

## REVIEW

# Progress toward understanding the contribution of alkali generation in dental biofilms to inhibition of dental caries

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Alkali production by oral bacteria is believed to have a major impact on oral microbial ecology and to be inhibitory to the initiation and progression of dental caries. A substantial body of evidence is beginning to accumulate that indicates the modulation of the alkalinogenic potential of dental biofilms may be a promising strategy for caries control. This brief review highlights recent progress toward understanding molecular genetic and physiologic aspects of important alkali-generating pathways in oral bacteria, and the role of alkali production in the ecology of dental biofilms in health and disease.

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## INTRODUCTION

Dental biofilms, the microbial communities that colonize the surfaces of the teeth, exist in a dynamic equilibrium with host defenses and are generally compatible with the integrity of the tissues they colonize.<sup>1–4</sup> A strong correlation is evident between the compositional and metabolic changes of the dental biofilms and the transition from oral health to disease states, including dental caries and periodontal disease.<sup>2,5</sup> In the case of caries, frequent acidification of dental biofilms favors the emergence of an acidogenic and aciduric microflora, including mutans streptococci and *Lactobacillus* spp., which ferment dietary carbohydrates rapidly and lower the pH to values that accelerate tooth demineralization.<sup>1–3,6–7</sup> As might be predicted, the increase in the proportions of aciduric organisms appears to occur at the expense of species that are less aciduric and often associated with dental health.<sup>8–10</sup> Importantly, many of the organisms that are associated with dental health are able to use arginine or urea to generate ammonia via the arginine deiminase system or urease enzymes, respectively.<sup>1</sup> Alkali production by these bacteria can positively affect the balance between remineralization and demineralization of the tooth and may also help to prevent the emergence of a cariogenic microflora.<sup>11–14</sup>

A substantial body of evidence suggests that the modulation of the alkali-generating potential of dental plaque may be a promising strategy for caries prevention. In light of the emerging recognition of the inverse correlation between alkali generation and caries, additional research efforts have been focused on the molecular biology, physiology, ecological impact and clinical relevance of alkali production in dental biofilms. The aim of this review is to highlight some of the more recent advances in these areas and immediate research needs.

## Primary sources of alkali generation in dental biofilms

The two primary routes for alkali generation in dental plaque are the hydrolysis of urea by urease enzymes and the metabolism of arginine

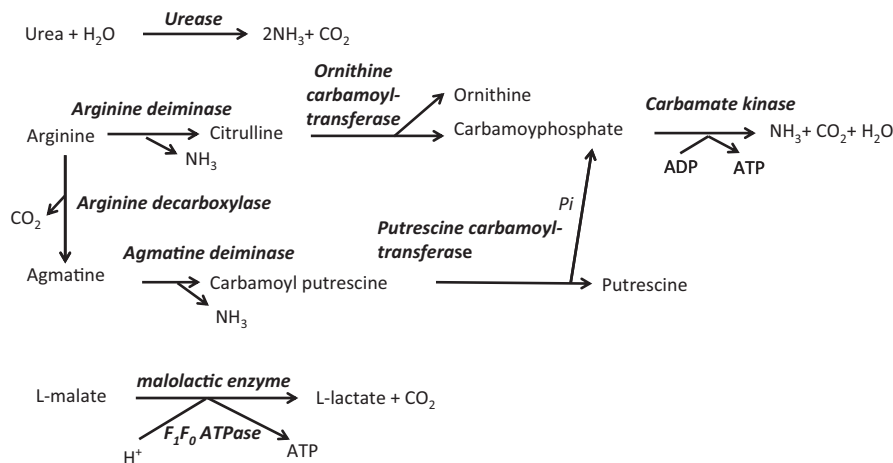
via the arginine deiminase system (ADS).<sup>15–18</sup> Urea is provided continuously in salivary secretions and gingival exudates at concentrations roughly equivalent to those in serum, which range from about 3 to 10 mmol·L<sup>-1</sup> in healthy humans. Urea is rapidly converted to ammonia and CO<sub>2</sub> by bacterial ureases (Figure 1), which are produced by a small subset of oral bacteria that includes *Streptococcus salivarius*, *Actinomyces naeslundii* and oral haemophili.<sup>19–22</sup> Arginine is abundant in salivary secretions as polypeptides, and free arginine concentrations in ductal saliva average around 50 μmol·L<sup>-1</sup>.<sup>18</sup> Arginine in the oral cavity is catabolized primarily by the ADS to release ornithine, ammonia and CO<sub>2</sub> (Figure 1). Unlike urea breakdown by urease enzymes, arginine catabolism by the ADS provides bacteria with adenosine triphosphate (ATP).<sup>15</sup> ADS-positive bacteria are abundant members of the normal oral flora that colonizes the teeth and soft tissues and include *Streptococcus sanguinis*, *Streptococcus gordonii*, *Streptococcus parasanguis* and *Streptococcus mitis*. Certain *Lactobacillus* and *Actinomyces* species, other oral streptococci and some oral spirochetes have been also identified as arginolytic.<sup>15,23–25</sup>

Agmatine catabolism may also have a significant effect on oral biofilm ecology, albeit not in the way that arginine or urea metabolism does.<sup>26</sup> Agmatine can be produced in dental biofilms from arginine by bacterial arginine decarboxylase enzymes, but it also occurs naturally in foods, such as rice, milk and beer.<sup>27–28</sup> Agmatine has been measured at concentrations of 0.75 μmol in dental plaque and 0.2 μmol in saliva when the samples were normalized to protein concentration (e.g. 0.75 μmol per mg of protein in plaque).<sup>29</sup> The primary route for agmatine utilization is through the agmatine deiminase system (AgDS), which is highly similar to the ADS, with end products being putrescine, ammonia, CO<sub>2</sub> and ATP (Figure 1). A survey of bacterial genomes and/or functional studies revealed that the AgDS is present in multiple oral bacteria, including *Streptococcus mutans*, *Streptococcus*

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**Figure 1** Summary of alkali-generating pathways in the oral cavity. See text for details.

*sobrinus*, *Streptococcus downei*, *Streptococcus rattus*, *Streptococcus uberis*, *Streptococcus mitis* and *Streptococcus cricetus*, as well *Lactobacillus salivarius* and *Lactobacillus brevis*.<sup>29</sup> Notably, some of these species are strongly associated with dental caries and express the AgDS at relatively high levels. Only *S. sanguinis* and *S. salivarius*, which are associated with dental health, can also generate ammonia *via* the ADS or urease, respectively, in addition to the AgDS.<sup>29</sup> These oral bacteria were reported to be highly capable of generating ammonia through the ADS or urease, but had much lower capacities for agmatine utilization. Therefore, the AgDS may not play as important role in the physiology of these oral commensals as it does in cariogenic streptococci. In fact, it has been postulated that the AgDS, unlike the ADS or urease, does not produce sufficient quantities of alkali to impact the pH of oral biofilm. Rather, the AgDS may actually enhance the acid tolerance of certain cariogenic organisms through ATP generation and raising of the cytoplasmic pH, which could enhance the ability of the bacteria to continue to catabolize carbohydrates at low pH values.<sup>26</sup>

It should also be noted that malate metabolism through malolactic fermentation (MLF) has recently been identified as another pathway to reduce the extent of acidification of dental biofilms, including *S. mutans*. Through MLF, L-malate can be catabolized to L-lactate and CO<sub>2</sub> by the malolactic enzyme<sup>30–31</sup> (Figure 1). Although the MLF does not produce ammonia, it has been proposed to contribute to cytoplasmic alkalinization, which has been postulated to support the generation of ATP by means of the F<sub>1</sub>F<sub>0</sub>-ATPase acting in the synthetic mode.<sup>30–31</sup>

## GENETICS AND REGULATION OF ALKALI GENERATION

### Urease

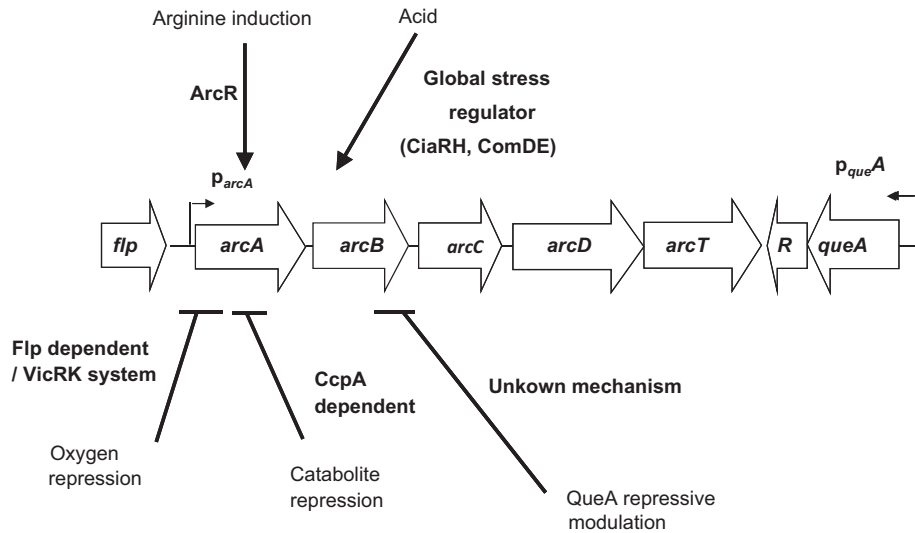
The genetics, biogenesis, biochemistry and effects on the host of bacterial ureases have been reviewed elsewhere,<sup>32–33</sup> while a previous review covered many of the specifics of oral bacterial ureolysis.<sup>15</sup> Briefly, urease is a nickel-containing oligomeric enzyme and the biogenesis of a functional urease requires at least seven gene products that are usually encoded in operons. The urease apoenzyme is composed of  $\alpha$ ,  $\beta$  and  $\gamma$ -subunits encoded by the *ureC-A* and *-B* genes. The subunits are assembled into an ( $\alpha\beta\gamma$ )<sub>3</sub> oligomeric complex with six-nickel ions coordinated at the active site. Four additional genes, *ureDEFG*, encode a chaperone complex that facilitates the incorporation of nickel and CO<sub>2</sub> at the active site.<sup>34–35</sup> The expression of oral bacterial ureases is often regulated by multiple inputs.<sup>36–39</sup> Commonly, the presence of urea or limitation for

a nitrogen source can induce urease gene transcription. In some bacteria, urease expression is repressed at neutral pH values, but under acidic conditions the urease genes become activated. Urease gene expression can also be sensitive to carbohydrate availability and rate of growth.<sup>36–38</sup>

### Arginine metabolism

The ADS is widely distributed among prokaryotes, and the primary structures of the enzymes in the system have been conserved during evolution. The genes encoding the ADS are commonly arranged in an operon (For example, see Figure 2), although the gene order varies among species.<sup>40–44</sup> The *arcA* gene encodes arginine deiminase, which hydrolyzes arginine to generate citrulline and ammonia. The *arcB* gene encodes a catabolic ornithine carbamyltransferase, which converts citrulline to ornithine and carbamoylphosphate, and *arcC* encodes the catabolic carbamate kinase that transfers a phosphate group from carbamoylphosphate to ADP to generate ATP, CO<sub>2</sub> and ammonia. Many organisms also harbor an arginine/ornithine antiporter (*ArcD*) that is encoded in the same operon, and arginine aminopeptidases and transcriptional regulators are often encoded in ADS gene clusters.<sup>41,44</sup> Of note, the oral commensal *S. gordonii* was identified to be the only ADS-positive bacterium containing a *queA* homologue associated with the ADS gene cluster. *QueA* is a predicted S-adenosylmethionine:tRNA ribosyltransferase-isomerase responsible for queosine modification of tRNAs<sup>45</sup> and *queA* is cotranscribed with the gene for the ADS transcriptional regulator. It has been postulated that the association of *QueA* with the ADS is indicative of a link between translational efficiency and arginine catabolism.

The regulation of the ADS genes has been studied extensively in certain oral streptococci and in a variety of non-oral species.<sup>41–43,46</sup> In all cases examined so far, the ADS is subject to regulation by multiple environmental stimuli, although the modes and mechanisms of control vary between species. ADS expression in most bacteria, including oral streptococci, is induced by arginine and low pH. Similarly, the operon is sensitive to carbon catabolite repression (CCR) and down-regulated in response to elevated oxygen levels, although the sensitivities to CCR and oxygen vary among species.<sup>47–48</sup> Interestingly, interspecies interactions, including coaggregation of *S. gordonii* with *A. naeslundii*, substantially enhanced ADS activity in *S. gordonii*, possibly by activating arginine biosynthesis and ADS expression.<sup>49</sup>



**Figure 2** Summary of ADS regulation in *S. gordonii*. Arrows indicate a positive influence on expression levels, while plain lines indicate a negative effects. See text for more detail.

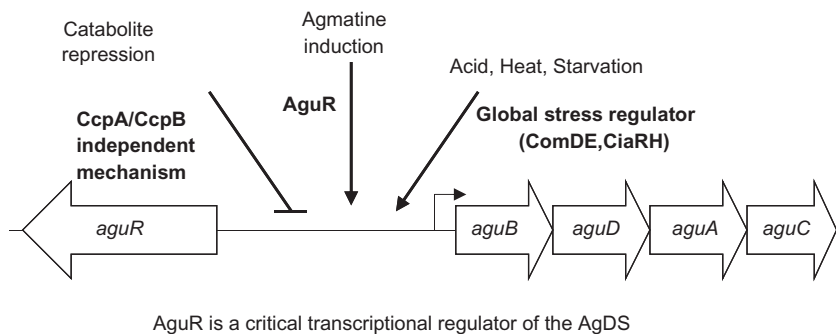
In *S. gordonii*, it was determined that ArcR is involved in the induction of the ADS genes by arginine and that CCR of the operon occurs primarily through CcpA (Figure 2).<sup>41,47</sup> An Fnr-like protein and the two-component system VicRK are required for the induction of the ADS in *S. gordonii* under anaerobic conditions (Figure 2).<sup>47</sup> Other two-component systems including CiaRH, ComDE and VicRK were found to be involved in the induction of the ADS in *S. gordonii* under acidic conditions (Figure 2).<sup>50-51</sup> QueA was also found to have a negative effect on *arc* gene transcription, possibly by impacting the translational efficiency of ADS genes or ADS regulatory genes (Figure 2).<sup>45</sup>

### AgDS

The AgDS does not appear to be as widely distributed in microbes as the ADS. Besides the oral species identified, only a few non-oral species, including *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Lactobacillus hilgardii* and *Helicobacter pylori* have been reported to be AgDS-positive.<sup>52-55</sup> Like the ADS genes, the AgDS genes are typically encoded in an operon consisting of *aguBDAC*<sup>26</sup> (Figure 3). Free agmatine can enter the cell *via* an agmatine-putrescine antiporter (AguD) and is hydrolyzed to *N*-carbamoylputrescine and ammonia by the agmatine deiminase enzyme encoded by *aguA*. The *N*-carbamoylputrescine is then metabolized by the putrescine carbamoyltransferase, encoded by *aguB*, to yield putrescine and carbamoylphosphate.

Finally, carbamate kinase, the product of the *aguC* gene, transfers a phosphate group from carbamoylphosphate to ADP to generate ATP, CO<sub>2</sub> and NH<sub>3</sub>. The putrescine generated can be exchanged for agmatine *via* the antiporter.<sup>26</sup> The *aguR* gene, which is located upstream of, and in the opposite orientation to, the *agu* operon in *S. mutans* encodes a transcriptional activator of the *agu* genes.<sup>56-57</sup>

In oral streptococci, AgD activity is generally lower than arginine deiminase or urease activity,<sup>26</sup> although there is some variation among AgDS-positive species. Comparable levels of AgD activity were detected in *S. mutans* and *S. rattus*, but over 50-fold lower activity was observed in *S. cricetus* and *S. sobrinus*.<sup>29</sup> In *S. mutans*, AgDS activity is growth-phase dependent, as well as inducible by agmatine and certain environmental stresses, including low pH and heat shock (Figure 3).<sup>26,56-57</sup> Expression of the AgDS genes in *S. mutans* was also sensitive to CCR and influenced by CcpA and a putative CcpB orthologue (Figure 3).<sup>26</sup> The AgDS is also inducible by low pH and agmatine through a predicted interaction between AguR and AguD. In particular, it has been proposed that agmatine stimulates AguR binding to its target upstream of the *agu* operon, and that acidic conditions favor a configuration of AguR that enhances binding to its target.<sup>57</sup> In the absence of exogenous agmatine, the interaction of AguR with AguD may prevent an interaction of AguR with its substrate and/or its target.<sup>57</sup> Multiple two-component systems, including CiaRH, ComDE and



**Figure 3** Summary of AgDS regulation in *S. mutans*. Arrows indicate a positive influence on expression levels, while plain lines indicate a negative effects. See text for more detail.

VicRK, were disclosed to be involved in the induction of the AgDS genes by low pH, and CiaRH was also shown to contribute to optimal expression of the AgDS under thermal stress (Figure 3).<sup>58</sup>

### CONTRIBUTION OF ALKALI GENERATION TO BACTERIAL PHYSIOLOGY AND BIOFILM ECOLOGY

As reviewed previously, oral organisms harvest significant benefits from alkali generation in the form of protection against acid damage and enhancement to cell growth.<sup>15</sup> Dental biofilms are complex ecosystems with hundreds of metabolically and physiologically diverse species, and there is believed to be considerable competition for nutrients. The ability of oral species to metabolize urea, arginine or agmatine at low pH could impart an advantage to these organisms through alkalization of the cytoplasm by ammonia. The generation of ATP by the ADS would also enhance acid tolerance by providing energy for proton extrusion, growth or maintenance.<sup>26,41,59–63</sup>

There is reason to believe that the contributions of the ADS, urease and AgDS to oral biofilm pH homeostasis and ecology may be quite different. For example, in some oral commensals, such as *S. salivarius* and *S. gordonii*, urease and the ADS, respectively, clearly can provide the bacteria with protection from environmental acidification.<sup>19,47</sup> At the same time, other less-aciduric species in the biofilm community benefit from arginine or urea breakdown because sufficient ammonia is sufficient to stabilize the pH of the local environment.<sup>19,47</sup> However, the caries pathogens *S. mutans* and *S. sobrinus* have no urease or ADS, but carry an AgDS that is expressed *in vitro* at a lower level than the ADS or urease is in oral commensals.<sup>26,29</sup> Further, agmatine, which arises primarily from the decarboxylation of arginine, is present at concentrations much lower than that of arginine in the oral cavity.<sup>18</sup> Consequently, ammonia generation from agmatine in dental biofilms probably does not cause significant alkalization of the environment. However, it could enhance the ability of *S. mutans* to continue to engage in glycolysis at low pH values, thus enhancing acid production. Similarly, MLF may not have much of a beneficial effect on biofilm pH, and like the AgDS, the benefits may be restricted to enhancing the growth and metabolism of the species that possess them.

### OBSERVATIONS ON THE RELATIONSHIP OF ALKALI GENERATION TO CARIES STATUS

Evidence continues to accumulate from *in vitro* studies and clinical observations that caries risk is associated with reduced alkali-generating capacity of the oral microbiome.<sup>11,17–18,64–68</sup> For example, indirect evidence that the oral metabolism of urea may enhance caries resistance was obtained from a study with chronic renal failure patients, who rarely develop caries even with carbohydrate-rich diet, but who also produce some 10- to 50-fold greater salivary urea levels than healthy subjects.<sup>67,69</sup> In another study, higher resting pH values in the dental plaque of caries-free (CF) individuals compared with those of caries-active (CA) individuals was noted, and the increased pH was shown to be correlated with elevated ammonia released in plaque.<sup>17</sup> Elevated levels of free arginine in saliva were revealed to be strongly correlated with caries resistance.<sup>18</sup> Noticeably, a potent inhibitory effect on caries development was observed in rats that were infected with a genetically-engineered strain of *S. mutans* expressing the urease genes of *S. salivarius*.<sup>16,68</sup> Specifically, rats colonized by the *S. mutans* strain expressing high levels of urease had fewer caries lesions and lower caries severity scores than those of control animals.

Investigators have now begun exploring whether there are clinical correlations between oral alkali-generating capacity and caries susceptibility. In studies with young adult subjects, higher urease and

ADS activity levels were detected in pooled supragingival plaque and whole saliva of CF individuals compared to those of CA individuals.<sup>64,66</sup> More recently, a positive correlation between oral arginine metabolism and dental health was also observed in children<sup>70</sup> when supragingival plaque was collected from specific tooth sites. Ammonia generation *via* plaque urease activity has also been correlated with reduced risk<sup>71</sup> for dental caries in a longitudinal study with children. A few recent studies have begun to explore the effects of including arginine in confections of oral health care products. Acevedo *et al.*<sup>72–73</sup> demonstrated in randomized clinical trials that toothpaste<sup>67</sup> and sugarless mints<sup>68</sup> containing arginine bicarbonate can have potent anti-caries effects. A very recent report showed that addition of arginine-bicarbonate to mouth rinse at concentrations as low as 2% can effectively raise the plaque pH above the critical pH for enamel dissolution following a sucrose challenge.<sup>74</sup>

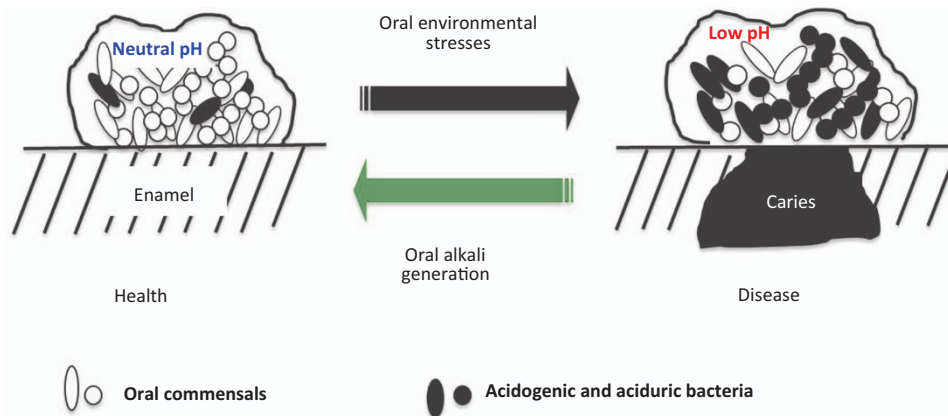
One of the major challenges facing caries researchers today is to understand the microbiological and molecular basis for the differences in alkali-generating potential in the dental biofilms of CA versus CF individuals. Although a number of studies have shown positive correlations of arginolytic species, e.g. *S. sanguinis*, with health and a decrease in the proportions of these species in caries, analysis of the oral microbiome has not revealed consistent changes in cultivable, non-cultivable or cultivated arginolytic taxa in CF and CA subjects.<sup>75</sup> Likewise, real-time PCR quantification showed no differences in the percentage of certain known arginolytic organisms in dental plaque of CF versus CA groups.<sup>64</sup> Thus, there may be reasons that CA subjects have higher ADS activity than CF subject other than simple decreases in the proportions of ADS-positive bacteria in carious tooth sites. For example, there may be additional arginolytic species beyond those previously identified that effect the bulk of arginolysis, although this seems unlikely as the microbiome data have been carefully analyzed. Alternatively, the oral environment of the host could influence the arginolytic capacity of the oral bacteria by affecting the expression of the genes or the activity of the enzymes involved in alkali production. Finally, there may be high- and low-producing strains of known ADS-positive species that are abundant in dental plaque but cannot be differentiated on the basis of 16S sequence, and health may be associated with colonization by high-producing strains. These high-producing strains may have constitutionally high expression or express comparatively high activity in response to pH, oxygen, carbohydrate and substrate availability versus the low-producing strains.<sup>25,76</sup>

### EPILOGUE

Alkali generation is widespread among oral species and is important in the physiology, ecology and pathogenicity of dental biofilms. A substantial body of evidence from microbiological, genetic, biochemical analyses and clinical studies has accumulated to confirm that the modulation of the alkaligenic potential of dental biofilms is a promising strategy for caries control. One of the strengths of alkali production as a strategy for control of caries is that it attacks the problem in two very important ways (Figure 4). First, it directly increases the pH of dental plaque, which tips the balance in favor of remineralization and away from demineralization. Secondly, alkali generation favors the persistence of health-associated bacteria while discouraging the outgrowth of those cariogenic bacteria that depend on low pH to gain an ecological advantage, e.g. *S. mutans*.

Despite recent progress in this area, there remain major gaps in our knowledge on the microbiological and ecological basis for differences in the alkaligenic potential of dental plaque of different populations. In





**Figure 4** The role of alkali generation in caries prevention. Dental biofilms in a healthy host displays a balance in pH and microflora conducive to a favorable demineralization/reminerization dynamic. With repeated acidification and exposure to other stresses, a shift in the composition and biochemical activities of the microflora creates conditions where demineralization is favored over remineralization, allowing for the initiation and/or progression of a caries lesion. Alkali generation by oral biofilm bacteria directly impacts plaque pH while preventing the emergence of a cariogenic microflora.

addition, we currently have a poor understanding of the otogeny of the alkaligenic microflora. Likewise, additional clinical studies are needed: (i) to confirm that the supplementation of arginine to plaque bacteria is in fact effective against caries in adults and children; (ii) to ensure that arginine does not diminish the impact of fluoride in the oral cavity; and (iii) to optimize the formulations for caries control. Other areas worthy of investigation include exploring probiotic applications to enhance oral arginolysis and prevent the development of caries lesions. Collectively, this information will facilitate the rationale design of strategies that rely on alkali production for caries risk assessment and interventions.

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