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Ecological approaches to oral biofilms: control without killing

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Abstract

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

244/250 words

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

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

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

The composition and metabolism of the oral microbiota in health

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

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

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

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

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

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

Benefits of the oral microbiota

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

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

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

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

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

The composition and metabolism of the oral microbiota in disease.

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

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

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

Oral disease: control without killing?

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

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

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

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

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

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

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

This mode of action may be most appropriate for 'over-the-counter' products that are used repeatedly and in an unsupervised manner, and one that is consistent with the 'ecological approach' to control dental disease. At sub-lethal concentrations, many of these agents could target key virulence traits of oral bacteria, such as adhesion to surfaces, and sugar transport/acid production in relation to dental caries, or protease activity in periodontal disease, while also 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,

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

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

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

Concluding remarks

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

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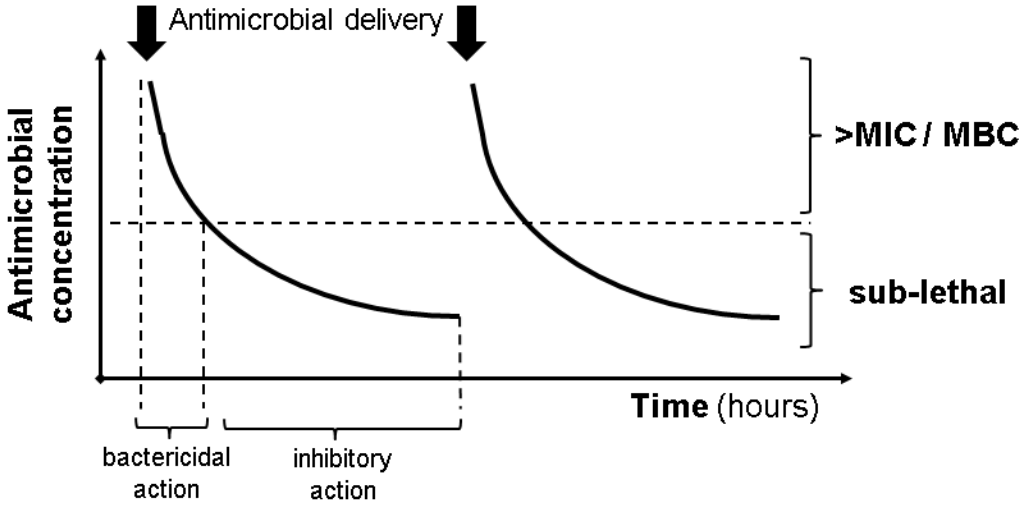

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535 Figure 1. Pharmacokinetics of antimicrobial agents delivered to the mouth [Marsh,
536 2010].

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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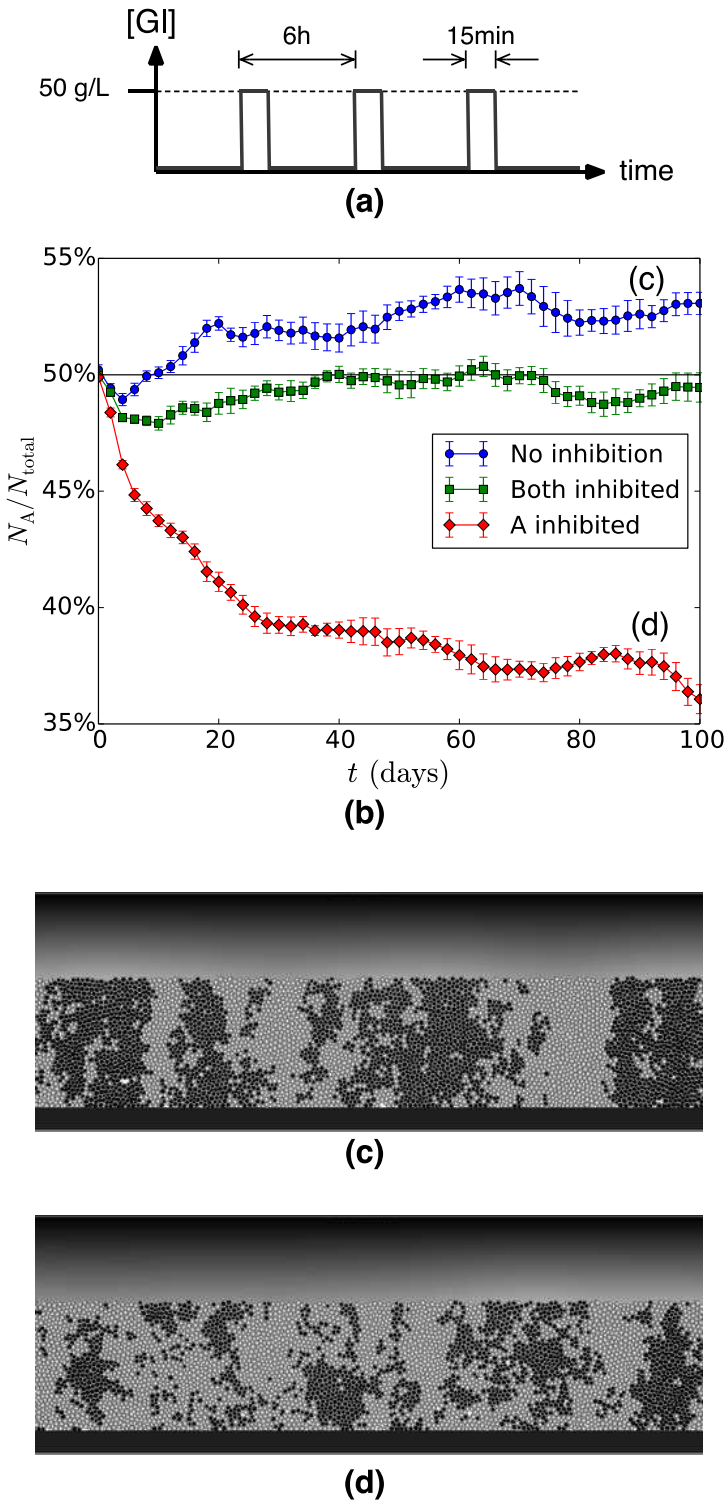

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547 Figure 2: *In silico* biofilm modelling studies of two bacterial populations that differ in
548 their acid tolerance, with population A being an aciduric population and population
549 NA being non-aciduric.

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

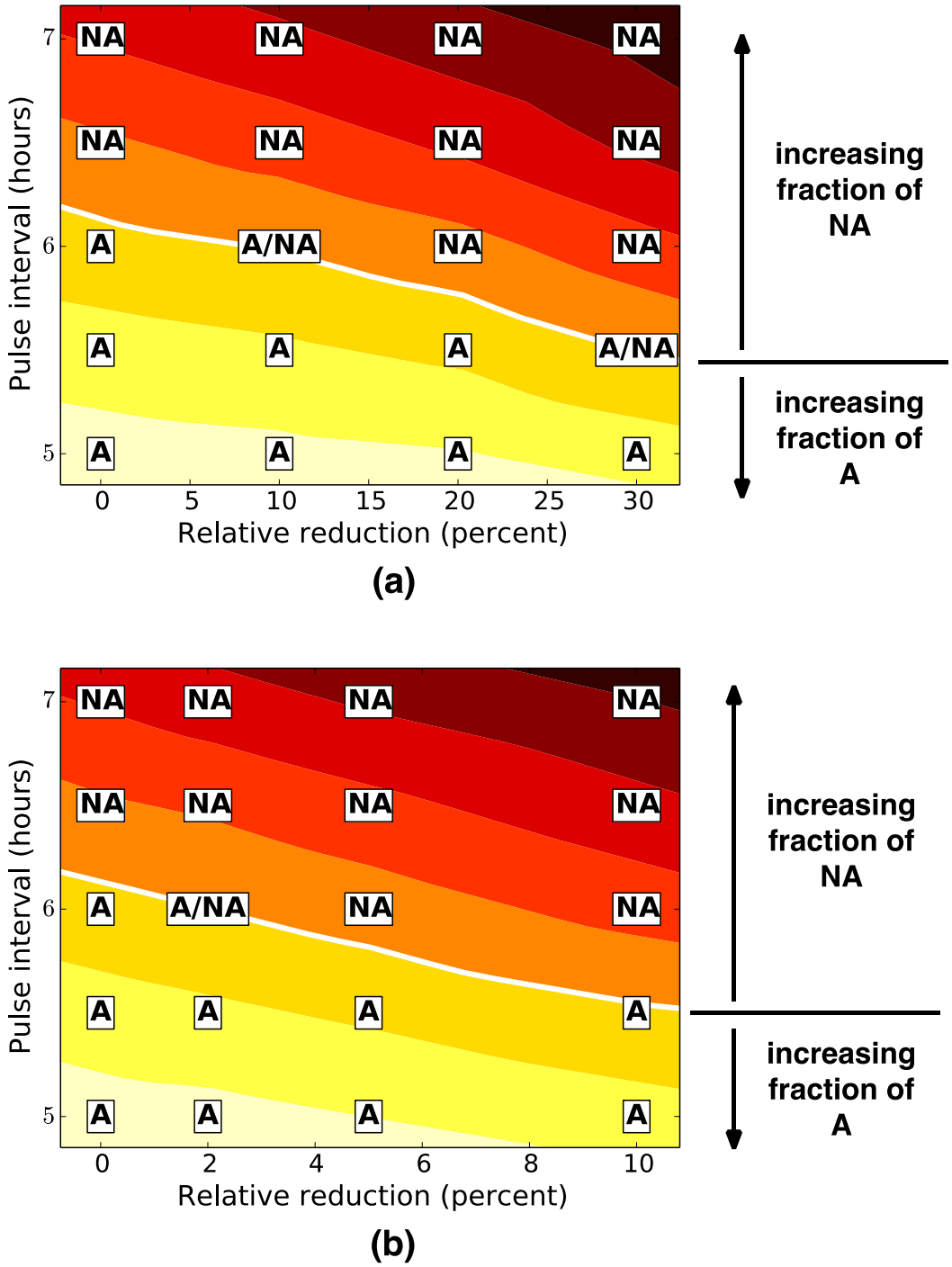

Figure 3: *In silico* biofilm modelling studies of two bacterial populations that differ in their acid tolerance, with population A being an aciduric population and population NA being non-aciduric.

Contour plots showing the composition of the biofilm as both the glucose pulse interval and the degree of growth inhibition were varied, where the inhibition was applied to the growth of (a) both bacterial populations, and (b) population A only. The labels 'A', 'NA' and 'A/NA' (the latter meaning roughly 50% A and NA) denote the dominant species at t=100 days, and darker shades correspond to higher fractions of NA. The thick white line in each figure gives the `tipping point' between A and NA 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.