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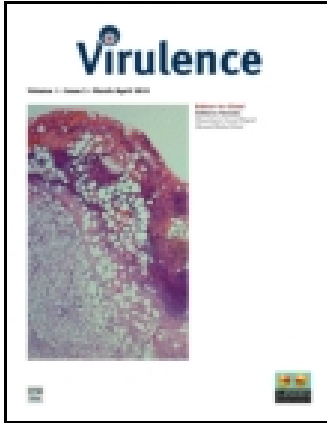
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### Non-conventional therapeutics for oral infections

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# Non-conventional therapeutics for oral infections

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**Keywords:** antimicrobial peptides, caries, detergents, enzymes, light-activation, nanoparticles, periodontitis, probiotics

As our knowledge of host-microbial interactions within the oral cavity increases, future treatments are likely to be more targeted. For example, efforts to target a single species or key virulence factors that they produce, while maintaining the natural balance of the resident oral microbiota that acts to modulate the host immune response would be an advantage. Targeted approaches may be directed at the black-pigmented anaerobes, *Porphyromonas gingivalis* and *Prevotella intermedia*, associated with periodontitis. Such pigments provide an opportunity for targeted phototherapy with high-intensity monochromatic light. Functional inhibition approaches, including the use of enzyme inhibitors, are also being explored to control periodontitis. More general disruption of dental plaque through the use of enzymes and detergents, alone and in combination, shows much promise. The use of probiotics and prebiotics to improve gastrointestinal health has now led to an interest in using these approaches to control oral disease. More recently the potential of antimicrobial peptides and nanotechnology, through the application of nanoparticles with biocidal, anti-adhesive and delivery capabilities, has been explored. The aim of this review is to consider the current status as regards non-conventional treatment approaches for oral infections with particular emphasis on the plaque-related diseases.

## Introduction

The oral cavity provides habitats for a wide diversity of microorganisms including bacteria, yeasts and viruses; members of all groups being associated with oral infections. Bacteria are the predominant components of this resident microflora, and the diversity of species found in the oral cavity reflects the wide range of endogenously derived nutrients, the varied types of habitat for colonization including surfaces on the teeth, mucosa and tongue, and the opportunity to survive as a biofilm.<sup>1,2</sup> However, the relationship between this microflora and the host can be disrupted in a number of ways, resulting in the development of disease of the

oral structures. These are mainly localized and include dental caries (dental decay), gingivitis, periodontitis (gum disease), candidiasis, endodontic (root canal) infections, orthodontic infections and peri-implantitis.<sup>3</sup>

Most bacterial infections within the oral cavity are polymicrobial in nature, and it is quite unusual to find any that are clearly due to a single species. The relative contribution of different bacterial components in such infections is thus difficult to determine. Oral infections may arise either from an endogenous source, i.e., one yielding microorganisms normally found in the mouth, such as the main plaque-related diseases of dental caries and periodontal disease, or from an exogenous source yielding microorganisms not normally found as part of the oral microflora (e.g. syphilis, tuberculosis). Plaque-related diseases are probably the most common bacterial diseases occurring in man. Dental caries is a destructive condition of the dental hard tissues that, if unchecked, can progress to inflammation and death of vital pulp tissue, with eventual spread of infection to the periapical area of the tooth and beyond. The disease process involves acidogenic plaque bacteria, including *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus* spp., *Actinomyces* spp and *Bifidobacterium* spp.,<sup>3,4</sup> whereas periodontal diseases can involve both the soft and hard tissues and are the most common inflammatory destructive conditions that affect man. They are initiated by components of the plaque that develops on the hard root surface adjacent to the soft tissues of the supporting periodontium and may be confined to the gingiva (gingivitis) or extend to the deeper supporting structures with destruction of the periodontal ligament and the alveolar bone that supports the teeth (periodontitis). Such loss of attachment, with associated periodontal pocket formation, may ultimately lead to loosening and loss of the affected teeth. *Porphyromonas gingivalis*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* are regarded as the major pathogens in advancing periodontitis.<sup>5</sup> Furthermore it has been recently suggested that there is an association between the oral microbiota and systemic disease, such as cardiovascular disease and complications during pregnancy.<sup>6,7</sup>

The prevention of dental caries and the periodontal diseases is traditionally targeted at the mechanical or non-specific control of dental plaque as this is the precipitating factor. This is carried out to maintain plaque at levels compatible with health and so prevent the breakdown of microbial homeostasis which increases disease risk. However, the individual response of the host and other confounding factors can influence disease initiation and progression. Antimicrobial and antiplaque compounds in oral care products represent a valuable complement to mechanical plaque control. Such strategies should ideally prevent plaque

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biofilm formation without affecting the biological equilibrium within the oral cavity, which is inhabited by up to 1000 different species of bacteria at  $10^8$ – $10^9$  bacteria per mL saliva or mg dental plaque.<sup>8</sup> However, with ever increasing antibiotic resistance and a public desire for more 'natural' therapies, there is an increased need to minimise antibiotic use and develop novel treatments for oral diseases that do not involve conventional antimicrobial agents.

## Dental Plaque Disruption

### Enzymes

A number of oral streptococci, including *Streptococcus mutans*, produce a range of water-soluble and insoluble glucan extracellular polysaccharides *in vivo*.<sup>9</sup> The different types of glucans are synthesized by different glucosyltransferases, particularly  $\alpha$ -(1→3)-linked glucans (synthesized by GTF B) and  $\alpha$ -(1→6)- and  $\alpha$ -(1→3)-linked glucans (synthesized by GTF C).<sup>9</sup> The water insoluble types ( $\alpha$ -1→3) are thought to contribute to the cariogenicity of the plaque at a site through its physical retentive nature, its ability to provide a large diffusion volume for dietary sugars and as a substrate for further adhesion of bacteria. Consequently a desirable goal would be to disrupt this feature of the plaque biofilm with glucanase enzymes (dextranase and mutanase). Both dextranases and mutanases are able to suppress the accumulation of dental plaque in human volunteers, in animals or *in vitro* depending upon the experimental system employed.<sup>10–12</sup> Such enzymes would be applied directly to dental plaque where GTFs are still active, which raises the question of their likely longer term efficacy. However, these enzymes have been found, to influence glucan synthesis by GTFs themselves in terms of linkage remodelling and branching and this appears to have an impact on the formation, maturation and physical properties of the glucans present.<sup>13</sup> Consequently there is reason to believe that such enzymes could be a valuable addition to the control of the plaque biofilm.

Another approach to enzymic disruption of biofilms is the use of deoxyribose nucleases (DNAases). While it has been known since the 1940's that bacteria can release DNA extracellularly,<sup>14</sup> it is only relatively recently that biofilms have been found to contain a significant amount of intact extracellular DNA (eDNA). This is present either because of cell death or active secretion, or both, and there is now strong evidence that this DNA contributes to the matrix of the biofilm, an important structural component of its scaffold.<sup>15</sup> It seems logical then that application of exogenous DNAases could degrade this scaffold and so provide a means of disrupting the integrity of the biofilm and/or its growth. Indeed, a number of workers recently have shown that DNAase treatment of *in vitro* biofilms can weaken the structure and result in release of bacteria.<sup>16–18</sup> However, DNAases are relatively expensive to produce currently and for application on a large clinical scale improvements in production methods will be required. Nonetheless, enzymic disruption of the plaque biofilm does look a promising way forward for the future.

### Detergents

An alternative to enzymic disruption of the biofilm matrix is chemical disruption and a number of ionic detergents can bind to bacteria and their matrix, which could be effective biofilm disrupting agents. While cationic detergents have been used in commercial products for some years, often referred to as pre-brushing mouth washes, there have been conflicting claims about their effectiveness. Despite the numerous claims and counterclaims, recent systematic reviews of the literature have failed to show clear advantages of detergent mouthwashes over good oral hygiene instruction on the removal of dental plaque.<sup>19,20</sup> However, it is possible that the failure of these products to realize their potential has been due to the mode of their delivery, since the tendency is for the agent to bind largely to the surface of the biofilm. One of the most commonly used agents in such pre-brushing mouthwashes is cetylpyridinium chloride and recent work has concentrated on exploring alternative delivery methods. These methods include incorporation of cetylpyridinium chloride within cholesterol liposomes<sup>21</sup> and in nanoemulsions.<sup>22</sup> Although not yet tested clinically, the nanoemulsions look particularly effective since *in vitro* testing has shown inhibitory effect on *S. mutans* biofilms<sup>23</sup> and using a multispecies biofilm grown in an artificial mouth system, showed good penetration into the biofilm. Furthermore, it was superior to chlorhexidine in reducing the depth of artificial carious lesions and in mineral loss in this system.<sup>22</sup> Perhaps combinations of nanoemulsions of detergents and enzymic disruption agents, to aid even more effective penetration, would provide optimum plaque disruption.

### Light-activated Killing

The fact that bacteria can be killed by light in the presence of a sensitizing agent was discovered 100 years ago by Reitz in 1908<sup>24</sup> but it is only recently that the possibility of using this technology for the control of oral infection has been considered.<sup>25</sup> The process of killing microorganisms with light depends upon the generation of cytotoxic singlet oxygen and OH radicals (reactive oxygen species; ROS), which are formed by the excitation of a photolabile agent or sensitizer. The result of excitation is that the sensitizer moves from its electronic ground state to a triplet state that then interacts with cell components to generate ROS.<sup>26</sup> One of the particular values of light-activated killing is that resistance to the action of singlet oxygen is less likely to become a major concern and spread widely, unlike that experienced with chemical antimicrobial agents. Despite the general truth of this, recent data have shown that spontaneous mutants that are resistant to photoactivated killing can arise.<sup>27</sup>

A sensitizer should bind avidly to the bacterial cell and/or be taken up by it, thus a number of sensitizers are highly charged molecules,<sup>28</sup> the most commonly tested of which have been tricyclic dyes, (e.g., methylene blue, erythrosine), phenothiazine dyes (e.g. toluidine blue O), tetrapyrroles (e.g., porphyrins) and furocoumarins (e.g. Psoralen). Table 1 shows a number of the sensitizers that have been used in recent studies, some of which are

**Table 1.** Example photosensitisers used in recent studies

Sensitizer	Reference
Phenalen 1	29
Indocyanine green	30
Methylene blue	31,32
Toluidine blue ortho	33
Malachite green	34
Eosin-Y	35,36
Rose bengal	35,36
Curcumin	36
Nile blue derivatives	37
BIODIPY derivatives	38
Dicationic 5,15-diarylporphyrins	39
Haematoporphyrin monomethyl ether	40
Meso-substituted porphyrins	27
Radachlorin	41

tried and tested agents while others are newly synthesized variants of previously used categories of agents (e.g., porphyrins).

A sensitizer ideally should absorb light at red to near-infrared wavelengths because longer wavelengths tend to be more penetrative. However, red light sources with narrow wavelength bandwidths tend to be expensive, whereas LED light sources (blue or red) are commonly available in dental surgeries so finding sensitizing agents that are compatible with such light sources would be an advantage for use against oral infections. These dental LED lights are used to cure resin-based composite restorative materials. A dental light was used to assess killing of *S. mutans*, *A. actinomycetemcomitans* and *Enterococcus faecalis* by a commercial preparation of haematoporphyrin sensitizer (Photosan).<sup>42</sup> The Photosan dye was readily taken up by the Gram-positive species but not by *A. actinomycetemcomitans* unless 10% EDTA was used to aid entry of the dye through the outer membrane. Around 3 to 4 log<sub>10</sub> reduction in viable count was achieved, which demonstrates the potential of these commonly used light sources for photoactivated killing. However, others have reported that dental LEDs are not as effective at killing *A. actinomycetemcomitans* as the more powerful indium-gallium-aluminum-phosphate lasers.<sup>41</sup> Inefficient killing of Gram-negative species relative to Gram-positive by photoactivation has been known for almost 30 years<sup>43</sup> as well as the value of adding a membrane-disrupting agent to enhance access of the photo-sensitizer. However, other effective approaches include using agents with strong cationic charge to enhance binding to Gram-negative bacteria<sup>44</sup> and liposome encapsulation to enhance delivery by fusion with the membrane.<sup>45,46</sup> In each case though, the degree of killing will still depend upon the efficiency of the photosensitizer being used.

The majority of work on light-activated killing has been performed using suspensions of planktonic bacteria; however a crucial requirement for application to many disease states, including the dental plaque-related diseases, is the ability to kill microorganisms growing as biofilms. In this phenotype, microorganisms are known to be much more resistant to killing by chemical agents, including antibiotics, and so assessment of efficacy should always include assays on biofilms. Over twenty years ago, Wilson et al<sup>47</sup> showed that oral bacteria could be killed by low power

laser radiation in the presence of methylene blue both as planktonic suspensions and when growing as a biofilm on an agar surface. Whether these are true biofilms has been the subject of debate for some years, but more recently *in vitro* biofilm-grown *S. mutans* cells were killed by up to 3 log<sub>10</sub> fold when treated with erythrosine and white light (500–650 nm).<sup>48</sup> Photoactivated killing has also been assessed against *E. faecalis* biofilms grown for 4 days and 4 weeks within the root canals of extracted teeth and while this did reduce bacterial viability, maximal improvement was gained when it was combined with chemomechanical treatment. Nonetheless, dental LEDs with common blue light-absorbing photosensitizer dyes can eradicate *E. faecalis* biofilm, although requiring increased sensitizer concentration over that required to kill planktonic bacteria.<sup>36</sup>

There are a number of other aspects that should be considered in relation to the therapeutic use of light-activated killing of biofilms on host surfaces: (1) direct toxicity of the sensitizer, (2) indirect toxicity of the sensitizer in terms of “by-stander” damage to adjacent host cells, (3) penetration into the biofilm, and (4) light exposure time required to kill bacteria within *in vivo* biofilms. Answers to all of these questions are not readily available or universally applicable to all bacteria. However, in relation to (1) and (2) it is known that sensitizing agents can give rise to phototoxic effects on keratinocytes and fibroblasts<sup>49</sup> and phototoxic reactions in experimental animal oral tissues have been recorded, although the effect appears to be short lived.<sup>50</sup> The photosensitizer erythrosine has an advantage over some other dyes because it is currently used in dentistry to visualize dental plaque *in vivo*, and so its lack of direct toxicity on the host is well established. For phototherapeutic use in periodontitis, the dye needs to be applied subgingivally prior to fiber-optic laser light activation. However, in disease the periodontal site has a marked flow of serum into the pocket (gingival crevicular fluid) and most photosensitizers lose a degree of activity in the presence of extraneous protein and some have virtually no effect in the presence of serum, blood or saliva. This is because the agents complex with proteins and host cells in the crevicular fluid which effectively competes for binding to bacteria. Despite these theoretical limitations, a commercial PDT system designed for use in periodontal patients (Periowave, Ondine Biopharma, Vancouver, Canada) has been shown to provide some clinical advantage when combined with conventional root surface debridement (RSD) compared to RSD alone.<sup>51,52</sup> However, ideally mono-therapy by PDT for periodontitis would be advantageous as PDT mono-therapy has been shown to provide not only some improvement in clinical signs but also reduction of local proinflammatory cytokines and reduction in numbers of *P. gingivalis* and *A. actinomycetemcomitans*.<sup>53</sup> Nonetheless, the modest clinical improvements achieved so far might be enhanced if methods for better retention of the photosensitizer at the site could be found.

An approach that might achieve this is the use of nanoparticles to deliver the ROS-generating system. Such systems may have better sustainability in the subgingival environment by being taken up into the plaque biofilm.<sup>54</sup> Nanoparticles that contain a conventional sensitizer and have antibacterial action have been known for some time, for example the

polysiloxane polymers containing embedded methylene blue and gold nanoparticles described by Perni et al.<sup>55</sup> However, more recently similar systems have been developed that are independent of a chemical sensitizer. Chong et al.<sup>56</sup> produced nanoparticles of a boron-dipyrromethene polymer that is cationic and that on photoactivation with white light generates ROS at the particle surface.

### Selective killing

Another approach for phototherapy is to take advantage of an 'intrinsic sensitizer' within the target bacteria, rather than relying on the vagaries of dye binding and penetration. Several studies have indicated the use of porphyrins as potential antimicrobial agents and these can interfere with bacterial heme uptake systems as well as generate ROS. Porphyrins are metal-chelating groups and the oral anaerobes belonging to the *Porphyromonas* and *Prevotella* genera include species that accumulate heme compounds on their cell surface in the form of a black pigment. Soukos et al.<sup>57</sup> found that light in the wavelength range 380–520 nm rapidly and selectively killed oral black-pigmented bacteria in pure cultures. They hypothesized that the killing effect was due to photo-excitation of porphyrins on the surfaces of these bacteria, so making the effect quite specific. A similar phenomenon has been found with *A. actinomycetemcomitans* in which blue light without sensitizer reduced the viable count of a suspension by 5 log<sub>10</sub>, which appeared to be due to endogenous flavin-like or porphyrin-like compounds.<sup>58</sup> In contrast there was no effect of blue light on *Escherichia coli* because no singlet oxygen species was generated presumably because no similar endogenous photosensitisers were detected.<sup>58</sup> Exploiting natural porphyrins as photosensitisers certainly has value if it can be successfully applied to subgingival plaque in patients with periodontitis. Indeed, light applied to the oral surfaces of periodontitis patients shows a selective reduction of 4 black-pigmented species.<sup>57</sup>

### Functional inhibition

As summarised above and for obvious reasons, the approach most often taken for treating infection is to attempt to kill or eradicate the offending organisms. However, where the identity of the offending organism is less clear, for example in chronic infections associated with complex mixtures of organisms (viz. periodontitis), an alternative approach to therapy that maintains an element of homeostasis of the population would be beneficial. One approach, as explained later, is to replace the pathogenic strain with a non-pathogenic strain ('replacement therapy'). Another is to consider a disease process in terms of the "virulence burden" that the host experiences, regardless of the identity of the contributing organisms. Limiting the functionality of these critical virulence factors would allow the host's defense mechanisms to deal with the organisms present as a collection. The concept moves away, therefore, from targeting specific organisms or groups of organisms and toward targeting their products. Of course, a disadvantage of this approach is that knowledge of the principal virulence factors involved is a necessity.

### Microbial proteases

One important class of bacterial virulence factors that are a potential target for such a therapeutic approach is extracellular proteases. Some of these have broad specificity, e.g. trypsin-like proteases,<sup>59,60</sup> while others are very specific (e.g., IgA protease, which cleaves the hinge region of the immunoglobulin molecule).<sup>61</sup> There are 4 main classes of proteases; (1) serine proteases, (e.g., trypsin-like, elastase), (2) cysteine proteases (e.g., gingipains), (3) Aspartic proteases, (e.g., *Candida albicans* Saps), and (4) metallo-proteases (e.g., microbial keratinases). Individually or collectively these classes of enzymes play critical roles in infection, both in terms of direct tissue destruction and more subtle effects, such as activation of host proteases,<sup>62</sup> inactivation of host protease inhibitors<sup>63</sup> and disruption of cytokine networks. Another important class of proteases in terms of microbial physiology is the caseinolytic proteases that are not extracellular. These are termed Clp proteases and they regulate protein quality and turnover including damaged proteins and transcriptional regulators.<sup>64</sup> Clp-dependent proteolysis has been implicated in expression of extracellular virulence factors, such as cytotoxins, and in resisting adverse conditions inside host cells. Loss of Clp functionality has been shown to render some pathogens more sensitive to innate host defenses, including host antimicrobial peptides.<sup>65</sup> Thus, bacterial proteases are a useful target for therapeutics not only to prevent their direct and indirect effects on host tissue and systems, but also because proteases fulfil a nutritive and defensive role for the bacteria.<sup>66,67</sup> **Table 2** shows a series of example target proteases of pathogenic bacteria, including certain oral organisms.

### Protease inhibitors

Inhibitors of therapeutic value are usually thought of as inorganic/synthetic agents; however, they could also be "host products" appropriately manipulated. A known example of a host product is salivary histatin 5, which is an inhibitor of both host and bacterial proteases implicated in periodontal disease.<sup>68</sup> This antimicrobial (host defense) peptide also shows general inhibitory effect against a range of organisms but particularly fungi<sup>69,70</sup> and the domain responsible for this anti-protease activity resides in a 14 residue C-terminal sequence. The peptide is known to have an effect at mucosal surfaces that are readily accessible to saliva, but periodontal sites are not normally penetrated by salivary secretions. Thus, histatin would have to be used as a therapeutic agent directly applied subgingivally in a suitable vehicle.

The main classes of inorganic or synthetic inhibitors are chelators, oxidizing agents, thiol-blocking agents, heavy metal ions, methanethiosulfonates and organo-mercurials. Chelators, such as EDTA, are common inhibitors of metalloenzymes, while lanthanides inhibit Ca<sup>2+</sup>-requiring proteases, because Ln<sup>3+</sup> ions replace Ca<sup>2+</sup> and form an inactive enzyme-substrate complex.<sup>71,72</sup> However, lanthanides also activate the conversion of trypsinogen to trypsin.<sup>73</sup> A recently discovered inhibitor of Clp proteases was identified by high throughput screening as N-(1-(2-aminoethyl)-1H-tetrazol-5-yl)-3-chlorobenzamide and which is termed F2.<sup>74</sup> This inhibitor has pleiotropic effects on the bacterial cell

**Table 2.** Selected examples of bacterial proteases, their preferred cleavage sites and example inhibitors

Enzyme	Source	Cleavage site	Inhibitor
<b>Serine and Cysteine proteases</b>			
Glutamyl endopeptidase I	<i>Staphylococcus aureus</i>	Glu-Phe or Glu-Val	None
Exfoliative toxin A	<i>S. aureus</i>	Glu-X	
IgA1-specific protease	<i>Haemophilus influenzae</i> <i>Neisseria meningitidis</i>	Pro-Ser, Pro-Thr	None specific
C5a peptidase	<i>Streptococcus agalactiae</i> <i>Streptococcus pyogenes</i>	His-Lys	None specific
Trepolisin	<i>Treponema denticola</i>	Phe-X	None specific
Prolyl tripeptidyl-peptidase	<i>P. gingivalis</i>	X-Y-Pro-X	None specific
OmpTn	<i>E. coli</i> <i>Yersinia pestis</i>	Arg-Arg, Arg-Lys	thiol-blocking agents
Clostripain C11	<i>Clostridium histolyticum</i>	Arg-X, Lys-X	oxidizing agents, thiol-blocking agents, heavy metal ions
Gingipain R			
Gingipain K	<i>P. gingivalis</i>	Arg-X Lys-X	thiol-blocking agents 1-(3-phenylpropionyl) piperidine-carboxylic acid-[4-amino-1(S)-(benzothiazole-2-carbonyl)butyl] amide,
Sortase	ubiquitous in Gram-positive bacteria	Leu-Pro-X-Thr-Gly	methanethiosulfonates or organo-mercurials
Clp peptidases	Ubiquitous	Met-Ala	ATP-dependent
Metalloproteinase			
Pseudolysin	<i>Pseudomonas aeruginosa</i>	Phe-X or Gly-Leu	EDTA, EGTA, phenanthroline
Vibrio collagenase	<i>Vibrio parahaemolyticus</i>	X-Gly	
Clostridium collagenases	<i>Clostridium perfringens</i>	X-Gly	sulfonylated amino acid hydroxamates
Aeruginolysin	<i>Ps. aeruginosa</i>	Leu-Gly or Gly-Gly	
Mirabilysin	<i>Proteus mirabilis</i>	Leu-Gly (in IgA)	
Fragilysin	<i>Bacteroides fragilis</i>	Leu-Gly Gly-Leu	EDTA, DTPA
Sialoglycoprotein endopeptidase	<i>H. influenzae</i>	Arg-Asp	

because the Clp protease regulates a wide range of genes<sup>75</sup> and also F2 has been shown to increase the effectiveness of bacterial killing by human whole blood, indicating that this compound can augment innate immune defenses.<sup>74</sup> Consequently the therapeutic effect of F2 may be enhanced at tissue sites where high levels of antimicrobial peptides are produced e.g. periodontal sites.

An important role for Clp proteases in the virulence of *P. gingivalis* has been identified. First, ClpC and ClpXP expression is elevated in biofilms and the ClpC and XP proteases are necessary for internalization into epithelial cells. ClpB is required for intracellular survival.<sup>76</sup> Consequently, inhibitors of the Clp proteases, such as F2, have potential applications in the treatment of periodontitis.

#### *P. gingivalis* gingipain proteases

The gingipain proteases of the periodontal pathogen *P. gingivalis* are a particularly interesting target in relation to periodontitis. There are 2 major gingipains, arginine-specific gingipain (RgpA & B) and a lysine specific gingipain (Kgp). Despite the fact that the Rgps are in approximately a 3-fold excess over Kgp,<sup>77</sup> the latter appears to be the most important enzyme for virulence and for nutrient assimilation. The specific mechanism (s) by which this enzyme is involved in these functions is not absolutely clear but a *kgp* mutant has been shown to be unable to accumulate haemin at the cell surface (loss of black pigmentation) which results in reduced oxygen tolerance and reduced virulence factor expression.<sup>78,79</sup> In addition, the inability to sequester iron may lead to the down regulation of other traits required for maximal expression of virulence *in vivo*. Consequently, Kgp appears to be a useful target enzyme for direct inhibition and work by Curtis et al.<sup>80</sup> developed and tested an inhibitor specific

for Kgp (1- (3-phenylpropionyl) piperidine-3 (R, S)—carboxylic acid—[4-amino-1 (S)-(benzothiazole-2-carbonyl) butyl] amide (A71561)). This agent showed no inhibition of the Rgps, and did not inhibit growth of *P. gingivalis* on complex media; however, pretreatment of *P. gingivalis* with this agent prior to infection in a murine model significantly reduced pathogenicity. In contrast, pretreatment of *P. gingivalis* with an Rgp inhibitor, leupeptin, did not affect virulence.

Despite this, it is known that the RgpP gingipains act in concert with Kgp gingipain to contribute to the entire virulence of the organism, particularly through disruption of the host complement system.<sup>81</sup> Therefore, a single agent that inhibits both gingipain proteases has clinical therapeutic potential. Kataoka et al.<sup>82</sup> recently developed such a dual inhibitor through structure-based drug design. It has potent antibacterial activity against *P. gingivalis in vitro* and reduced gingival inflammation in a beagle dog periodontitis model.

## Probiotics and Prebiotics

### Probiotics

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. Probiotics have been used successfully to control gastro-intestinal diseases and appear to act through colonisation resistance and / or immune modulation. Likewise, studies also suggest that probiotics have the potential to modify the oral microbiota. However these may only be successful over the short term. Experimental studies and clinical trials have demonstrated that certain gastrointestinal bacteria, including *Lactobacillus* and *Bifidobacterium* spp,

may also control the growth of oral microorganisms, including cariogenic streptococci associated with disease.

Mechanisms of probiotic action within the oral cavity can possibly be suggested from gastrointestinal studies<sup>83</sup> whereby the introduction of microorganisms as a therapeutic tool for the prevention and treatment of dental caries and periodontal disease could possibly act as follows within the oral environment.

1. Direct interactions within dental plaque. These could possibly include the disruption of plaque biofilm formation through competition for binding sites on host tissues and other bacteria, and competition for nutrients. The production of antimicrobial compounds that inhibit oral bacteria may also be a significant mechanism. It is known that lactic acid bacteria produce a range of antimicrobial agents including organic acids, hydrogen peroxide, low molecular weight antimicrobial peptides, bacteriocins and adhesion inhibitors.<sup>83</sup>
2. Indirect probiotic actions within the oral cavity, including the modulation of aspects of both innate and specific immune function. Within this context, it is possible that lactic acid bacteria can interact with immunocompetent cells, such as macrophages and T-cells, leading to cytokine production and subsequent effects on overall immunity.<sup>83</sup>

*Lactobacillus rhamnosus* CG,<sup>84</sup> *L. casei*,<sup>85</sup> *L. reuteri*<sup>86</sup> and *Bifidobacterium* DN-173 010<sup>87</sup> have all demonstrated the potential to alter colonisation of cariogenic bacteria and thus prevent dental caries. The oral administration of probiotics has also been explored in the control of periodontal disease. Krasse et al.<sup>88</sup> demonstrated a reduction in plaque levels and gingival inflammation with the application of *L. reuteri* to subjects with moderate to severe gingivitis. To achieve optimal effects, simultaneous use of multiple species may be required as described using *S. oralis*, *S. uberis* and a lactic acid-deficient variant of *S. rattus*, whereby the probiotic mouthwash was able to markedly affect the levels of cariogenic bacteria in saliva and periodontal pathogens in subgingival plaque.<sup>89</sup> However, although available data indicates an effect of probiotics on the oral microbiota, a more limited effect on clinical periodontal outcome measures is observed. There is a need for clinical trials where probiotics are used as adjuncts to standard periodontal treatment.

'Replacement therapy' based upon biotechnological approaches has also been investigated. Techniques include gene inactivation to remove metabolites that may harm the host and the incorporation of genes to encode for antimicrobial compounds, for example bacteriocins that inhibit the growth of strains of the same species. Genetically modified bacteria, for example *S. mutans*, are being considered for replacement therapy in the control of dental caries. Using recombinant DNA methodology, a strain of *S. mutans* was made lactate dehydrogenase deficient by the deletion of virtually all of the genetic sequence encoding this activity. To then compensate for the resulting metabolic imbalance, an alcohol dehydrogenase gene from *Zymomonas mobilis* was introduced. No detectable lactic acid during growth was produced from the resulting clone. This strain was also significantly less cariogenic than the parent strain in

gnotobiotic- and conventional-rodent models of disease. In addition it was found to colonise the teeth of conventional rats to the same extent as the parent strain using both aggressive-displacement and preemptive-colonisation approaches. The clone was shown to be genetically stable and did not revert to producing acid in both *in vivo* and *in vitro* test systems.<sup>90</sup>

### Prebiotics

Prebiotic substances, non-digestible oligosaccharides and other selectively fermented food ingredients have been used to improve gastrointestinal health and are now receiving interest in relation to oral applications. The main mechanism by which prebiotics act is assumed to be via facilitating proliferation of commensal bacteria, with resulting probiotic effects.<sup>91</sup> Some prebiotics may also exert effects on the host mucosal immune and inflammatory systems, independent of their effects on commensal bacteria.<sup>91</sup> Examples of prebiotics include inulin-type fructans, maltodextrin, fructooligosaccharides and galactooligosaccharides. It is also known that the oral microflora of the child is influenced to a large extent by diet. Within this context it is of interest that human milk contains oligosaccharides that have prebiotic characteristics.<sup>92</sup>

### Antimicrobial Peptides

Antimicrobial (host defense) peptides (AMPs) are a diverse group of molecules and include defensins, cathelicidins, histatins, neuropeptides, peptide hormones, and many other proven and putative peptides. These peptides are produced by many tissues and cell types; with phagocytic and epithelial cells as the predominant source. Peptide concentrations from these cells increase significantly following infection or injury. Human saliva and gingival crevicular fluid contains at least 45 individual antimicrobial proteins and peptides that can be classified into different functional classes<sup>93</sup> (Table 3). The majority of AMPs fall within the cationic peptide group and are generally defined as being 12-50 amino acids in length, with a net positive charge of +2 to +7 and up to 50% hydrophobic amino acids. This provides for an amphipathic design, consisting of spatially separated hydrophobic and charged regions, and permits intercalation of the peptide with microbial membranes. Direct antimicrobial activity against microorganisms has been considered, until recently, their primary function. However, many peptides may have no direct inhibitory activity at their physiological concentrations found within oral fluids. Conversely, AMPs may have an antimicrobial effect at the epithelial surface of the gingivae or at the secretion site from neutrophils where the local concentration could be higher than that found in oral fluids.

There is now increasing evidence that AMPs are multifunctional molecules of fundamental importance in host defense, modulating between aspects of the innate and adaptive immune systems. Recently, it has become apparent that AMPs stimulate a wide range of effects relevant to inflammation, innate immunity and adaptive immunity. This includes effects on innate immune cells, including neutrophils and epithelial cells, and in those cells



**Table 3.** Functional classes of antimicrobial peptides and proteins (with examples) found in the oral cavity

Cationic peptides	Bacterial agglutination and adhesion	Metal ion chelators	Peroxidases	Protease inhibitors	Activity against bacterial cell walls
Adrenomedullin β defensins 1-3	β-2-microglobulin Fibronectin	Calgranulins A & B Lactoferrin	Lactoperoxidase Myeloperoxidase	Cystatins Secretory leukoprotease inhibitor protein	Lysozyme Peptidoglycan recognition proteins
Cathelicidin (LL-37) Histatins 1 and 3 Neutrophil (α) defensins 1-4	Proline-rich proteins	Psoriasin Transferrin			

that bridge the innate and adaptive immune systems, including monocytes, macrophages and other antigen-presenting cells. These peptides have been shown to modify cellular functions such as chemotaxis, apoptosis, gene transcription and cytokine production. In addition, they have been shown to have roles in the stimulation of wound healing and angiogenesis.<sup>94</sup> The use of AMPs to control oral infections including periodontitis may therefore depend upon direct antibacterial, anti-inflammatory and/or immune modulatory actions.

Functional and structural diversity of AMPs and other innate defense molecules may well be necessary to help protect the oral epithelia from infection and maintain the balance of commensals and opportunistic pathogens required for health. Furthermore, AMP expression is partly regulated by the oral microbiota,<sup>95</sup> with the commensal bacteria often inducing peptide expression above that demonstrated with the more pathogenic species. Differences in AMP expression between periodontitis patients and healthy subjects has been identified using proteomic analyses.<sup>96</sup> Understanding the role of individual AMPs in oral disease may lead to the development of disease biomarkers and new therapies. AMPs also differ markedly in their antimicrobial activity against different oral species.<sup>97</sup> For example, the antimicrobial effects of adrenomedullin can vary considerably, with both fully resistant and sensitive (minimum inhibitory concentrations down to < 0.1 µg/ml) oral anaerobic species / strains.<sup>98</sup> When such MIC values are compared to concentrations of AM found in the gingival crevicular fluid (GCF) of patients with periodontal disease (approx. 1-2 µg/ml),<sup>99</sup> it is clear that adrenomedullin has the potential to influence the growth of the oral microflora *in vivo*.

Systems are being developed to target AMPs against given oral species using a targeting peptide, linker region and antimicrobial peptide component.<sup>100,101</sup> Other approaches include the modification of natural peptides to generate peptides with more favorable efficacy/toxicity profiles.<sup>102</sup> Alternatively, peptide mimetics have been designed and synthesized which retain the biological activity of an AMP but are advantageous as regards production costs, possess favorable therapeutic index and show stability under physiological conditions.<sup>103</sup> For example, mimetics based upon the defensin structure have demonstrated a high therapeutic index in pre-clinical studies.<sup>104</sup> With regards to the oral cavity, the development of novel antimicrobials should allow control of pathogens without loss of beneficial commensals.<sup>105</sup> Approaches that stimulate or restore the normal expression patterns of AMPs, rather than being used as exogenous therapeutic agents, may be

particularly useful in the prevention of periodontal disease. For example, through the use of receptor activation or through the use of protease inhibitors to improve the longevity of AMPs or related receptors.<sup>106</sup> As with other agents, a combination of AMP use and mechanical debridement is likely to be most successful in the control of periodontitis.

## Nanotechnology

Nanotechnology represents the ability to image, manipulate and model functionalities on the nanometer scale.<sup>107</sup> This discipline includes the use of nanoparticles which can be classified as particles of a size less than 100 nm. Properties of nanoparticles, for example, their active surface area, chemical reactivity and biological activity, are often far removed from those of a greater size. These characteristics should allow them to closely interact with microbial surfaces, and thus elicit an antimicrobial effect that is not solely due to released components. Metallic and other nanoparticles are now being combined with polymers and other base materials, and coated onto surfaces to ultimately provide a variety of potential applications within the oral cavity. The use of nanotechnology offers the possibility to control the formation of oral biofilms through the application of nanoparticles with biocidal, anti-adhesive and delivery capabilities.<sup>108</sup>

Nanoparticle based implant coatings should also offer both osteoconductive and antimicrobial functionalities to prevent dental implant failure. Such implant systems are increasingly being used to replace missing teeth, and most integrate with bone without complications. The condition peri-implantitis is a major cause of dental implant failure whereby the induced inflammatory changes in the soft tissues surrounding the implant lead to a progressive destruction of the supporting bone.<sup>3</sup> Current forms of treatment are often inadequate, with chronic infection often requiring implant removal and expensive resective and regenerative procedures in an attempt to restore and reshape the supporting tissue.<sup>109</sup>

### Antimicrobial nanoparticles and control of oral biofilms

Metals have been used for centuries as antimicrobial agents. Silver, copper, gold, titanium and zinc have attracted particular attention, each having different properties and spectra of activity. Indeed, many oral products, including toothpastes, now incorporate powdered (micron-sized) zinc citrate or acetate to control

the formation of dental plaque.<sup>110</sup> With respect to nanoparticulate metals, the antimicrobial properties of silver<sup>111</sup> and copper<sup>112</sup> have received the most attention. Both of these have been coated onto or incorporated into various test materials, including the denture material Poly (methyl methacrylate) (PMMA).<sup>113</sup>

Nanoparticulate metal oxides have been of particular interest as antimicrobial agents as they can be prepared with extremely high surface areas and unusual crystal morphologies that have a high number of edges, corners and other potentially reactive sites.<sup>114</sup> On the other hand, certain metal oxides are now coming under close scrutiny because of their potential toxic effects to eukaryotic cells.<sup>115</sup> Oxides under consideration as antimicrobial agents include those of copper, zinc, titanium and tungsten. Studies have shown that some nanoparticulate metal oxides, such as ZnO, have a degree of selective toxicity to bacteria with a minimal effect on human cells at the concentrations employed.<sup>116</sup> Bacteria are far less likely to acquire resistance to metal nanoparticles than they are to other conventional and narrow-spectrum antibiotics. This is thought to occur because metals may act on a broad range of microbial targets, and many mutations would have to occur in order for the microorganisms to resist their antimicrobial activity.<sup>117</sup>

Quaternary ammonium poly (ethylene imine) (QA-PEI) antimicrobial nanoparticles have also been developed and incorporated into dental composite resins.<sup>118</sup> This approach may be particularly beneficial when compared to the currently used composite resins for hard tissue restoration, which are known to possess several disadvantages including development of biofilms on both teeth and the restorative material.

#### Anti-adhesive nanoparticles and oral biofilm control

Particles of a nano and micro size based upon the element silicon have been designed to rapidly deliver antimicrobial and anti-adhesive capabilities to the desired site within the oral cavity.<sup>119</sup> Companies use silica (often classed as 'micro fine', but with a particle size within the definition of nanoparticles) in toothpastes, and some have actively sought new directions in this area through the use of porous silicon and nanocrystalline silicon technology to carry and deliver antimicrobials, for example, triclosan. Other systems based upon silica have been investigated with respect to the control of oral biofilms. The use of nitric oxide-releasing silica nanoparticles to eradicate biofilm growth has been described. Bioactive glasses of the  $\text{SiO}_2\text{-Na}_2\text{O-CaO-P}_2\text{O}_5$  system have been shown to possess antimicrobial activity through the release of ionic alkaline species over time.<sup>120</sup> Those in the form of amorphous nanoparticles, with a size range of 20 to 60 nm, may show an advantage over micron-sized material as the decrease in glass particle size should increase the active exchange surface of glass and surrounding liquid. This should then substantially increase ionic release into suspension and enhance antimicrobial efficacy. Chitosan, a biopolymer derived by the deacetylation of chitin occurring in the exoskeleton of crustaceans is positively charged and soluble in acidic to neutral solution, enabling it to bind to mucosal surfaces. Both chitosan nano- and microparticles have been investigated as a potential platform for local delivery of drugs within the oral cavity.<sup>121</sup>

The application of nano-scaled hydroxyapatite (nHA) particles has been shown to impact on oral biofilm formation and can also provide a re-mineralization capability. Biomimetic approaches based upon HA nanocrystals which resemble the structure at the nano-scale of abraded dental enamel crystallites, in theory should allow adsorbed particles to interact with bacterial adhesins, reduce bacterial adherence and hence impact on biofilm formation.<sup>122</sup> A number of oral health care products, including toothpastes and mouth rinses, have been developed containing nano-sized apatite particles with and without protein-based additives.<sup>123,124</sup> It is suggested that the efficacy of these compounds can be attributed to the size-specific effects of the apatite nanoparticulates. Casein phosphopeptide (CPP)—amorphous calcium phosphate (ACP) nanocomplex (Recaldent™/MI Paste™) is a particular technology based upon ACP and stabilized by casein phosphopeptide (CPP).<sup>125</sup> Use of this technology has demonstrated anticariogenic activity under both *in vitro* and *in vivo* test conditions. With reference to dental implants, numerous companies market synthetic HA materials as the 'optimal' osteoconductive implant coating available and some companies have developed nano-scaled varieties. Furthermore, combined nHA and nano zinc oxide (nZnO) coatings have shown much potential as regards antimicrobial activity and biocompatibility.<sup>116</sup>

#### Biocompatibility of nanoparticles within the oral cavity

Although the development and application of nanotechnology is of considerable interest, knowledge regarding the possible toxicity of nanotechnology products to humans is limited. In order to fully understand the mechanism of toxicity, a thorough knowledge of the toxico-kinetic properties of nanoparticles is required. Toxicology and biodynamic studies do suggest that silica, silicon, and chitosan nanoparticles are relatively safe if introduced *via* the oral route.<sup>126</sup> The safe use of nanotechnology and the design of nanomaterials for biological applications involve a thorough understanding of the interface between these materials and biological systems.<sup>127</sup> The nanoparticle characteristics of most importance as regards interaction with biological systems, whether mammalian or microbial, are chemical composition, surface function, shape and number of sides, porosity and surface crystallinity, size heterogeneity, roughness, and hydrophobicity or hydrophilicity.<sup>128</sup> In order to help prevent aggregation of nanoparticles, stabilizing (capping) agents that bind to the entire nanoparticle surface can be used; these include water-soluble polymers, oligo- and poly-saccharides, sodium dodecyl sulfate, polyethylene glycol and glycolipids.<sup>129</sup> An understanding of the interface between biological systems and nanomaterials should enable design features to be used to control the exposure, bio-availability and biocatalytic activities. A number of possible approaches are starting to be identified including changing the ability to aggregate, application of surface coatings, and altering charge density and oxidative state. However this may well compromise the intended selective toxicity of antimicrobial nanoparticles. It remains to be determined how potential mammalian toxicity issues will fully impact on the use of nanotechnology in the control of oral infections.

## Conclusions

Further studies, including investigations into the cost effectiveness, specificity, safety aspects and patient acceptance of using non-conventional approaches in the control of oral infections will be required. Such strategies should ideally reduce plaque levels without affecting the overall biological equilibrium within the oral cavity. Selective light-activated killing, functional inhibition of specific virulence factors and microbial replacement therapy offer a more targeted approach, whereas the use of plaque disrupting agents, antimicrobial

peptides and nanoparticles are relatively more general. Approaches that offer complementary modes of action will increase antimicrobial effectiveness when used in combination, particularly when improved delivery systems are employed. The increasing consumer demand for effective and safe oral care products will help to further drive the need to investigate non-conventional therapeutics.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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