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Mouthwash Effects on the Oral Microbiome: Are They Good, Bad, or Balanced?

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ABSTRACT

This narrative review describes the oral microbiome, and its role in oral health and disease, before considering the impact of commonly used over-the-counter (OTC) mouthwashes on oral bacteria, viruses, bacteriophages, and fungi that make up these microbial communities in different niches of the mouth. Whilst certain mouthwashes have proven antimicrobial actions and clinical effectiveness supported by robust evidence, this review reports more recent metagenomics evidence, suggesting that mouthwashes such as chlorhexidine may cause "dysbiosis," whereby certain species of bacteria are killed, leaving others, sometimes unwanted, to predominate. There is little known about the effects of mouthwashes on fungi and viruses in the context of the oral microbiome (virome) in vivo, despite evidence that they "kill" certain viral pathogens ex vivo. Evidence for mouthwashes, much like antibiotics, is also emerging with regards to antimicrobial resistance, and this should further be considered in the context of their widespread use by clinicians and patients. Therefore, considering the potential of currently available OTC mouthwashes to alter the oral microbiome, this article finally proposes that the ideal mouthwash, whilst combatting oral disease, should "balance" antimicrobial communities, especially those associated with health. Which antimicrobial mouthwash best fits this ideal remains uncertain.

Introduction

The oral cavity is home to arguably one of the most well-studied ecosystems in the human body. This ecosystem hosts communities of aerobic and anaerobic bacteria, fungi, archaeae, protozoae, and viruses on abiotic surfaces such as the tooth, dental implants, and dental restorations as well as biotic environments, such as the subgingival crevice, the dorsum of the tongue, hard palate, attached gingiva, buccal mucosa, tonsils, and alveolar mucosa. The subgingival crevice provides 12 cm^{21} of surface area for bacterial colonisation,[2] whilst both keratinised and nonkeratinised surfaces of the oral mucosa constitute more than 200 cm^2.[1] In total, oral colonisation niches constitute a space that is larger than the size of one's palm.

Since the oral cavity forms the gateway to the body, the oral microbiome is subject to constant perturbations arising from frictional forces, food consumption, oral hygiene practices, and lifestyle, including habits such as smoking, vaping, and alcohol consumption. The adult oral microbiome is established as a steady-state community mostly within the first year of life[3] and, through immune education, creates a state of dynamic homeostasis with the host immune system.[4] This immune recognition of, and tolerance to, the resident microbiome, along with well-entrenched interbacterial interactions, endows this open ecosystem with the ability to resist change, a property known as resilience. Indeed, it is recognised that the oral microbiome returns to baseline following routine dental prophylaxis (reviewed by Teles et al, 2013)[5] and that subgingival and supragingival biofilms return to nearly 90% of their original compositional structure following repeated episodes of gingivitis.[6] It is now recognised from several body systems that temporal stability and resilience of the microbiome are critical to maintaining human health.[7,8]

Until recently, much of the literature had focussed on the effectiveness of mouthwashes in the context of their ability to be bactericidal in vitro against bacteria pathogenic for oral disease.

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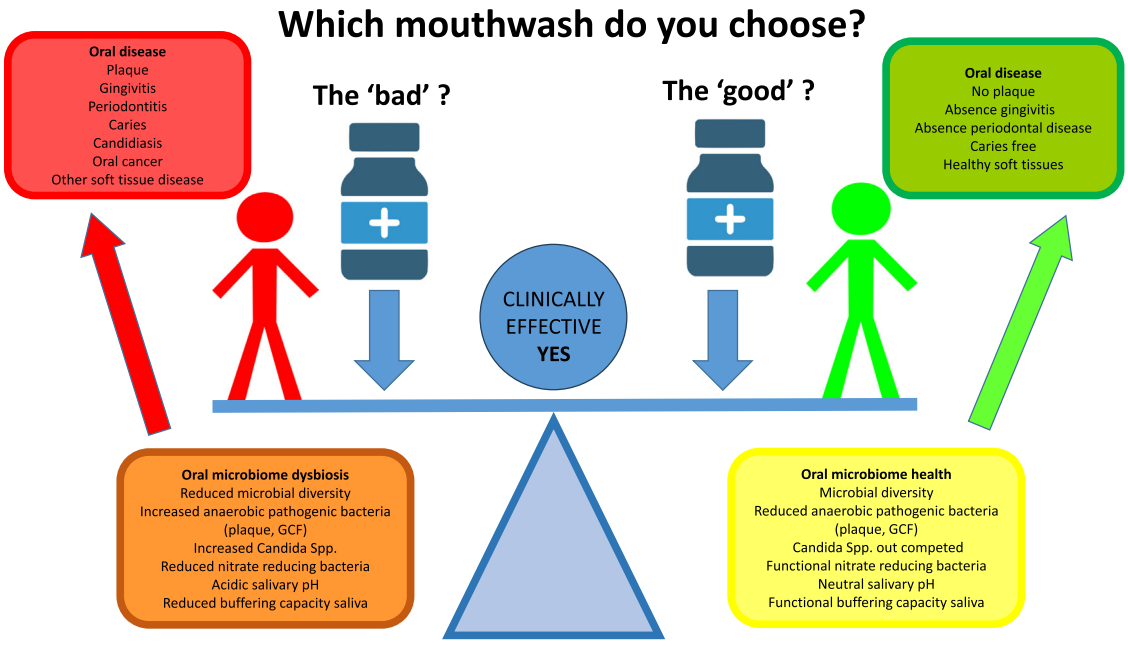

Fig – Summary of the "good" and the "bad" effects of mouthwashes on the oral microbiome and clinical signs and symptoms of oral disease. (This addresses the research question: Having determined your mouthwash is clinically effective, does mouthwash used indiscriminately tip the "balance" from oral health to dysbiosis?)

This has been a largely successful approach, but it is rather simplistic in the advent of our increased understanding of the oral microbiome. Oral hygiene practices are probably the single greatest factor that have shaped the oral microbiome of modern man.[9] The goal of these procedures is to maintain the plaque biofilm in an immature state with high proportions of early bacterial colonisers, which are mostly aerobic or facultative species.[10] For centuries, oral hygiene has been achieved through mechanical brushing, interdental cleaning, and nonsurgical therapy. However, these techniques have developed alongside the aforementioned traditional views, supporting the concept that removing pathogenic species from the oral cavity with antiseptic mouthwashes should also constitute good oral hygiene. We now understand that the oral microbiome exists as a complex and diverse community of bacteria, fungi, archaeae, protozoae, and viruses in oral health. Despite this, most studies of the oral microbiome refer to bacteria; the antibacterial effect of mouthwashes on the oral microbiome can be both advantageous and deleterious, depending on whether there is a "good" shift towards oral "health," with diversity, or "bad" shift towards "disease," with a predominance of species. There are many different factors that can induce this shift (Figure). Hence, this article in this supplement will compare the effects of a number of the most commonly used mouthwashes on the oral microbiome, and leading on from this, the next article in this supplement will then introduce the causal links between the oral microbiome and systemic health, with a focus on cardiovascular disease.

Bacteria and the oral microbiome (bacteria)

Early studies on the antibacterial effects of mouthwashes were based on culturing single species of bacteria. However, in recent years, the development of sequence analysis of either small amplicons, such as the 16S ribosomal RNA gene, the whole genome community (metagenome), or the community of RNA (metatranscriptome) has allowed for a more comprehensive exploration of the oral microbiome,[11] enabling us to enumerate in vivo the shifts in diversity and proportions of bacterial species in different oral niches in response to mouthwashes. Such studies are far fewer than the traditional culture-based, single-species investigations; thus, we will combine all levels of evidence to investigate their effects on bacteria, fungi, and viruses as well as the resistance genes within these microbial communities for most commonly used mouthwashes.

Chlorhexidine

Numerous in vitro studies have demonstrated that 0.01% to 0.2% chlorhexidine glucoronate (CHX) has a potent bactericidal effect on single species and multispecies cultures containing *Streptococcus mitis*, *Fusobacterium nucleatum*, *Porphrymonas gingivalis*, and *Aggregatibacter actinomycetemcomitans*.[12] Chlorhexidine also decreases bacterial diversity[13] and vitality in saliva[14] and on the tongue.[15] Chlorhexidine mouthwashes reduce plaque and gingivitis,[16] as described in the previous article, and chlorhexidine may be used as an adjunct to manage periodontal disease in certain countries (European Periodontology Guidelines).[17] Whilst this could be considered an advantage in reversing microbial dysbiosis, studies in healthy individuals demonstrate that certain species, namely *Veillonella*, *Actinomyces*, *Haemophilus*, *Rothia*, and *Neisseria*, are also inhibited by chlorhexidine.[18] These health-associated oral bacteria in saliva perform the important function of reducing dietary nitrates to nitrite, contributing the

maintenance of cardiovascular health via the release of nitric oxide.[18] Due to the nonspecific nature of its antimicrobial activity, chlorhexidine mouthwashes may thus negate the beneficial effects of nitrate-rich diets that are mediated via the oral microbiome.[13]

Hydrogen peroxide

Hydrogen peroxide is a bleaching and oxidising agent known to be bactericidal in vitro.[19] Several in vivo studies suggest that 1.5% hydrogen peroxide reduces the incidence of gingivitis and bleeding scores, but it is less effective at reducing plaque levels (reviewed by Muniz et al, 2020) and, as discussed in another article in this supplement, there is uncertain evidence for its effectiveness against caries and periodontal diseases. In relation to this, 1% hydrogen peroxide slightly decreased plaque concentrations of obligate anaerobes that are associated with periodontal disease, such as *Fusobacterium* and *Veillonella*, to a significantly lesser extent than chlorhexidine.[20] Very little antimicrobial action was observed against *Streptococcus mutans*, the organism associated with dental caries.[21] However, to our knowledge there are no reported studies investigating the global impact of hydrogen peroxide on the oral microbiome.

Cetylpyridinium chloride

Cetylpyridinium chloride (CPC) is a type of quaternary ammonium compound (QAC) and a broad-spectrum antimicrobial compound found in mouthwashes, usually at a concentration of 0.05% to 0.07%. CPC was effective in reducing plaque and the levels of anaerobic species of bacteria in plaque and saliva[22]; however, evidence from microbiome studies remains limited. One study using 16S sequencing reported that levels of *Porphyromonas*, *Corynebacterium*, *Abiotrophia*, TM7, and other known periodontal pathogens did not increase in supragingival plaque over 21 days of induction of experimental gingivitis in the presence of CPC, along with nonsignificant increases in gingival inflammation and bleeding from baseline.[23]

Povidone iodine

Povidone iodine contains the polymer polyvinyl pyrrolidine and elemental iodine, which together act as an antimicrobial agent via oxidation and destruction of vital cellular components.[24] A concentration of 10% povidone iodine demonstrates appreciable bactericidal activity against *P gingivalis*, *A actinomycetemcomitans*, *F nucleatum*, *Tannerella forsythia*, *Prevotella intermedia*, and *Streptococcus anginosus*,[25] as well as 3 strains of Gram-positive and 4 strains of Gram-negative bacteria[26] in vitro. However, a clinical study reported reduction in quantities of plaque without significant alterations in proportions of different bacteria in the microbiome.[27] In summary, the effects of povidone iodine on the oral microbiome have not yet been fully described.

Essential oils

Numerous essential oils used in mouthwashes, notably carvacrol, thymol, and eugenol, have demonstrated bactericidal effects against oral pathogens.[28,29] In vitro, an essential oil blend was able to eradicate *Staphylococcus aureus* and *Streptococcus* biofilms on hydroxyapatite discs to a greater extent than chlorhexidine.[30] Essential oils are also reported to reduce plaque and bleeding scores when used as an adjunct to tooth brushing (reviewed by Alsheri et al, 2018[31]; Sharma et al, 2004[32]). However, when used as an adjunct for treatment of periodontal disease, plaque scores and bleeding on probing did not reduce any more than with the placebo, nor did the levels of *A actinomycetemcomitans*, *P gingivalis*, *T forsythia*, *T denticola*, *P intermedia*, *Fusobacteriae*, or *S mutans*.[33] These results are equivocal, and there appear to be no studies investigating the effects of various essential oils on the oral microbiome in vivo.

Alcohol

Alcohol (ethanol) is found in many mouthwashes and has been discussed in depth elsewhere in this supplement. However, being antimicrobial, it must be considered whether it is the essential oils or the ethanol affecting the oral microbiome.[34] There is a general consensus, that alcohol kills both "good" and "bad" bacteria within the oral microbiome indiscriminately. Indeed, it appears that alcohol can decrease the abundance of commensal bacteria in heavy drinkers and increase the abundance of *Actinomyces*, *Leptotrichia*, and *Neisseria* (some of these genera contain oral pathogens).[35] However, no studies have reported the effects of mouth rinsing on oral microbiome communities. Progressively more alcohol-free mouthwashes are becoming available over-the-counter (OTC), one reason being that alcohol may increase salivary acetaldehyde,[36] arguably linked to oral cancer in the context of mouthwashes.[37]

Sodium fluoride

Sodium fluoride mouthwashes are used based on their moderate anticariogenic effectiveness as previously described, involving remineralisation of enamel; in vitro, 200, 400, and 1400 ppm sodium fluoride prevented demineralisation without affecting biofilm composition and growth on sintered hydroxyapatite or bovine enamel disks.[38] Lower concentrations of fluoride mouthwash (0.05% sodium fluoride), however, may not affect plaque scores or gingival inflammation in vivo.[39] Sodium fluoride mouthwash also had little effect on *P gingivalis*, *P intermedia*, *F nucleatum*, and *A actinomycetemcomitans* using ex vivo cultures of bacteria from the tongue.[40] Thus, further metagenomics analysis is warranted, as despite being widely used to manage dental caries, at the current time the effects of different concentrations of sodium fluoride mouthwash on the oral microbiome is unknown.

Probiotics

Probiotics are live bacteria that are administered to the host to orchestrate a healthy microbiome. The commonly used

bacteria in OTC mouthwashes include *Lactobacillus* and *Bifidobacterium*. Evidence on the effectiveness of probiotics as a mouthwash is limited. *L salivarius* NK02 in mouthwash led to reduced plaque and bleeding scores in patients with periodontitis after 28 days of use, alongside reduced counts of pathogenic *A actynomycetancomitans* and increased commensal bacteria in saliva and gingival crevicular fluid.[41] A mouthwash containing freeze-dried powder from *Lactobacillus rhamnosus* and *Bifidobacterium* species also reduced colonies of *S mutans* cultured ex vivo from the saliva of children[42]; however, there is no evidence on the effectiveness of probiotics and the oral microbiome as a whole, which could be an interesting area for future research in the context of a "balancing" effect.

Propolis

Propolis is made from the waxy substance bees use to seal their hives. It has been reported to exhibit antimicrobial activity in vitro against species which predominate in periodontitis, gingivitis, caries, plaque, recurrent aphthous ulcers, and pharyngitis[43] and will be explored in more detail elsewhere in this supplement, addressing more novel compounds and future directions. However, lower plaque and bleeding scores[44-46] and lower levels of *S mutans* in saliva have been reported in vivo with propolis mouthwash,[47] suggesting clinical effectiveness. Five days of rinsing with II-ethanolic extract of propolis type-3 3% also reduced counts of microbial counts of volatile sulphur compound-producers, associated with periodontal disease,[48] but as with most other mouthwashes there is limited information on the oral microbiome.

Fungi and the oral microbiome (mycobiome)

Communities of fungal species within the oral cavity (oral mycobiome) remain a relatively new concept.[49] *Candida spp*, as commensals, form an important part of a balanced microenvironment on oral hard and soft tissues; however, gene-based sequencing of 18S, 5.8S, and 28 rRNA has revealed that many other fungal species may exist in a health oral microbiome, for example, *Malassezia spp, Caldosporium*, and *Aspergillus*.[49,50] *Candida spp* may predominate during oral dysbiosis,[51] then associated with oral disease such as candidosis of the tongue and denture-associated stomatitis. However, there remains insufficient information on the effects of different antimicrobial mouthwashes on the oral mycobiome. There are suggestions that 0.2% chlorhexidine can inhibit the growth of *C albicans* in vitro and can also be fungicidal.[52,53] There are mixed results relating to oral biofilms, which can be more complex when the oral mycobiome is considered, as certain periodontal pathogens, such as *P gingivalis* and *A actinomycetemcomitans* can become more invasive and virulent in the presence of *Candida spp*.[54] Results have been mixed using in vitro models of oral biofilms. In some studies, chlorhexidine appears to inhibit single species biofilms containing *C albicans* and *S Mutans*.[55] In others, alcohol-free commercial mouthwashes (CHX, sodium fluoride, essential oils, CPC, and triclosan) failed to impair the ability of *C albicans* to form biofilms.[56] There is also uncertain evidence that chlorhexidine is able to reduce the number of colony-forming fungal species on dentures.[57] More research is thus required in this area.

Viruses and the oral microbiome (virome)

The human oral microbiome also contains viruses (oral virome), comprising RNA or DNA, being either single-stranded or double-stranded. Metagenomics analysis of the virome is a novel area of research where methodology remains in development. However, metagenomics studies so far have revealed that bacteriophages constitute the vast majority of viruses in the "healthy" oral cavity, for example, Caudovirales and Microviridae.[58] The oral virome also contains some eukaryotic viruses, for example, herpes simplex virus, cytomegalovirus, and Epstein-Barr virus from the Herpesviridae family, Orthomyxoviridae-like influenza A virus (IAV) or Coronaviridae such as human coronavirus (HCoV) or severe acute respiratory syndrome coronavirus 2[59-63] (SARS-CoV-2). In contrast to the effects of antiseptic mouthwashes on the oral bacteriome, much less is known on the effects of mouthwashes on the oral virome. Due to the membrane-disrupting mechanism of action of most antiseptics described above, when mouthwashes are used in vitro, it is generally expected that enveloped viruses are more prone to inactivation by mouthwashes as compared to non-enveloped viruses.[64,65] Envelopes or membranes are a rare feature amongst bacteriophages and exist in only about 4% of the described isolates.[66,67] Accordingly, a recent study showed that antiseptic formulations containing QACs such as benzalkonium chloride had virtually no virucidal effects towards MS2 bacteriophage in vitro.[68] More research is needed assessing the effects of mouthwashes on the virome, where there are viral loads in the oral cavity leading to clinical signs of disease.

In vivo, however, a recent systematic review reported that chlorhexidine exhibited some virucidal effects against herpes simplex virus-1 and IAV but moderate to no efficacy against HCoV and SARS-CoV-2.[69] In a randomised controlled clinical trial, a commercially available essential oil blend reduced viral loads in saliva of patients with active herpes labialis infection for at least 30 minutes after use. More recently, since the outbreak of the COVID-19 pandemic, numerous in vitro as well as clinical studies have been conducted examining mouthwashes as part of a bundle of measures for infection control in dental practice.[70,71] Initially, there was interest in their use as preprocedural rinses during the COVID-19 pandemic. During a short period of research, so far, whilst most in vitro studies showed high virucidal efficacy against SARS-CoV-2 for QACs, CPC, povidone iodine and essential oils, lower virucidal activity was shown for chlorhexidine and hydrogen peroxide[65,70]; translation of these results into clinics remains complicated.[71] Nevertheless, clinical evidence is mounting that mouthwashes containing CPC could temporarily decrease the infectivity of SARS-CoV-2.[72-76]

In summary, there is a lack of knowledge on potential effects of antiseptic mouthwashes on the oral virome.[77] Since the ratio of viruses to human cells is thought to be 100:1,[60,62,78] further research into the virome and phageome

of the oral cavity and into possible effects of mouthwashes seems essential.

Mouthwashes and antimicrobial resistance (resistome)

The oral resistome is defined as "all the resistance genes within the microbiome."[79] The use of metagenomics and next-generation sequencing has allowed for the analysis of the diversity of antibiotic resistance genes and resistance profiles of bacteria without the need for traditional isolation of individual species.[79] When assessing the effect of mouthwashes on the resistance profiles of the oral microbiome, studies to date mostly use isolation and cultivation of individual species and will be summarised here. However, metagenomic investigations of oral antibiotic resistance genes from the consistent use of mouthwashes and their clinical implications is an area for future research.

Chlorhexidine

There are two predominant mechanisms conferring resistance to chlorhexidine. The first is multidrug efflux pumps, allowing the microorganism to export chlorhexidine and other antibiotics from the cytoplasm and out of the cell.[80] The second mechanism is cell membrane changes that prevent chlorhexidine from binding to the target site.[81] The mechanism underlying cross-resistance between chlorhexidine and antibiotics is not yet determined, although plasmids containing resistance genes often carry resistance to both chlorhexidine and antibiotics.[81]

The earliest evidence for antimicrobial resistance in oral bacteria came in the early 1970s, with reports of reduced susceptibility of oral streptococci to chlorhexidine after prolonged use (reviewed by Cieplik et al).[80,82,83] An in vitro study of 315 isolates of subgingival bacteria demonstrated that strains of *S mitior, S sanguis*, and *Capnocytophaga* demonstrated resistance to 0.2% chlorhexidine.[84] Another in vitro study demonstrated 2 to 4-fold increased minimum inhibitory concentrations of 2 isolates of *P gingivalis* following subinhibitory concentrations of chlorhexidine, although repeated exposure did not significantly alter these concentrations.[80,85]

Saleem et al[86] demonstrated that chlorhexidine-resistant bacteria were also resistant to various antibiotics, including ampicillin, kanamycin, gentamicin, and tetracycline, underpinning the presence of multidrug resistance in dental plaque.[80,86] Yamamoto et al[87] isolated a plasmid from a MRSA strain that conferred resistance to CHX, several antibiotics, and benzalkonium chloride. Recently, a high prevalence of biocide resistance genes isolated from MRSA strains were positively correlated with resistance to chlorhexidine and mupirocin in Portuguese hospitals.[88] <AQ: Should "biocide resistance genes" be "biocide-resistant genes?">Wand et al[89] showed that isolates of the opportunistic pathogen *Klebsiella pneumoniae* can develop resistance to chlorhexidine through sharing mechanisms that correlate with colistin resistance, the last-resort antibiotic, as well as vancomycin.

QACs

Multiple mechanisms explain development of resistance to QACs such as benzalkonium chloride and CPC. These include upregulated efflux pumps, outer membrane alterations targeting QAC-binding sites, and biodegradation.[90] The increased production of efflux pumps is particularly problematic as they confer co-resistance to other antibiotics.[90] In addition, exposure to low concentrations of QACs induces bacterial oxidative stress, leading to production of superoxide and hydrogen peroxide. In response, bacteria adapt through DNA mutations and increasing gene transfer, thereby transferring resistance to other bacteria.[90]

In vitro, *Enterococcus faecalis* demonstrated higher MIC in response to repeated exposure of chlorhexidine, but not CPC, although cell surface changes were noted to both CPC and chlorhexidine in the resistant bacteria.[91] There were no significant changes to the MIC noted for *S mutans*.[91] Another in vitro study reported significant increase in minimum inhibitory concentrations after exposure to low concentrations of CPC and CHX for *A actinomycetemcomitans, F nucleatum, P gingivalis, P intermedia, S mutans*, and *S sobrinus*.[92] Furthermore, cross-resistance to other antiseptics and antibiotics was also observed, mostly due to changes in cell surface, modification of protein expression, and adaptation of the bacteria to oxidative stress.[92] In contrast, a prospective, placebo-controlled study showed that twice-daily rinsing with CPC for 6 weeks did not produce any significant alterations to the oral microbiome.[93,94]

Current literature is ambivalent on the clinical significance of developing resistance to chlorhexidine and QACs.[94] In addition, is it unclear whether the oral cavity is a potential reservoir for horizontal gene transfer that will increase resistance to both QACs and chlorhexidine and other antibiotics through co-resistance.[94] In summary, our knowledge of the impact of mouthwashes on oral bacteria is garnered largely from in vitro studies or those examining a targeted suite of organisms. Studies that investigate shifts in bacterial, fungal, and viral populations or acquisition and transfer of antimicrobial resistance amongst the residents are woefully lacking. Oral organisms exist as biofilms, and knowledge from in vitro studies on planktonic organisms cannot be extrapolated to oral health. As a case in point, the cationic nature of chlorhexidine causes it to bind avidly to the surface of plaque but prevents its penetration.[95] In support of this, its efficacy is at least 13-fold lower against biofilms than planktonic mixtures of the same species.[96] Considering these limitations of currently available literature, it is recommended that mouthwashes be limited to specific indications and periods of use that are supported by appropriate lines of evidence.[81]

Conclusions

Whilst having bactericidal and bacteriostatic effects against pathogenic species in vitro and clinical effectiveness against plaque and gingivitis in vivo, OTC mouthwashes may concurrently cause dysbiosis of the oral microbiome. Most studies on clinical effectiveness and antimicrobial research have been undertaken with chlorhexidine which, whilst clinically

effective, may also reduce the diversity of oral bacteria within various niches of the oral cavity, killing potentially both the "good" species associated with heath and the "bad" species associated with disease (Figure). Whether this has relevance for systemic health is discussed in another article. Much less is known about the effects of other currently available OTC mouthwashes on the oral microbiome, and hence these are difficult to summarise accurately. Thus, further metagenomics are urgently needed before recommendations can be made.

Viruses and fungi also have an important function in health and disease and may be affected by antiseptic use, but are poorly understood in the context of the oral microbiome. There are also risks that overuse of certain antiseptic mouthwashes, including chlorhexidine, may contribute to bacterial resistance, supporting this tailored approach. However, the message is not that all mouthwashes are "bad," but rather that dental practitioners should be seeking to advise antiseptics that maintain a "balanced," healthy, and diverse microbiome when they are used to manage any microbial-induced oral disease. There are some early suggestions that mouthwashes containing probiotics and propolis could be more "balancing," but this is controversial, and thus this will be discussed later in this supplement.

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