297 relation to dental caries, or protease activity in periodontal disease, while also
298 generally slowing bacterial growth [ten Cate and Marsh, 1994]. For example, zinc

299 can inhibit sugar transport, acid production and protease activity, while low levels of
300 Triclosan can also inhibit acid production by oral streptococci and protease activity
301 by *P. gingivalis* [Brading and Marsh, 2003; Cummins, 1992]. Unlike the principles
302 behind the use of antibiotics in medicine, oral care products could function
303 prophylactically to stabilise the normal oral microbiota under conditions that may
304 otherwise have predisposed a site to caries or gingivitis, thereby maintaining the
305 benefits of the resident microbiota for the host (control without killing).

306

307 ***In silico* modelling can demonstrate the effect of sublethal modulation.**

308 Following on from the findings described above, we have initiated *in silico* modelling
309 studies to quantify the impact of subtle changes in:

310 (a) bacterial growth rate, for example, by sub-lethal concentrations of antimicrobial
311 agents,

312 (b) the environment, for example, by varying the frequency of sugar (glucose)
313 exposure, and

314 (c) bacterial metabolism, for example, by interfering with rates of acid production
315 and the terminal pH reached.

316 Our *in silico* plaque model is based on a computational approach that has been
317 developed for over a decade and applied to biofilms in diverse environments [Wang
318 and Zhang, 2010]. It is a hybrid scheme that couples discrete particles representing
319 cells or cell aggregates, to continuous fields representing dispersed phases such as
320 nutrients and metabolites. Metabolic reactions localised at particle centres convert
321 between dispersed phases at rates given by standard parameterised forms, leading
322 to cellular growth and division. For the plaque model, we considered two acidogenic
323 bacterial populations, designated 'A' and 'NA' that are aciduric and non-aciduric,

324 respectively, as quantified by differing half-concentrations in the Monod acid
325 inhibition factor for glycolysis to lactic acid. When provided with regular sugar pulses
326 representing dietary intake, one population came to dominate the plaque
327 composition, either A or NA, depending on a variety of factors including the
328 frequency of glucose pulses. Note that increasing the fraction of one population
329 necessarily decreases the fraction of the other. Full details, including other factors
330 affecting the final biofilm composition, are given elsewhere [Head et al., 2014].

331 We used this mathematical model to generate predictions for the changes in
332 biofilm composition when the growth rates of one or both populations were reduced
333 by a constant factor, this being the simplest manner to simulate inhibition by a
334 putative antimicrobial agent at sub-lethal levels. Figure 2 shows the biofilm
335 composition with time when neither population, both populations, or just the aciduric
336 population had their metabolic activity reduced by only 10%. It is clear from Figure 2
337 that inhibiting population A led to growth being biased towards the NA-dominated
338 state. Inhibiting both populations had a similar although weaker effect, resulting from
339 the reduced overall acid production selecting for the NA population. Figure 3 shows
340 how the composition of the simulated plaque (measured after 100 days) varied with
341 both the frequency of glucose pulsing and the magnitude of the inhibition, with the
342 latter applied to both populations or just population A. It is evident from this figure
343 that, in both cases, sub-lethal inhibition favoured the growth of the NA population
344 over the A population, compared to the same frequency of glucose pulsing without
345 inhibition. We have also demonstrated elsewhere that varying the buffering capacity
346 of the plaque fluid can similarly modulate the biofilm composition [Marsh et al.,
347 2014b, a]. All of these measured trends demonstrated that environmental
348 alterations, such as those achieved by a putative external agent that reduces the

349 rate or frequency of fall in environmental pH, or inhibits bacterial growth rate, can
350 beneficially modulate biofilm community dynamics without requiring any form of
351 direct lethal antimicrobial action.

352

353 **Concluding remarks**

354 There is increasing evidence that the natural oral microbiota has a symbiotic
355 or mutualistic relationship with the host, and delivers important benefits. However,
356 on occasions, this symbiosis can be perturbed and disease can occur (dysbiosis).
357 Oral care should be focussed on the remediation of factors responsible for dysbiosis
358 while maintaining the resident microbiota at levels compatible with health. Many oral
359 care products are formulated with antimicrobial agents to augment mechanical
360 plaque control. Although these agents are often described as having a broad
361 spectrum of antimicrobial activity, it has been argued that, under the conditions of
362 use in the mouth, they may also function effectively at sub-lethal concentrations by
363 interfering with bacterial traits associated with disease. This type of ‘controlled’
364 antimicrobial approach would maintain the beneficial activities that the host derives
365 from the presence and metabolism of the resident oral microbiome, while reducing
366 the activity of the microbial factors (e.g. acid production; proteolysis) that drive the
367 changes in the balance in the biofilm that lead to dysbiosis (control without killing).

368

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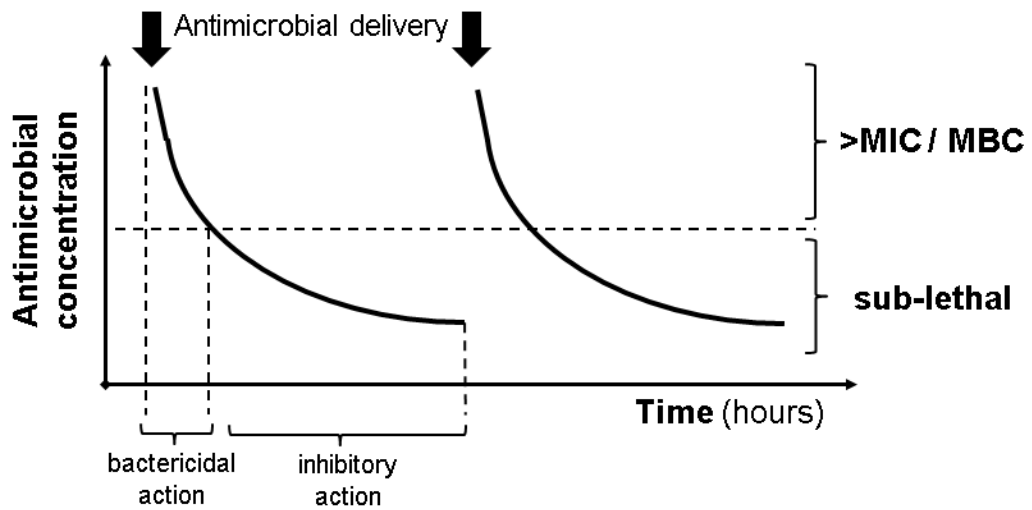
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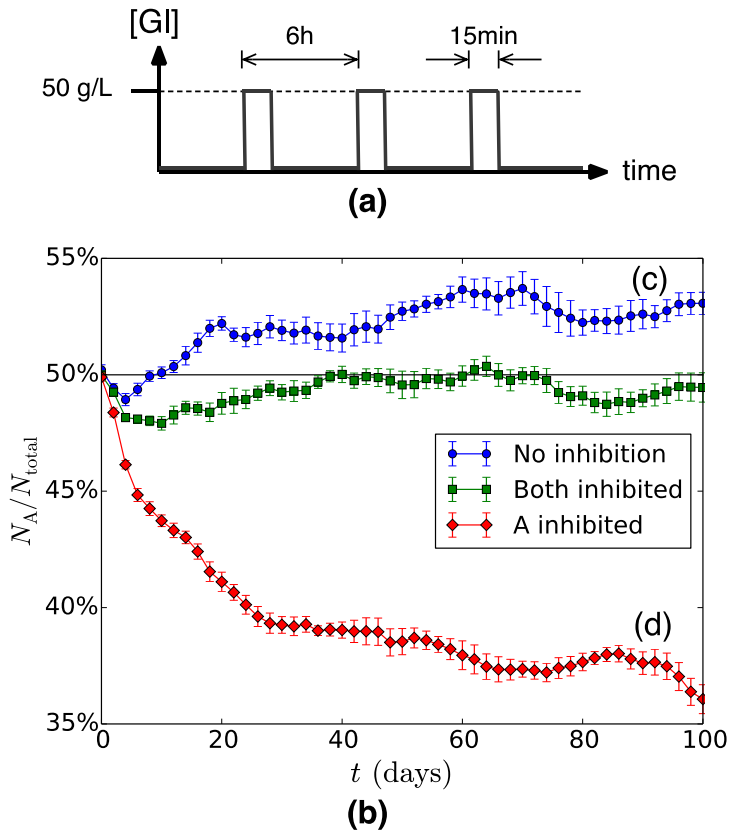
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535 Figure 1. Pharmacokinetics of antimicrobial agents delivered to the mouth [Marsh,
536 2010].

537 A schematic representation of the change in concentration over time of an
538 antimicrobial agent from an oral care product following the delivery to the mouth on
539 two occasions. The agent may be present above its MIC/MBC level for a relatively
540 short period before it is lost from the mouth. The agent may be present for longer
541 periods at sub-lethal concentrations; agents may still exert beneficial effects by
542 inhibiting traits associated with disease. The dynamics of the curve will vary for each
543 antimicrobial agent.

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547 Figure 2: *In silico* biofilm modelling studies of two bacterial populations that differ in
 548 their acid tolerance, with population A being an aciduric population and population
 549 NA being non-aciduric.

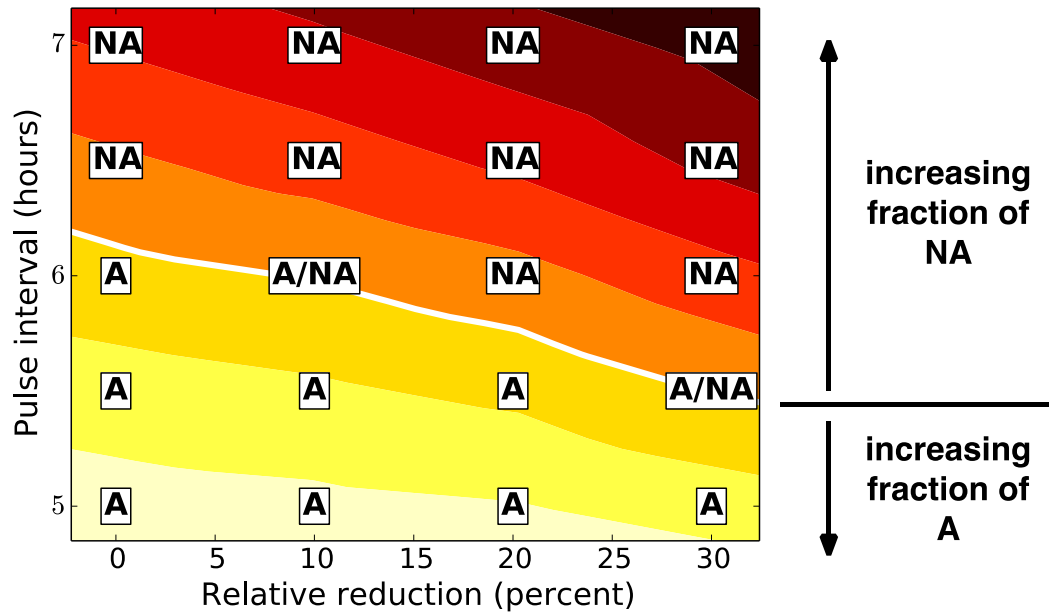
550 (a) Schematic of the glucose pulsing protocol (not to scale). During a pulse the
 551 glucose concentration was fixed at 50 g/L, which was far higher than the half-
 552 concentrations for nutrient uptake for both A and NA. The pulse duration was fixed at

553 15 minutes, and the pulse interval was varied; the example here has a pulse interval
554 of 6 hours.

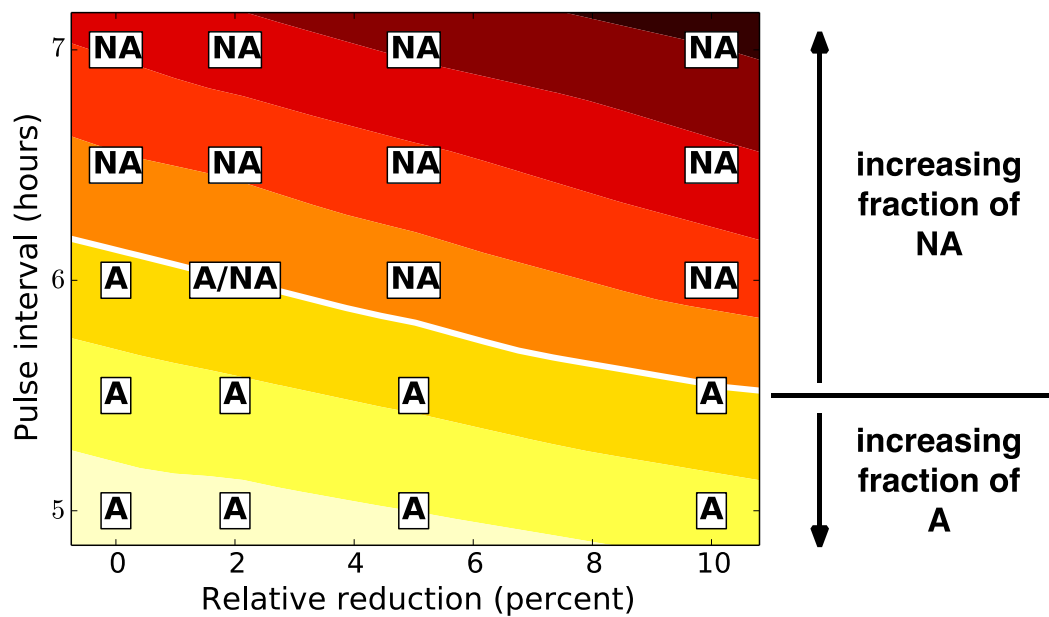
555 (b) The variation in biofilm composition over time for when neither, both, or just the
556 aciduric population (population A) had their growth rates reduced by 10%. N_A/N_{total} is
557 the fraction (%) of population A (*i.e.* the more aciduric bacterial population), and the
558 glucose pulse interval was 6 hours.

559 (c) and (d) show images from the model at $t=100$ days for (c) no inhibition and (d)
560 10% inhibition of population A. Light (dark) grey discs correspond to cells of NA (A)
561 respectively, and the continuous field in the background represents the lactic acid.

562



(a)



(b)

563

564 Figure 3: *In silico* biofilm modelling studies of two bacterial populations that differ in
 565 their acid tolerance, with population A being an aciduric population and population
 566 NA being non-aciduric.

567 Contour plots showing the composition of the biofilm as both the glucose pulse
 568 interval and the degree of growth inhibition were varied, where the inhibition was
 569 applied to the growth of (a) both bacterial populations, and (b) population A only. The
 570 labels 'A', 'NA' and 'A/NA' (the latter meaning roughly 50% A and NA) denote the
 571 dominant species at $t=100$ days, and darker shades correspond to higher fractions of
 572 NA. The thick white line in each figure gives the 'tipping point' between A and NA
 573 dominated states, which varied in response to more frequent glucose pulses with

574 increased bacterial growth inhibition. The shading of the arrows to the right is not
575 significant.