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Effects of chlorhexidine mouthwash on the oral microbiome

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Abstract

Introduction/Objectives: Chlorhexidine (CHX) is a commonly used ~~antimicrobial~~ mouthwash with potent ~~anti-microbial/bactericidal~~ effects useful for the management of oral disease. However, we are moving away from the view of simply 'killing' bacteria, towards managing oral microbial ecosystems (oral microbiome), as an integrated system, to promote oral and systemic health. Here, we aimed to review the effects of CHX mouthwash on the balance of microbial communities in the mouth *in vivo* in oral health and disease.

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Sources and study section: The hierarchy of evidence was applied, with systematic reviews and randomised controlled trials consulted where available and case controlled studies being described thereafter. Search terms for each subject category were entered into MEDLINE, PubMed, Google Scholar and the Cochrane database. Metagenomics studies were focussed on to provide unique overview of the oral microbiome as an integrated system.

Data: Evidence was limited, but several next generation sequencing case-controlled studies suggested that in an integrated system, CHX may cause a shift towards lower bacterial diversity and abundance, in particular nitrate-reducing bacteria *in vivo*. CHX also appeared to alter salivary pH, lactate, nitrate and nitrite concentrations in saliva. Evidence regarding the effects of CHX on the oral microbiome during oral disease is still emerging.

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Conclusions: CHX alters the composition the oral microbiome. However, as CHX use remains widespread in dentistry to manage oral disease, urgent research using metagenomics studies of microbial communities *in vivo* are still needed to determine CHX mouthwash is 'good', 'bad' or otherwise for bacteria ~~within microbial communities~~, in the context of oral and systemic health.

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Introduction

Oral bacteria have been traditionally associated with the presence of oral disease; however, this view has substantially changed since next-generation sequencing techniques have become cheaper and databases more accessible for researchers to explore the oral microbial ecosystem in a more integrated fashion. Consequently, research over the last two decades has shown a much more complex oral microbial community than previously thought (1). We have thus moved beyond the approach of simply 'killing' bacteria by antimicrobials as being the answer to managing oral disease to appreciate that the collective genome of all the bacteria, fungi and viruses that reside on, or within, our mouth are essential for oral and systemic health of the human host (2). Most of these microorganisms thus exist in our oral cavity in a symbiotic capacity, maintaining relationships with the host that are based on mutual benefits (3). Not only do they not cause harm, but also the commensal populations may keep pathogenic species in check by minimising their adherence to mucosal surfaces (4). Bacteria do not become successful pathogens, causing infection and disease, until they breach the barrier of commensals (4). Overall, it means we may have to re-think the evidence-based practice we are using as clinicians, when using anti-microbial agents orally, such as mouthwashes, and employing a more holistic approach to use will require understanding of the oral microbiome.

Certain bacteria within the oral microbiome are associated with gum disease (gingivitis and periodontitis) and tooth decay (caries) (5, 6). Although the host immune response is also key. Dental caries is associated with an overgrowth of bacterial species including those within the Firmicutes phylum such as *Streptococci mutans* and *Lactobacilli*, that can use dietary carbohydrates to produce lactic acid, that in the long term, can cause dissolution of tooth enamel and dentine (7). Recent studies using next-generation sequencing techniques have also revealed other species within Proteobacteria and Bacteroidetes phyla are involved in the pathophysiology of dental caries (8). but further studies are required to investigate the oral microbiome in individuals with increased dental decay, including the effects of antimicrobials.

Gingivitis is a mild form of gum disease whereby suboptimal oral hygiene promotes the establishment and maturation of dental and periodontal biofilms, conferring an environment conducive to excessive growth of certain commensal bacteria (9). Higher colonisation of bacteria, such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Treponema denticola* within the gingival crevice and saliva are associated with initial inflammatory

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response or 'gingivitis' (10, 11). In susceptible individuals, gingivitis then may progress to periodontitis, which is a more severe form of gum disease where an established dysbiosis leads to progressive inflammation and unfavourable host responses, resulting in damage to the soft tissues and supporting bone. The end result of this process is tooth loss. Abundance of *Prevotella melaninogenica*, *Rothia mucilaginosa* and *Fusobacterium* in saliva have been associated with more severe periodontitis (using the system of periodontal disease classification used prior to 2017) (12, 13). but similar to caries, more studies are required involving the oral microbiome analysis in varying stages of gum disease with and without antimicrobial agents such as mouthwashes.

Chlorhexidine (0.2%, CHX) mouthwash is arguably one of the most widely used ant-microbial agent used by oral health care practitioners, as well as the public with and without oral disease, to reduce bacterial load within the oral cavity on the pretext of preventing and managing oral disease. Thus, our research question asks: what are the effects of CHX mouthwash on the oral microbiome in relation to oral health and disease (using recent next-generation genome sequencing data as evidence to demonstrate an integrated system)?. More established mechanisms detail that CHX is bactericidal, by increasing cell membrane permeability, leading to the loss of intracellular components, including nucleotides, due to cell lysis (reviewed elsewhere) (14, 15). Indeed, CHX is used with some success for the management of periodontal disease, particularly as an adjunct to oral hygiene and non-surgical therapy (20). CHX mouthwash can also reduce dental plaque formation and gingivitis when used in patients *in vivo* (11). Despite this, very little is known about its effects on the oral microbiome and systemic health. Thus the evidence reviewed henceforth will investigate our hypothesis that CHX alters the oral microbiome composition within healthy mouths and during some of the most common oral diseases *in situ*, namely caries and periodontal disease. Prior to advances to genomic techniques, historical studies usually reported mechanisms of mouthwashes using isolated microbes *in vitro*, rather than the oral microbiome balance as a whole. However here, we will introduce diversity, emerging mechanisms and systematic outcomes that support our hypothesis that it is important to understand the mechanisms of CHX in the context of an integrated oral microbiome and whole body.

The aim of this study therefore, was to review the effects of CHX mouthwash on the balance of microbial communities in the mouth *in vivo* in oral health and disease,

using

next-generation genome sequencing data where ver possible. Initial scoping searches revealed insufficient evidence to perform a systematic review on this

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topic, hence the review was conducted in a narrative fashion, also highlighting where further original observational studies and randomised controlled trials are still needed to increase the evidence base.

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Oral microbiome

The microbiome is a term used to define the combined community of microorganisms that exist throughout the human body (16). The oral cavity contains one of the most diverse and unique microbiomes consisting of over 700 different species of bacteria, but also includes fungi, viruses, archaea, protozoa and other microorganisms (2, 17, 18). The mouth also has multiple habitats, including the teeth, gingival sulcus, tongue, cheeks, saliva, hard and soft palates, and tonsils, which are colonised by different communities of microorganisms (18, 19). Studies characterising the composition of the human microbiome are progressing rapidly, due to cheaper access to next-generation sequencing techniques and the expansion of databases of bacteria genetic sequences like the Human Oral Microbiome Database (HOMD) (20, 21), allowing us to go more in depth in describing bacterial communities colonising the mouth. The majority of research has also focussed on bacteria (as opposed to viruses and fungi), as bacteria arguably make up the most significant portion of the microbiome. However, this field is still in its infancy and we need continued effort to expand microbial genome databases to have a complete picture of what a healthy oral microbiome looks like.

~~Currently, only four studies have analysed the composition of bacteria within the oral microbiome using saliva samples in large populations (n>1,000) of young and older individuals from Japan, US and Spain (22-25). These studies have revealed that Firmicutes is the most abundant bacterial phylum in saliva (Table 1). However, the abundance of other phyla varied between the studies. These discrepancies may occur for several reasons. For example, different methodological approaches in DNA extraction and sequencing, and the analysis of different DNA regions. Apart from methodological issues, genetic and environmental factors can also modulate the composition and activity of the oral microbiome. From this viewpoint, diet has been suggested to be a key factor. However, we and others have not observed significant differences in the composition of oral bacteria, when comparing people following vegetarian and omnivore diets (26, 27). In contrast to this, a recent study reported differences in bacterial composition between the oral microbiome of vegetarians (vegans) and omnivores (28). Besides diet, smoking is another lifestyle factor which has a large impact on the oral cavity. Recent evidence has shown that it decreases abundance of species within Proteobacteria, Fusobacteria, SR1 and Cyanobacteria phyla (23, 29), while increasing the abundance of other species of Spirochaetes, Synergistetes, Tenericutes, Bacteroidetes and~~

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Actinobacteria (29). Oral care products (toothpaste, mouth rinses, cleaning aids) can also have a large impact in modulating the bacterial profile in the mouth since they reduce the accumulation of bacteria in different oral surfaces and thus the formation of biofilms (19). However, few studies describe the impact of these practices on the whole oral microbiome to date.

Regarding non bacterial microbes, previous studies in dentistry have focussed on *Candida albicans*, determining associations between *Candida albicans* and oral diseases, such as oral candidiasis, denture stomatitis, angular cheilitis and possibly dental caries (30, 31). Understanding of the role of fungi within the oral microbiome is limited, compared to bacteria; however, dysbiosis of *Candida* species during disease is also starting to be addressed using next generation sequencing technologies. Such studies have revealed a wider array of fungal organisms in the mouth than previously expected (18), but further research is needed *in vivo*. Similar complications in technical approaches have also led to scarce research on oral viruses amongst the microbiome. Thus far, the majority of oral viruses identified have been bacteriophages (32). Saliva, the oral mucosa and dental plaque all contain phage virions, able to infect and target specific oral bacteria associated with disease, such as Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria (33). As bacteriophages infect bacteria, not human cells, they can thus play a key role in modulating the oral microbial community. They provide an exciting and under explored area of research, with therapeutic potential, but detailed discussion is out of the scope of this review (34). Other non-phage viruses found within the oral cavity and linked with oral disease include: *Herpes viridae* (35), which cause herpetic ulcers (Herpes Simplex Virus 1 and 2); Epstein Barr Virus, which contributes to oral hairy leucoplakia (and may also play a role in periodontal diseases) (36); Human Papilloma Viruses (37), which may contribute to neoplastic transformation of epithelial cells in oral squamous cell carcinoma, and enteroviruses such as Coxsackie virus, which cause oral blisters and ulcers (36). The interactions of these particular viruses amongst the microbiome, however, also remain largely unexplored.

CHX and oral microbiome

The effects of CHX on isolated bacterial species associated with oral disease, such as *Streptococcus mutans* and *Porphyromonas gingivalis*, have been elucidated largely from *in vitro* culture methods, with the findings used to support CHX as an effective therapeutic agent (5). However, studies providing a broad view of the impact of CHX on the oral microbiome were missed until recently. This question has been partially addressed by two studies using

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next-generation sequencing techniques (38, 39). First, Tribble *et al* (38) investigated the effect of 0.12% CHX gluconate mouthwash, used twice daily for 7 days, on the abundance of bacteria colonising the tongue in healthy individuals. They found that CHX decreased species diversity and richness, whilst promoting a greater abundance of Gram-negative bacteria, especially within the Bacteroidetes (*Capnocytophaga*) phylum. Similar findings were reported by the same research group in previous experiments in rats (40). We have also analysed the effect of CHX digluconate (0.2%) mouth rinse, twice daily for 7 days, on the abundance of bacteria in saliva in healthy humans (39). Similar to the study by Tribble *et al* (38), we found that CHX lowered the diversity and richness of bacteria in saliva, but rather than increasing the abundance of Bacteroidetes, we observed a reduction of bacteria in this phylum, especially within the genus *Prevotellaceae*, whilst Proteobacteria (including *Neiseriaceae*) and Firmicutes (including *Streptococcus*) demonstrated an increase after using CHX (39).

Currently, it is difficult to explain whether these bacterial changes caused by CHX are promoting a healthier oral ecosystem, or, on the contrary, they harm it. However, data from our laboratory suggest that microbial changes caused by CHX could arguably be more detrimental than beneficial (39). We found a significant reduction in saliva pH and buffering capacity after using CHX for 7 days, twice daily (39). These changes were accompanied by an increase of lactate and glucose concentration in saliva (39). Similar findings have been reported by another recent study using *in vitro* methods (41). This is important because lower salivary pH is associated with demineralization of tooth enamel and erosion as well as increased risk of dental caries (42). Indeed, high concentrations of lactate in combination with low oral pH are important factors for tooth demineralisation and tooth decay, increasing the risk of dental caries (43, 44). Previous studies have also reported an increase in salivary pH shortly after using CHX (45), and using *in vitro* methods (46), however, our study was the first one to look at the effect of CHX on salivary pH after several days of use (39). We have confirmed the saliva pH-lowering effect of CHX mouthwash in other recent studies (39, 47). Importantly, this effect may be related to changes in the balance between lactate producer and lactate consumer bacteria (48). In agreement with this, we found that CHX lowered the abundance of some important species of lactate consumers, especially within the genus *Actinobacteria* and *Veillonella* alongside an increase of *Streptococcus* that contains several families of lactic acid producers (49). Furthermore, another recent study using *in vitro* methods reported a significant increase of *Streptococcus* (lactic acid producers) and lower abundance of *Veillonella* (lactic acid consumers) after short term exposure to CHX (41). The bacterium *Veillonella* has the ability to reduce nitrate into nitrite through the oral nitrate/nitrite/nitric oxide pathway (described below), which has been recently reported as a key mechanism to maintain oral pH within normal physiological ranges (50).

Regarding non-bacterial microbes, CHX can also act upon fungi and viruses. Research on fungi has been mainly focussed on *Candida albicans*, because it is directly implicated in oral candidiasis, which is an infection affecting the oral mucosa and tongue (51). However, this yeast is a common commensal in the human oral cavity, and like pathogenic bacteria, *Candida albicans* normally causes no harm, but has pathogenic potential when the microbial ecosystem is breached, usually following immunosuppression. CHX has shown a strong activity against *Candida albicans* on acrylic denture surfaces (52), oral epithelial cells (53) and oral mucosa (54). However, recent evidence has shown that some populations of *Candida albicans* can persist after using CHX, forming a multidrug-tolerant subpopulation that can lower the effectiveness of CHX over time (55). Studies investigating the gut microbiome also determined that broad-spectrum antibiotics promoted fungal growth and pathogenicity because they disrupted the microbiota by eliminating anaerobic bacteria in the [gut, which](#) could have otherwise inhibited the fungi (56, 57). This is concerning, in regards the use of CHX as a mouthwash; if it lowers the abundance of anaerobic bacteria, such as *Veillonella* (39), it could give *Candida albicans* or other yeast to predominate and cause disease. Although much more research is needed on this important question, we suggest that the use of CHX mouthwash should perhaps be avoided in immunocompromised patients, especially if fungal infections are a potential cause of morbidity and mortality (58, 59).

In regards to viruses, it has been suggested that CHX can also inactivate enveloped viruses, such as herpes simplex virus, human immunodeficiency virus (HIV), influenza virus and cytomegalovirus more effectively than non-enveloped types (60). Much like bacteria, CHX disrupts the lipid membrane structure of enveloped viruses, causing leakage of contents. Therefore, CHX has little virucidal activity on small non-enveloped viruses, such as enteroviruses, polio viruses and papilloma viruses (61). CHX also appears to be ineffective on the enveloped human coronaviruses, potentially including Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) (60). However, before even considering viruses in the context of the oral microbiome, there remains a lack of studies detailing the exact virucidal mechanism of CHX *in vivo*, or at a more molecular level to date, and further studies are required to determine the mechanism of action of CHX on Coronaviruses such as SARS-CoV-2.

CHX and antimicrobial resistance

Antimicrobial resistance (AMR) is the term used to describe the adaptations that micro-organisms develop to defeat drugs to kill them such as antibiotics, antifungals, antivirals,

antimalarials and anthelmintics. AMR can also be studied using metagenomics to assess resistance genes within the oral microbial community (resistome) (62), and has emerged as one of the principal public health problems of the 21st century; threatening the effective prevention and treatment of an ever-increasing range of infections (63). In regards to CHX, recent evidence suggests an increase of AMR in Gram-negative and Gram-positive species, due to either mutation or addition of genetic material, leads to changes in cell membrane structure and the function of ion pumps when CHX is used at low concentrations (64, 65). Bacterial spores are also widely resistant to CHX (61). Again, this is relevant especially in immunocompromised patients, as it may challenge treatment against potential infections that are unresponsive to antimicrobials. However, no studies have yet investigated the oral resistome in response to CHX.

What we do know so far in the context of oral health, is that the dental plaque biofilm provides a reservoir for the growth and dissemination of multi-drug resistant bacteria, posing a threat to immunocompromised patients (66-68). The effectiveness of CHX mouthwash against dental plaque formation has been successfully demonstrated since 1970, but generally after shorter term exposures (5, 6). Nevertheless, repeated short term exposure to 0.12% CHX, whilst similarly inactivating oral bacteria initially, can also thereafter lead to a rapid regrowth in biofilm formation (41). Longer and repeated CHX exposures also resulted in the development of more pathogenic bacterial variants, such as with *Streptococcus mutans* and *Porphyromonas gingivalis* (69, 70). This is relevant because these species are related to caries and some types of gum disease, respectively. Together the findings indicate that CHX has a temporal effect on oral bacterial biofilms, possibly falling short of keeping microbial numbers under control in the longer term, due to resistance. It would be interesting in future studies to evaluate this using metagenomics.

Regarding fungi, like bacteria, CHX may be effective for inhibiting the growth of *Candida Auris* within oral biofilms at short term, but longer term may be less effective due to the development of multidrug resistance (71, 72). Viruses can also act as portals for AMR, by conferring resistance genes to resident plaque bacteria (73), and this resistance can be transmitted, via the microbiome, between persons in close contact (74). However, it is uncertain whether CHX used as a mouthwash could lead to resistance of oral viruses to either antiseptics or antibiotics over time. Consequently, further studies are needed to investigate the impact of CHX on microbial resistance genes and metagenomics could provide the ideal approach. Current evidence suggests some concerning effects of CHX on AMR. This is very important because if, at the same time as decreasing microbial diversity, CHX creates a favourable niche for a

larger proportion of multidrug resistant oral microorganisms (resistome), it has the potential to promote the existence of more systemic infections that are resistant to antimicrobial therapy.

CHX and oral nitrate/nitrite/nitric oxide pathway

Returning to the 'normal' microbiome, by shifting the composition of the oral microbiome, CHX can also affect important physiological pathways modulated by oral commensal bacteria. An example of this is the oral nitrate/nitrite/nitric oxide pathway, where inorganic nitrate from saliva is reduced into nitrite by oral bacteria (75, 76). This reaction is driven by bacteria due to the lack of effective nitrate reductase enzymes in mammalian cells (77). Importantly, the formation of nitrite by oral bacteria has been elucidated as an important mechanism to balance oral acidity (78). In agreement with this, alongside genomic analyses, we have reported a consistent reduction in salivary pH when oral nitrite synthesis was inhibited with CHX mouthwash (26, 39, 47). This was due to a stronger effect against nitrate-reducing species, mainly within the genus *Prevotellaceae* (39). Other recent microbiome studies have also observed that feeding oral bacteria with food rich in nitrate raises salivary pH, which confirms the relevance of nitrite synthesis by oral bacteria in managing oral pH (79, 80). This has also been confirmed in *in vitro* studies (50). Consequently, treatments compromising the ability of oral bacteria to reduce salivary nitrate into nitrite, such as CHX mouthwash, could also concurrently compromise the health of soft and hard oral tissues, due to increased acidity in the oral ecosystem (Figure 1).

Last but not least, the oral nitrate/nitrite/nitric oxide pathway plays a key role in maintaining nitric oxide (NO) homeostasis (81). It is an important causal mechanism that directly links oral health (via the oral microbiome) to cardiovascular health and is starting to gain much attention. Once nitrite is formed in the mouth, it is rapidly absorbed, and in the stomach is protonated to form nitrous acid, which decomposes further to form NO and other nitrogen oxides (82, 83). A small portion of nitrite is also absorbed into the bloodstream where it can be reduced to nitric oxide through several pathways involving haemoglobin, myoglobin and xanthine oxidoreductase (84-90). Given the strong effect of CHX mouthwash against nitrate-reducing bacteria, we and others have used it to inhibit the oral nitrate/nitrite/nitric pathway to investigate the role of this pathway on blood pressure regulation (Figure 1). Importantly, the majority of studies, but not all (26, 91), have reported an increase in blood pressure in healthy and hypertensive individuals after blocking this pathway with CHX (38, 39, 47, 92, 93). Furthermore, a recent observational study reported a raise in blood pressure in people using antibacterial mouthwash, including CHX, twice a day or more frequently (94). These findings clearly show the key role that the oral microbiome has on blood pressure regulation probably

through the modulation of nitric oxide synthesis, which is an essential molecule for cardiovascular control and immunity (95, 96). Further research is thus needed to investigate this concerning clinical effects of CHX mouthwash, as well as to find alternative treatments for managing oral health without compromising cardiovascular health.

Conclusion

CHX has been extensively used in dental practice over the last four decades, for treating oral disease, as well as reducing plaque formation, due to its antimicrobial effects against oral pathogens. There is no doubt that it 'kills' bacteria successfully. However, the use of next generation sequencing techniques has allowed us to broaden our view about the effect of CHX mouthwash on the oral microbial ecosystem *in vivo*. Such research suggests that CHX may cause a shift towards less bacterial diversity, and lower abundance of bacteria essential for maintaining oral and systemic health. However, at present, there are insufficient studies investigating the effects of CHX on the oral microbiome, particularly in disease. Hence systematic review of this topic cannot yet be completed, to conclude whether CHX is 'good', 'bad' or otherwise in an oral microbial community context, despite its widespread use. Alongside continuing use of CHX, urgent research is also needed for discovering a variety of mouthwashes to treat disease, that do not concurrently cause oral dysbiosis, instead looking towards restoring or promoting an oral microbial ecosystem associated with health.

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Study	Sample size (country)	Type of population	Average age (years)	Encoding region sequence (sequencing platform)	Abundant phylum	Main findings
Takeshita et al (2016)	2,343 (Japan)	Older adults	63 ± 11	V1-V2 (Ion Torrent)	1. Firmicutes 2. Actinobacteria 3. Bacteroidetes 4. Proteobacteria 5. Others	Lower phylogenetic diversity was associated with better conditions for oral health, including a lower plaque index, absence of decayed teeth, less gingival bleeding, shallower periodontal pockets and not smoking.
Wu et al (2016)	1,204 (US)	Smokers and non-smokers	70 ± 6	V3-V4 (Roche FLX)	1. Firmicutes 2. Actinobacteria 3. Bacteroidetes 4. Proteobacteria 5. Fusobacteria 6. Others	Smokers had lower relative abundance of the phylum Proteobacteria compared with never smokers.
Fan et al (2018)	1,044 (US)	Non-drinkers and alcohol drinkers	68 ± 7	V3-V4 1 (Roche FLX)	1. Firmicutes 2. Fusobacteria 3. Actinobacteria 4. Proteobacteria 5. Bacteroidetes	Drinkers had decreased abundance of <i>Lactobacillales</i> , the major order in the Firmicutes phylum. Other taxa, some of which are potentially pathogenic within Proteobacteria and Actinobacteria phylum, were enriched with higher alcohol consumption.
Willis et al (2018)	1,319 (Spain)	Teenagers	13 - 15*	V3-V4 (Illumina)	1. Firmicutes 2. Proteobacteria 3. Bacteroidetes 4. Actinobacteria 5. Fusobacteria	Chemical composition of tap water changed the composition of the oral microbiome.

Table 1: Large human studies ($n > 1,000$) looking at the composition of the oral microbiome (bacteriome) in saliva samples (mean ± SD; *mean ± SD age was not provided in this study)

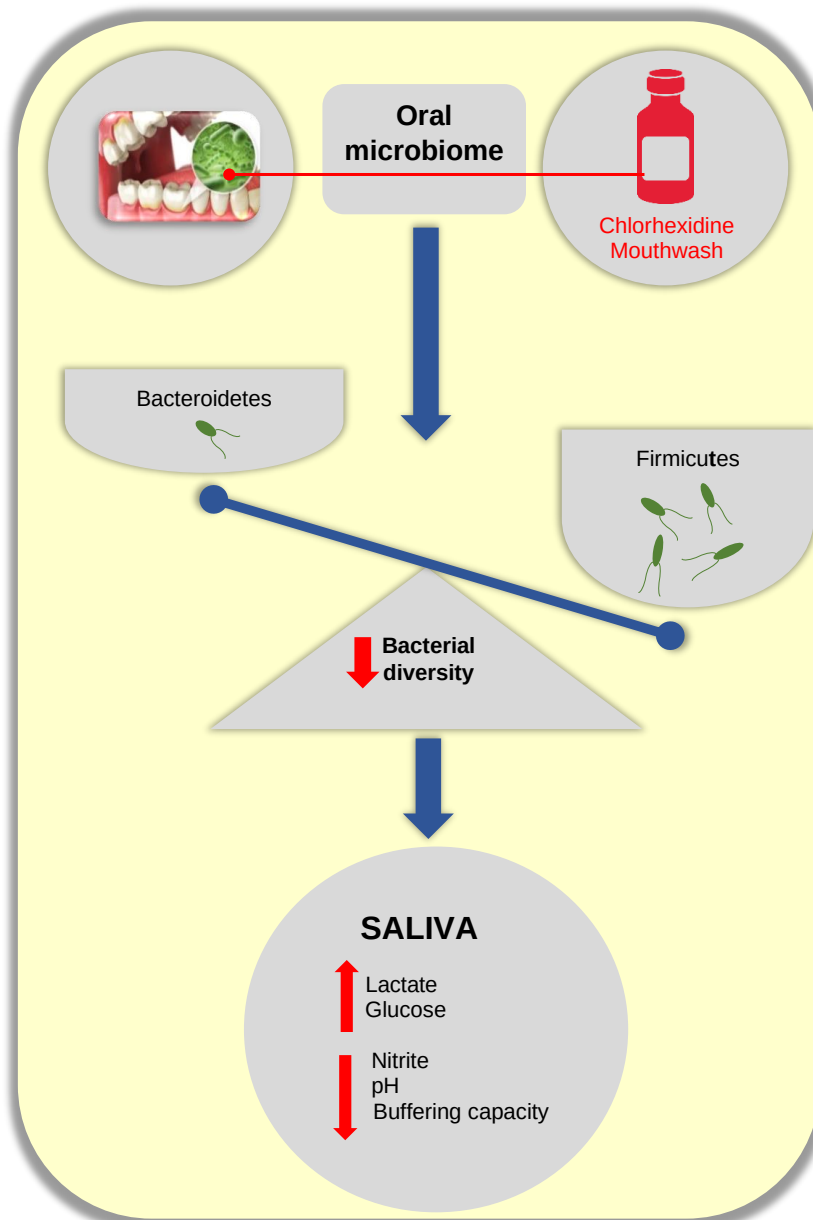


Figure 1: Effect of chlorhexidine mouthwash on the oral microbiome. Seven-days use of chlorhexidine mouthwash reduces oral bacterial diversity causing an increase of Firmicutes species abundance and a reduction of Bacteroidetes, which in turn, leads to acidification of saliva (39). Less nitrite in saliva then equates to less nitric oxide availability within the systemic circulation and increased blood pressure (91,92,98).