Review

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Streptococcus sanguinis biofilm formation & interaction with oral pathogens

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Caries and periodontitis are the two most common human dental diseases and are caused by dysbiosis of oral flora. Although commensal microorganisms have been demonstrated to protect against pathogens and promote oral health, most previous studies have addressed pathogenesis rather than commensalism. *Streptococcus sanguinis* is a commensal bacterium that is abundant in the oral biofilm and whose presence is correlated with health. Here, we focus on the mechanism of biofilm formation in *S. sanguinis* and the interaction of *S. sanguinis* with caries- and periodontitis-associated pathogens. In addition, since *S. sanguinis* is well known as a cause of infective endocarditis, we discuss the relationship between *S. sanguinis* biofilm formation and its pathogenicity in endocarditis.

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The mouth is the gateway of the human body and is in frequent contact with the external environment. Microbial communities in the mouth may be impacted by various environmental conditions [1]. When the homeostasis of oral microbiomes is disrupted, certain oral diseases may emerge. Two of the most prevalent diseases in the oral cavity are dental caries and periodontitis [2]. According to the 2016 global burden of disease study, periodontal disease was the 11th most prevalent human disease affecting 750,847 million people worldwide [2]. Published findings from the CDC estimate that half of Americans over 30 years of age have periodontal disease [3]. Caries of permanent teeth was the most prevalent disease, affecting 2.44 billion people and caries of deciduous teeth was the 17th most prevalent human disease worldwide [2]. In the USA alone, approximately 37% of children (2–8 years) have experienced dental caries in primary teeth and 58% of adolescents (12–19 years) have suffered dental caries in permanent teeth [4]. These oral diseases, if left untreated, lead to pain, dental abscesses, destruction of bone and other serious health problems. They have also been found to be strongly associated with an increase in mortality rate [5–7]. In addition, dental care in the USA represented about 5% of the country's spending on all healthcare, or US$111 billion, in 2012 [8]. The WHO reports dental caries as the fourth most expensive chronic disease to treat in most industrialized countries [9]. Given the extent of the problem, oral diseases are a major public health concern.

Using culture-independent approaches, primarily 16S rRNA gene-based cloning studies, it was estimated that the human oral cavity harbors approximately 700 prokaryote species, and more than half remain uncultivated once isolated from the complex oral environment [10]. The oral cavity is an ecologically unstable, saliva-bathed landscape providing numerous distinct habitats for bacteria to colonize, including the unique nonshedding surfaces of the teeth [11]. Some bacterial communities show a predilection for certain oral spaces and are commonly isolated from samples from particular sites. Colonization of the host within these niches is facilitated by the formation of biofilms, which may be defined as microbial communities embedded in a self-produced matrix of extracellular polymeric substances of bacterial origin [12]. Mature oral biofilms (dental plaque) have overall compositions that differ between niches and individuals, but have been shown to have a relative degree of species composition stability among the principal species [13–16]. However, it has been shown that the bacterial population profile is significantly different between a healthy oral cavity and one with oral disease. The initiation of chronic bacterially mediated periodontal

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diseases has now been identified as a compositional shift of dental plaque flora from predominantly Gram-positive facultative anaerobes to predominantly obligate Gram-negative anaerobes [17–19].

Characteristics of *Streptococcus sanguinis*

Streptococcus sanguinis, previously known as *S. sanguis*, is typically associated with healthy plaque biofilm [13–16,20–22]. It is a Gram-positive, nonspore-forming, facultative anaerobe. Like other streptococci, cell division of *S. sanguinis* occurs along a single axis, resulting in chains or pairs of cocci. *S. sanguinis* has generally been reported as being nonmotile. This has recently been challenged, as Gurung *et al.* reported that *S. sanguinis* strain 2908 is capable of surface-associated twitching motility facilitated by retractable type-IV pili [23,24].

In 2007, Xu *et al.* published the genome sequence of *S. sanguinis* SK36, which was originally isolated from human dental plaque [25]. The genome is a circular DNA molecule comprised of 2,388,435 bp, encoding 2274 predicted proteins [25]. There are 61 predicted tRNA genes producing all 20 amino acids and 50 putative carbohydrate transporters, including phosphotransferase system enzymes specific for transport of glucose, fructose, mannose, cellobiose, glucosides, fructose, lactose, trehalose, mannose, galactitol and maltose [25]. *S. sanguinis* seems to be able to utilize a broad range of carbohydrate sources for survival.

S. sanguinis is a pioneering colonizer, aiding in the attachment of succeeding organisms, and a key player in oral biofilm development [26–28]. Caufield *et al.* recorded the time of colonization of *S. sanguinis* in 45 infants. In their research, 25% of the infants had acquired *S. sanguinis* within 8 months of age, and 75% had *S. sanguinis* by 11.4 months; the median age of colonization by *S. sanguinis* was 9.0 months [27]. *S. sanguinis* is a commensal bacterium that is widely distributed in the oral cavity. It exists on tooth surfaces, oral mucosa surfaces and in human saliva [20,29,30]. As a facultative anaerobic species, *S. sanguinis* is abundant in both supragingival and subgingival plaque [15,31]. At different tooth locations, the biomass of *S. sanguinis* may differ significantly despite similarities in plaque mass [32]. It is present in high proportions at the lower incisor/canine sites of teeth, but in low proportions at the upper molar sites [32]. *S. sanguinis* has also been shown to form biofilm on different dental implant surfaces [33–35]. It is worth noting that the incidence of peri-implant complications significantly increases in patients with periodontitis [36]. Several studies demonstrate that plaque formation on dental implants results in peri-implant mucositis [37,38]. However, it is still not clear whether *S. sanguinis* promotes or reduces this effect.

Factors that affect biofilm formation in *S. sanguinis*

The attachment of *S. sanguinis* to the tooth surface

The first step in biofilm formation is the process of single cells attaching to a surface [39]. Fimbriae are involved in attachment to both animate and inanimate surfaces and in the formation of biofilms in many species of bacteria [40]. In 1985, Fachon-Kalweit *et al.* reported that fimbriae mediated the adhesion of *S. sanguinis* to saliva-coated hydroxyapatite (the main substance of the tooth surface) [41]. Okahashi *et al.* later identified three pilus proteins PilA, PilB and PilC in *S. sanguinis* SK36 [42]. A Δ*pilABC* mutant was defective in accumulation on saliva-coated surfaces and biofilm formation [42]. These investigators also showed that PilB and PilC bound to human whole saliva [42]. Moreover, PilC bound to multiple salivary components, one of which was found to be salivary α-amylase (Figure 1A) [42]. Tooth surfaces are coated with a large amount of salivary proteins [29]. Pilus binding to salivary components may help *S. sanguinis* attach to tooth surfaces and initiate biofilm formation in the oral cavity.

SsaB was first described as a saliva-binding protein that mediates attachment to saliva-coated hydroxyapatite via an uncharacterized pH-sensitive receptor [43]. Although SsaB has been demonstrated to be a lipoprotein [44], it is still not clear what targets SsaB binds to or, indeed, whether it is an adhesin at all, given that the evidence suggesting this function was indirect [43]. SsaB is also a virulence factor for infective endocarditis [45–49].

The glycoprotein serine-rich protein A (*SrpA*) has been found to mediate the binding of *S. sanguinis* to human platelets [50,51]. Recent studies analyzed the crystal structure of *SrpA* and revealed that *SrpA* bound to human sialoglycans [52,53]. The sialoglycan binding region of *SrpA* in *S. sanguinis* is homologous to two other sialoglycan-binding adhesins, GspB and Hsa in *Streptococcus gordonii* [54,55]. GspB and Hsa, which are alleles, have been shown to bind to human salivary proteins [54]. A *srpA*mutant has been shown to bind poorly to microtiter plates *in vitro* [56]; however, there is still no evidence demonstrating that *SrpA* mediates attachment or biofilm formation of *S. sanguinis* in the oral cavity.

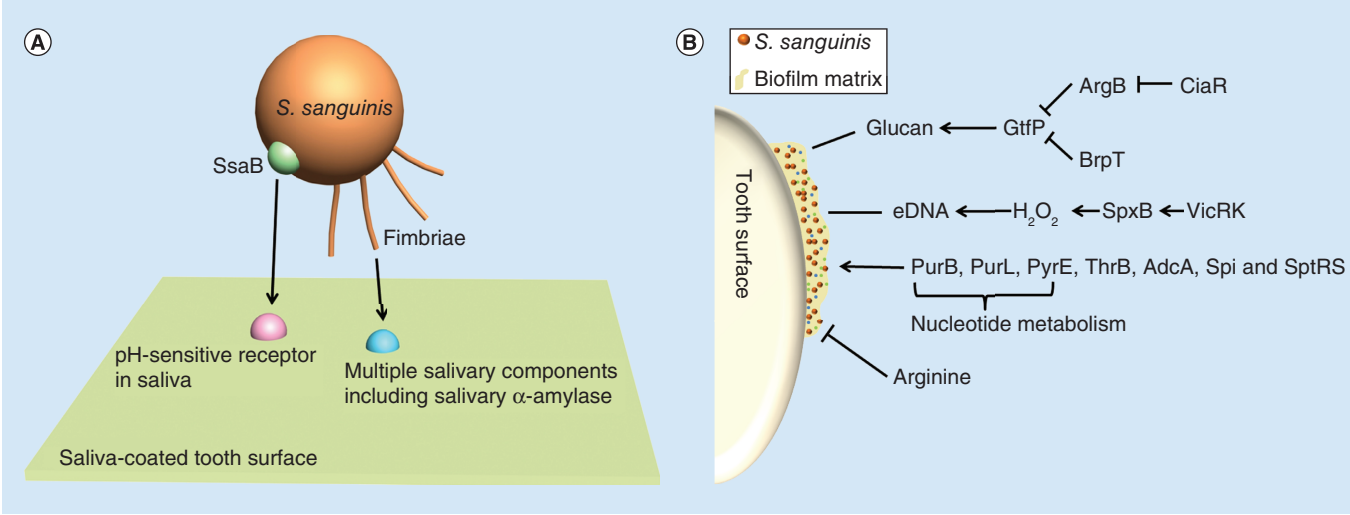

Figure 1. Impact factors of biofilm formation in *Streptococcus sanguinis*. (A) Pioneer *S. sanguinis* bacterium (orange) recognizing tooth surface salivary pellicle receptors (pink and blue) and forming initial bonds. Model shows recognition of multiple types of attachment receptors including long-range attachment, for example, fimbriae (orange) which can bind to multiple salivary components (blue) and SsaB (green) which may mediate attachment to saliva-coated hydroxyapatite via an uncharacterized pH-sensitive receptor (pink). **(B)** The response regulator CiaR of the CiaRH two-component system can inhibit the expression of ArgB which in turn leads to the upregulation of *gtfP*. Upregulation of *gtfP* can also be triggered by the deletion of BrpT. An increase in GtfP promotes the synthesis of glucan which enhances biofilm formation. The two-component system VicRK regulates the expression of pyruvate oxidase SpxB. Upregulation of SpxB will increase H_2O_2 and vice versa. Increased H_2O_2 induces cellular autolysis and subsequent eDNA release. Deletions in PurB, PurL, PyrE, ThrB, AdcA, Spi and SptRS, all show a decrease in biofilm formation. Exogenous L-arginine has been shown to decrease biofilm formation with mechanisms unknown.

The maturation of *S. sanguinis* biofilm

In most biofilms, <10% of the dry mass is composed of microorganisms, while the biofilm matrix can account for the remainder [40]. The most important components of biofilm matrix are polysaccharides, proteins, nucleic acids and lipids, which mediate cell–cell and cell–surface adhesion to form cohesive, 3D polymeric networks [40].

In *S. sanguinis*, glucans are the main biofilm polymer formed in the presence of sucrose and are composed mostly of α-1,6-linked and α-1,3-linked glucose [57]. The linkage of glucans made in the presence of sera, starch hydrolysates and dextran are different from that of control glucan [57]. The biofilm formation ability of *S. sanguinis* differs dramatically depending on the growth medium used [58]. These studies indicate that different media components can affect glucan levels and structure, affecting biofilm formation. In addition, the oxygen concentration also affects biofilm formation even when cultured in the same medium [58], perhaps due to changes in metabolic pathways affecting glucan biosynthesis.

Glucosyltranferases are responsible for the synthesis of adhesive glucans from sucrose [59]. The genome annotation shows that *S. sanguinis* SK36 contains two *gtf* genes, *gtfA* and *gtfP* [25], of which *gtfP* is the only gene shown to produce glucan, and it has also been reported to promote the adherence of *S. sanguinis* to saliva-coated hydroxyapatite and to increase biofilm formation (Figure 1B) [60–62]. The deletion of *gtfP* decreases the production of both water soluble glucan and water insoluble glucan [61] and as a result reduces biofilm formation [60–62]. The response regulator CiaR of the CiaRH two-component system and a transcriptional regulator BrpT have been reported to modulate the expression of the *gtfP* gene [61,62]. The deletion of *ciaR* reduces the transcription of *gtfP* through upregulating arginine biosynthesis genes, especially *argB*, leading to a defective biofilm formation [62]. The Δ*brpT* mutant promotes the expression of *gtfP* and displays increased biofilm formation ability [61]. These studies suggest that *gtfP* is a key gene for glucan synthesis and biofilm formation in *S. sanguinis*. However, because glucan production is affected by media composition [57,58], other genes may participate in glucan biosynthesis and biofilm formation.

Extracellular DNA (eDNA) is another essential component for biofilm formation in many species, including *Pseudomonas aeruginosa* [63] and *Staphylococcus aureus* [64]. In *S. sanguinis*, H_2O_2 produced by SpxB is able to cause DNA release which induces cell aggregation [65]. This phenomenon can be attenuated by DNase I treatment, indicating that eDNA promotes cell–cell binding and may contribute to the maturation of biofilms [65]. A *vicK* knockout mutation inhibits H_2O_2 production and eDNA release, which decreases biofilm formation [66]. In this

study, when biofilms were cultured in brain heart infusion (BHI) medium with 1% sucrose for 2 or 4 h, DNase I treatment significantly decreased biofilm formation [66], while another study reported that DNase I treatment had no effect when added after biofilms were allowed to form in biofilm medium for 24 h [62]. Although some studies showed that reduction of eDNA release and the decrease of biofilm formation appeared simultaneously in Δ*nox* or Δ*ccpA* mutants, the authors did not present direct evidence to show linkage between these two phenomena [67,68]. The different results may be due to the conditions used for biofilm formation, or from the duration of growth prior to addition of DNase I. It is possible that eDNA may play a more important role before the synthesis of a large amounts of glucans or in an environmental condition that lacks carbon sources required for glucan synthesis.

L-arginine can be secreted by salivary glands [69]. There are several studies showing that L-arginine decreases biofilm formation in *Streptococcus mutans* [70–72]. Zhu *et al.* reported that the biofilm formation of *S. sanguinis* was also reduced by exogenous L-arginine [62]. Furthermore, the deletion of genes involved in the arginine biosynthesis pathway promoted the expression of *gtfP*, increased the production of water insoluble glucan and enhanced biofilm formation in *S. sanguinis* [62]. However, the mechanisms by which exogenous L-arginine and arginine biosynthesis impact the expression of *gtfP* are not clear. Additionally, the question also remains whether or not exogenous arginine affects *S. mutans* and *S. sanguinis* biofilm formation by the same mechanism.

Several genes have been reported to impact biofilm formation by unknown mechanisms in *S. sanguinis*, including *purB*, *purL*, *pyrE*, *thrB*, *adcA*, *spi*, *sptR* and *sptS* [58,73,74]. It is interesting that genes (*purB*, *purL* and *pyrE*) related to nucleotide biosynthesis are involved in the biofilm formation network. It has been well studied that several nucleotides, such as cyclic di-GMP, cyclic di-AMP, cAMP and (p)ppGpp are widely used as small molecular signals for modulating biofilm formation in other bacteria [75–78]; however, this has not been demonstrated in *S. sanguinis*. More research should be done to explore whether any of these nucleotides regulate biofilm formation in *S. sanguinis*.

The relationship of *S. sanguinis* with dental caries

Description of dental caries

Dental caries is a chronic, transmissible disease that results in the demineralization of dental hard tissues [79,80]. It is caused by attachment of certain microbes to tooth surfaces through formation of the biofilm known as dental plaque, followed by metabolism of sugar into organic acids and, as a result, dissolution of enamel [17]. There are several genera of bacteria capable of inducing the acidic environment in oral biofilms, such as *mutans* streptococci [81], *Lactobacillus* spp. [82,83], *Bifidobacterium* spp. [84] and *Actinomyces* spp. [85]. *Candida albicans* also significantly contributes to caries pathogenesis [83,86–88]. Although not present in every case [89,90], *S. mutans* is one of the most common acid producers and is a significant impact factor in most cases of caries [20,21,85,91–93]. It is worth noting that there is always a complex biofilm present and caries is driven through the action of multiple species in many cases [14,20,93].

Evidence of inverse association of *S. sanguinis* with dental caries

By comparing colony numbers of caries and caries-free samples, Becker *et al.* determined that *S. sanguinis* was the only species identified that was significantly associated with dental health when comparing caries-active and caries-free children [93]. With the development of sequencing technologies, more and more 16S rRNA gene sequencing and metagenomic studies have been performed to discover bacterial species positively or negatively related to dental caries. *Streptococcus sanguinis* has been frequently if not consistently associated with oral health in these studies [13,14]. It has an inverse relationship with bacterial species that are caries associated and may have an antagonistic effect against cariogenic species [13–14,20–21]. *Streptococcus mutans* in particular is a well-studied example of a cariogenic species that competes with *S. sanguinis*.

Antagonism between *S. sanguinis* & *S. mutans*

In one study, oral colonization of infants with *S. sanguinis* was correlated with a significant delay in colonization with *mutans* streptococci [27]. The phenomenon of antagonism between *S. sanguinis* and *S. mutans* was first described in 1976 [94]. Kreth *et al.* further illustrated that the outcome of the interaction *in vitro* was dependent on environmental conditions, such as cell density, nutritional availability and pH [95]. When *S. sanguinis* and *S. mutans* were inoculated onto half-strength BHI plates, the previously inoculated species could inhibit the growth of the later inoculated species, but simultaneous inoculation by both species resulted in coexistence [95]. Furthermore, the authors showed that H_2O_2 produced by *S. sanguinis* repressed the growth of *S. mutans* [95], a conclusion that has also been supported by clinical research and *in silico* analysis [96,97]. Conversely, mutacins I and IV secreted by *S.*

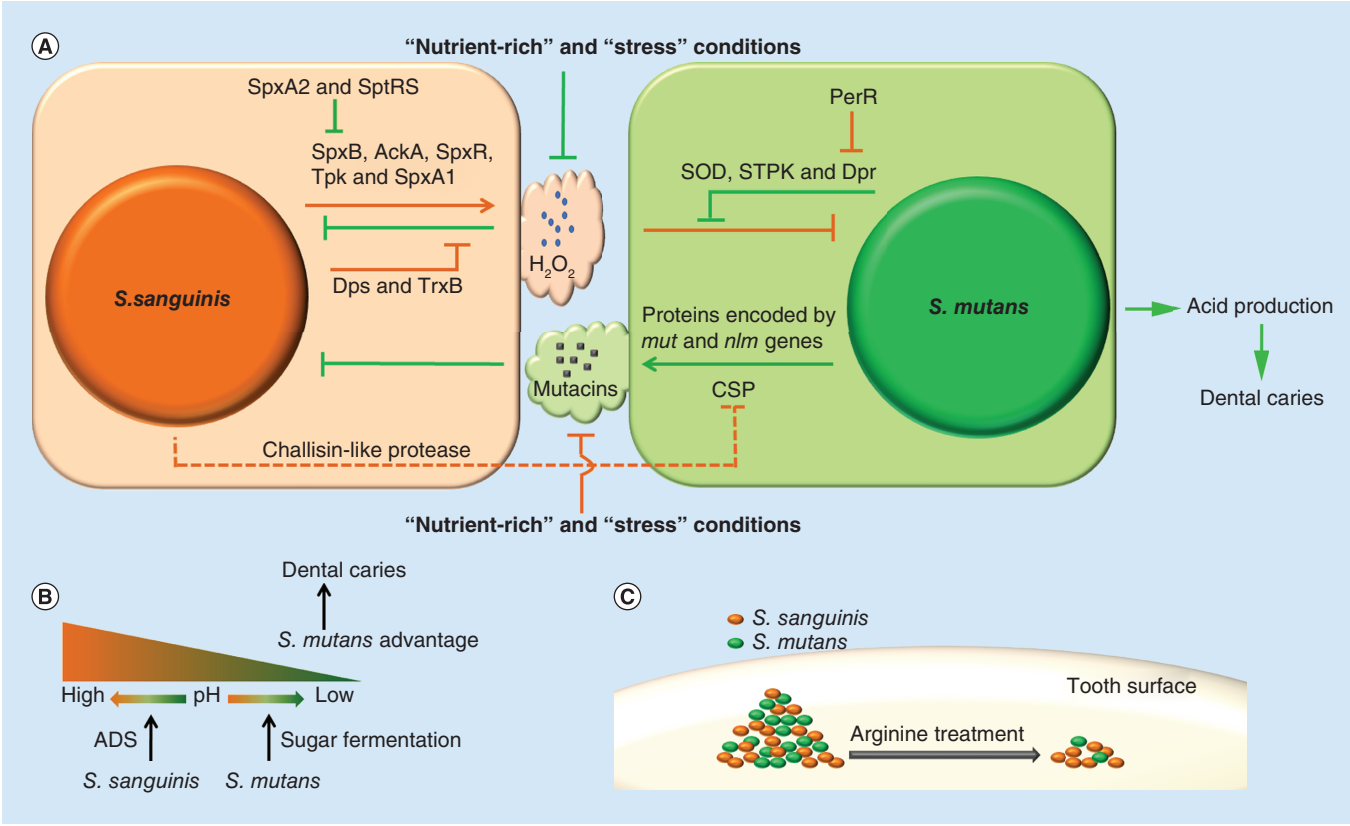

Figure 2. Mechanisms of antagonism between *Streptococcus sanguinis* and *Streptococcus mutans*. (A) H_2O_2 generated by *Streptococcus sanguinis* inhibits the growth of *Streptococcus mutans* and itself. Enzymes for reactive oxygen species degradation are produced by both species to increase their H_2O_2 resistance. Mutacins are synthesized by *S. mutans* to suppress the growth of *S. sanguinis*. CSP of *S. mutans* is necessary for mutacins production and can be inactivated by *S. sanguinis*. **(B)** *S. mutans* can generate acids from fermentable sugars to induce dental caries. However, the pH homeostasis may be maintained by the arginine deiminase system of *S. sanguinis* to prevent against dental caries. **(C)** L-arginine treatment decreases the biomass of *S. mutans* more than that of *S. sanguinis*. ADS: Arginine deiminase system; CSP: Competence-stimulating peptide; SOD: Superoxide dismutase; STPK: Serine/threonine protein kinase.

mutans suppressed the survival of *S. sanguinis* (Figure 2A) [95]. Because of defects in mutacin and H_2O_2 production, the competition between *S. sanguinis* and *S. mutans* disappeared in 'nutrient-rich' (BHI plus 1% sucrose, buffered to pH 7) and 'stress' (BHI at pH 5.5) conditions [95]. The 'nutrient-rich' condition may provide more carbon sources for glucan and acid production in *S. mutans* and facilitate its growth. At the same time, the inhibition of *S. sanguinis* H_2O_2 production in the 'nutrient-rich' condition may be another reason for an overgrowth of *S. mutans* and may contribute to the association of a high sugar diet with dental caries [1]. However, the mechanisms of mutual inhibition are not completely understood.

H_2O_2 is a type of reactive oxygen species (ROS) and can cause serious damage to cellular macromolecules, including proteins and DNA. In *S. sanguinis*, SpxB is a pyruvate oxidase that converts pyruvate to acetyl phosphate and H_2O_2 [98]. Oxygen is consumed in this reaction [98–101]. In addition, several other genes (*ackA*, *spxR*, *tpk* and *spxA1*) are also involved in the generation of H_2O_2 by mechanisms that are unknown but are likely related to SpxB activity [102,103], while *spxA2*, *sptR* and *sptS* suppress expression of the *spxB* gene and inhibit H_2O_2 production [74,104].

H_2O_2 produced by *S. sanguinis* is not only harmful to *S. mutans* but can also induce autolysis in *S. sanguinis* [65,105]. Because of the resulting release of eDNA, these genes may also impact biofilm formation. Dps and TrxB have been shown to contribute to H_2O_2 resistance in *S. sanguinis* [106]. Dps is a ferritin-like iron-binding protein that likely prevents the generation of toxic hydroxyl radicals produced by the interaction of Fe^{2+} with H_2O_2, thereby protecting cells from oxidative damage [107]. TrxB is a thioredoxin reductase that reduces oxidized thioredoxin [108]. Because thioredoxin participates in the formation of reduced disulfide bonds in oxidized proteins, TrxB attenuates the ROS damage [108]. The deletion of both *dps* and *trxB* severely decreased H_2O_2 resistance in *S. sanguinis* [106]. However,

deletion of the gene encoding the superoxide dismutase SodA did not affect H_2O_2 resistance in *S. sanguinis*, while the same mutation decreased H_2O_2 resistance in *S. gordonii* [106]. In addition, a glutathione peroxidase BasA was predicted to be a gene responsible for the degradation of H_2O_2 in *S. sanguinis* [97].

In *S. sanguinis*, genes related to the generation of H_2O_2 (*spxB*, *ackA*, *spxR*, *tpk* and *spxA1*) are all essential for repressing the growth of *S. mutans* [102,103]. To protect against H_2O_2 damage, *S. mutans* also has a H_2O_2 resistance system [109,110]. As in *S. sanguinis*, Dpr (a Dps-like protein) plays an important role in the resistance of *S. mutans* to ROS [107]. A Δ*dpr* mutant is hypersensitive to H_2O_2 and more readily killed by *S. sanguinis* and *S. gordonii* [109,110]. When co-cultured with *S. gordonii*, Dpr production is increased in *S. mutans* to protect against H_2O_2 damage [109]. The expression of *dpr* is negatively regulated by the peroxide regulator PerR [110]. As a result, deletion of *perR* promotes *dpr* expression and reduces susceptibility to H_2O_2 [110]. Superoxide dismutase (SOD) and a eukaryotic-type serine/threonine protein kinase also contribute to the ability of *S. mutans* to deal with ROS damage and coexist with *S. sanguinis* [110]. Since H_2O_2 producers such as *S. sanguinis* are prevalent in the oral cavity, Dpr, PerR, SOD and serine/threonine protein kinase may be essential for *S. mutans* survival in the presence of early colonizing oral streptococci.

As mentioned above, *S. mutans* produces mutacins that inhibit the growth of *S. sanguinis* and some other early colonizing oral streptococci [111]. For more information about mutacins, the interested reader is directed to the following review on this topic [112]. *Streptococcus sanguinis* has been shown to reduce mutacin production by inactivating the *S. mutans* competence-stimulating peptide (CSP), a quorum sensing signal inducing mutacin gene expression [113]. The mechanism by which this occurs in *S. sanguinis* may be similar to that of *S. gordonii*, which produces a challisin-like protease that degrades *S. mutans* competence-stimulating peptide [113].

Streptococcus mutans and other acidogenic organisms can generate acids from fermentable sugars, which are responsible for the pathogenesis of dental caries (Figure 2B) [114–116]. Because *S. sanguinis* is more sensitive to acidic conditions than *S. mutans* [117], the acid microenvironment condition generated by aciduric bacteria may result in decreased abundance of *S. sanguinis* prior to or concomitant with the development of dental caries. In the oral commensal community, some oral bacteria produce alkali from the metabolism of arginine via the arginine deiminase system (ADS), which protects against caries caused by *S. mutans* and other aciduric bacteria [118,119]. *S. sanguinis* is the most prevalent species in the oral cavity that contains the ADS [120]. It may utilize the ADS to maintain pH homeostasis and gain an advantage in competing with *S. mutans*.

Previous studies reported that a higher concentration of L-arginine existed in the saliva of dental caries-free individuals than that of caries-active individuals [121,122], which suggested that humans may use L-arginine as a weapon to fight against the acid producers in oral biofilm. L-arginine treatment directly reduces the amount of insoluble extracellular polysaccharide production [71,72], which significantly altered the architecture of the biofilm in *S. mutans* [72]. Although biofilm formation by *S. sanguinis* is also repressed by exogenous L-arginine [62,123], the addition of arginine reduces the biomass of *S. mutans* more than that of *S. sanguinis* within dual-species biofilms (Figure 2C) [123]. In other words, the L-arginine treatment enriches for *S. sanguinis* but decreases the abundance of *S. mutans*. In addition, treatment with 15 mg/ml of L-arginine (a clinically effective concentration) decreased the proportion of *S. mutans*, increased the proportion of *S. gordonii* and maintained the *Actinomyces naeslundii* proportion within biofilms [70]. Moreover, a recent study showed that combinatory use of arginine with fluoride could increase *S. sanguinis* levels further and suppress *S. mutans*, and thus significantly retard the demineralizing capability of saliva-derived oral biofilm [124]. These studies indicate that L-arginine treatment may be a promising ecological approach to caries management.

In summary, both epidemiological and *in vitro* studies suggest that *S. sanguinis* may suppress the generation of dental caries. Nonetheless, we still lack direct evidence to support a definite conclusion concerning the role of *S. sanguinis* in dental caries. The oral microbiome is exposed to a variety of environmental conditions that may affect this relationship. As an example, the abundance of *S. sanguinis* is decreased by smoking [125], which may also attenuate its ability to compete against pathogens. It will be of interest to identify new environmental factors leading to dysbiosis. Moreover, a better understanding of these factors may provide new strategies to prevent or treat dental caries.

The association of *Streptococcus sanguinis* with periodontitis

Description of periodontitis

Periodontitis is an inflammatory disease that compromises the integrity of the tooth-supporting tissues, including gingiva, periodontal ligament and alveolar bone. Over time periodontitis leads to periodontal ligament destruction,

loss of supporting alveolar bone and loosening of teeth [1]. In addition, periodontitis is associated with atherosclerosis, adverse pregnancy outcomes, rheumatoid arthritis, aspiration pneumonia and cancer [126–131]. Periodontitis is caused by persistent exposure of periodontal tissue to an ecologically unbalanced polymicrobial dental-plaque community [18,132–133]. In this community, periodontitis-associated microorganisms synergistically interact for enhanced colonization, nutrient procurement and persistence in an inflammatory environment [133,134]. Several bacterial species have been reported to participate in the periodontal disease pathogenesis, such as the 'red-complex' bacteria (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) [16,135–139], *Aggregatibacter actinomycetemcomitans* [138–140] and the orange complex bacteria (*Fusobacterium nucleatum*, *PeptoStreptococcus micros*, *Prevotella intermedia*, *Prevotella nigrescens*, *Eubacterium nodatum* and *Streptococcus constellatus*) [16,139,141,142]. The interested reader is directed to the following review on newly discovered pathogens associated with periodontitis [143]. More details about the pathogenesis of periodontitis can be found in several excellent reviews [18,141,144,145].

Evidence of the association of *S. sanguinis* with periodontitis

During the transition from periodontal health to periodontal disease, the components of microbiomes shift from mostly Gram-positive to mostly Gram-negative species [146]. *Streptococcus sanguinis* is one of the most important Gram-positive commensal bacteria in the oral cavity [147]. 16S rRNA sequencing data suggest that *S. sanguinis* has significantly increased abundance in healthy versus diseased subgingival microbiome samples and is a constituent of the core microbiome in periodontal health [15–16,22].

Gingival epithelial cells are stimulated by oral commensal bacteria to produce IL-8 and β-defensins, which may protect periodontal tissue against periodontitis-associated pathogens [148–155]. As a major component of the oral commensal microbiome, *S. sanguinis* may participate in this process. At the same time, the host response induced by *S. sanguinis* is much weaker than that induced by *P. gingivalis* or *F. nucleatum*. This may be one mechanism by which the host benefits from *S. sanguinis* competing with these pathogens in the oral cavity [156]. More details about host immune responses to oral microbial flora are shown in the following review [157].

Interaction of *S. sanguinis* with periodontitis-associated pathogens

Although suggested to be a health-associated bacterium by 16S rRNA sequencing studies, *S. sanguinis* may be a potential binding target for the localization of pathogens within dental plaque [158,159]. *Porphyromonas gingivalis* is thought to be the major etiologic agent in chronic periodontitis [135,160]. The fimbriae of *P. gingivalis* are able to bind to several streptococcal species, including *S. sanguinis*, *Streptococcus oralis*, *S. gordonii* and *Streptococcus parasanguinis* (Figure 3) [158]. Western blot analysis demonstrates that purified recombinant fimbrillin of *P. gingivalis* binds to cell-surface glyceraldehyde-3-phosphate dehydrogenases of these streptococci, indicating that *P. gingivalis* may utilize fimbriae to attach to these bacteria for biofilm formation [158]. *Streptococcus sanguinis* inhibits the transcription of the *mfa1* gene. This gene encodes a structural subunit of *P. gingivalis*-short fimbriae that mediates coadhesion between *P. gingivalis* and *S. gordonii* [161,162]. It is not clear whether Mfa1 promotes the attachment of *P. gingivalis* to *S. sanguinis*.

F. nucleatum is a periodontal pathogen associated with a wide array of human diseases involving chronic and aggressive periodontitis [163]. It has been reported to coaggregate with many oral microorganisms, such as *S. sanguinis*, *S. mutans* and *P. gingivalis* [159]. Moreover, it enhances the coaggregation between *S. sanguinis* and *P. gingivalis* [159]. Later studies discovered that an arginine-inhibitable adhesin RadD of *F. nucleatum* enhances the coaggregation with the Gram-positive 'early oral colonizers' including *S. sanguinis*, *Streptococcus oralis*, *S. gordonii* and *Actinomyces naeslundii* [164]. Deletion of *radD* decreases *S. sanguinis–F. nucleatum* dual-species biofilm formation, suggesting that the RadD adhesin plays an essential role in interspecies adherence and multispecies biofilm formation [164]. Another gene, *aid1*, has also been shown to enhance coaggregation [165]. Additionally, Aid1 function is dependent on RadD and is abolished in the presence of arginine [165]. An *in vitro* mixed-species oral microbiota system can be stimulated by *F. nucleatum* to produce H_2O_2, subsequently killing *F. nucleatum* [166]. However, H_2O_2 production and its killing effect are reduced when *F. nucleatum* is allowed to form coaggregates with *S. sanguinis* prior to addition to the mixed community [166]. Because a Δ*radD* mutant is defective in coaggregation, the H_2O_2 damage of the mutant is not attenuated by exposure to *S. sanguinis* [166]. These studies suggest that *S. sanguinis* may help *F. nucleatum* to survive in the oral cavity. Since the activities of both RadD and Aid1 are inhibited by L-arginine, this raises the interesting question of whether *S. sanguinis* increases L-arginine biosynthesis in response to *F. nucleatum* attachment.

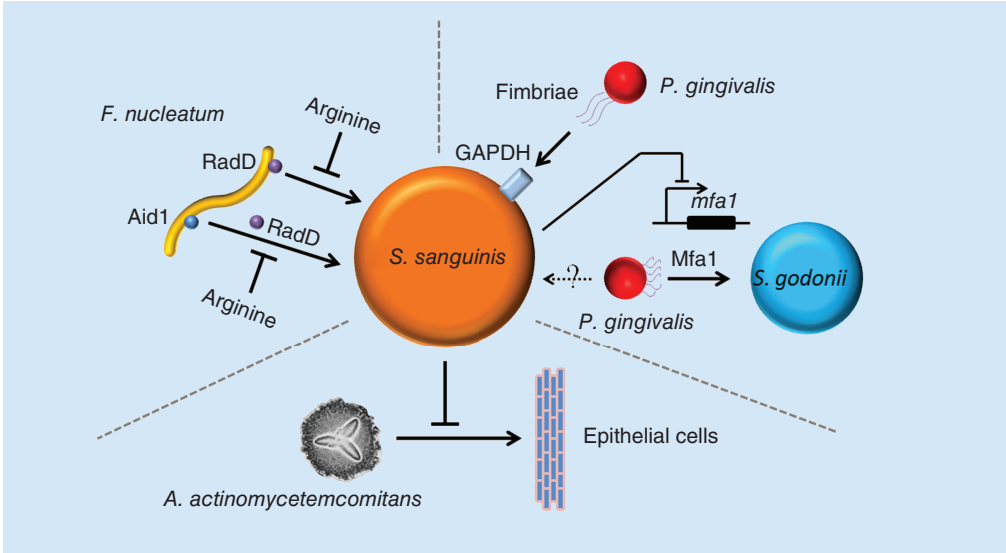

Figure 3. Interaction of *Streptococcus sanguinis* with periodontitis-associated pathogens. *Fusobacterium nucleatum* can attach to *Streptococcus sanguinis* via RadD or Aid1. Aid1 attachment is mediated by RadD. Both interactions can be inhibited by the presence of arginine. *Porphyromonas gingivalis* can attach to *S. sanguinis* by fimbriae to surface glyceraldehyde-3-phosphate dehydrogenases receptors. *P. gingivalis* can attach to *Streptococcus gordonii* using small fimbriae made from the adhesin Mfa1. Perhaps this mechanism is also utilized for attachment to *S. sanguinis*. However, it is known that *S. sanguinis* can suppress the expression of the *mfa1* gene. *Aggregatibacter actinomycetemcomitans* colonization of epithelial cells in a flow chamber will be repressed by the colonization of *S. sanguinis*.

Aggregatibacter actinomycetemcomitans is another well-studied pathogen associated with periodontal diseases such as localized aggressive periodontitis and, consequently, bone resorption [167,168]. Under flow conditions, the colonization of soft tissue surfaces by *A. actinomycetemcomitans* is reduced by several streptococci species, and especially by *S. sanguinis*, indicating the potential beneficial effects of *S. sanguinis* for preventing periodontitis caused by *A. actinomycetemcomitans* [169].

Welch *et al.* observed the spatial organization of complex natural microbiomes in oral samples by FISH [170]. Streptococci occupy a broad range of oral habitats [170]. At the periphery of the dental plaque samples, *Streptococcus* spp. appears at the distal tips of *Corynebacterium* spp. filaments, together with *Haemophilus* spp./*Aggregatibacter* spp. and *Porphyromonas* spp., and seems to mediate the association of *Porphyromonas* spp. with *Haemophilus* spp./*Aggregatibacter* spp. [170]. In addition, streptococci may create a microenvironment rich in CO_2, lactate and acetate and low in oxygen, which facilitates the survival of *Aggregatibacter* spp., *Capnocytophaga* spp., *Fusobacterium* spp. and *Leptotrichia* spp. at the inner layer of dental plaque [170]. This study gives direct evidence of colocalization between *Porphyromonas* spp. and *Streptococcus* spp. However, it is possible that the *Porphyromonas* spp. here does not include *P. gingivalis* because the periphery of dental plaque is recognized as a presumably aerobic environment that would not be suitable for the growth of *P. gingivalis* [170]. On the other hand, streptococci were not only visible at the bottom, but also appeared at the periphery of dental plaque, suggesting that streptococcal species are a spatially available target for initial attachment of pathogens.

Given our limited knowledge, we cannot draw any firm conclusions about the role of *S. sanguinis* in the pathogenesis of periodontitis. Based on 16S rRNA sequencing data, *S. sanguinis* seems to be associated with periodontal health. However, it is not clear whether *S. sanguinis* actually promotes oral health or merely survives better at healthy sites, making it an indicator of oral health rather than a cause. Moreover, it is also a binding target for *P. gingivalis* and *F. nucleatum*. In current periodontitis models, pathogens exist in the biofilm formed on tooth surfaces inside of the periodontal pocket, and then attack oral epithelial cells nearby [18,144]. Because of the complex conditions in the oral cavity, such as salivary flow, immune system response and bleeding, it is difficult to establish an *in vivo* model that begins with a biofilm of *S. sanguinis* formed in periodontal pocket and then entails an input pathogen to induce periodontitis. To simplify the problem, the first question we need to clarify

is whether the colonization of *S. sanguinis* on tooth surfaces impacts the attachment and biofilm formation of periodontitis-associated pathogens. The second question is how *S. sanguinis* responds to pathogen attachment.

Role of *S. sanguinis* biofilm formation in infective endocarditis

Apart from its role as a primary colonizer in the oral cavity, *S. sanguinis* is best known as a cause of infective endocarditis, an infection of the valves or endocardial lining of the heart [171]. Indeed, *S. sanguinis* was first known as '*Streptococcus* s.b.e.,' for 'subacute bacterial endocarditis', and was recognized as a cause of endocarditis well before it was identified as an inhabitant of the oral cavity [172]. In a recent review, oral streptococci including *S. sanguinis* were recognized as one of the top three causes of endocarditis, alongside two other genera of Gram-positive cocci: staphylococci and enterococci [173].

Endocarditis begins when the causal agent enters the bloodstream to cause a bacteremia and is then carried by the blood to the heart. For oral streptococci, attention is often focused on invasive dental procedures as a cause of bacteremia, although oral streptococci have also been identified as the second-most frequent cause of endocarditis in intravenous drug users and ICU patients [173]. After reaching the heart, *S. sanguinis* must then adhere to the endocardium. Given the importance of biofilm formation for adhesion in the oral cavity, it would be reasonable to suspect that biofilm formation might be important for adhesion to endocardial surfaces as well. Indeed, endocarditis is often considered an example of a biofilm-mediated disease [174]. There are likely at least three reasons for this association. First, endocarditis occasionally accompanies infections of implanted cardiac devices, such as pacemakers or defibrillators [171]. In these cases, infections are typically caused by species that have been shown to produce biofilms in systemic infections, such as *S. aureus* or *Staphylococcus epidermidis*, and biofilms are often found [175]. However, these infections are typically not caused by *S. sanguinis* or other oral streptococci [176]. Second, even when infection is of the endocardium rather than an implanted device, some causative agents such as *C. albicans* likely produce biofilms [177]. Third, the typical lesion found in streptococcal (as well as staphylococcal and enterococcal) endocarditis, which is called a 'vegetation' [171,178], has some properties in common with a biofilm. Vegetation is a nodule that is composed primarily of platelets and fibrin. In animal models, and presumably in many human patients, sterile vegetations form in response to endocardial damage and precede infection, which explains why rheumatic heart disease, congenital heart conditions that create turbulent blood flow, and certain cardiac surgical procedures such as valve replacements put patients at high risk for subsequent endocarditis [171,179]. In a previous study employing an oral streptococcal isolate and a rabbit model of endocarditis in which cardiac catheterization was used to create minor endocardial damage prior to bacterial inoculation [178], infected vegetations were found to be comprised of bacterial microcolonies enclosed within a matrix of platelets and fibrin. This matrix is likely responsible for protecting embedded bacteria from phagocytic killing [178] and for the relatively long duration of antibiotic treatment that is required for cure [180]. These properties are typical of biofilm infections. Nevertheless, as indicated above, biofilm has been defined as an 'aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance adhere to each other and/or to a surface' [181]. Because the platelets and fibrin are not self produced by the infecting bacterium, a vegetation does not fit this definition of a biofilm.

Despite this lack of evidence, or perhaps because of it, other approaches have been used to address whether biofilm formation fitting the standard definition might be important for endocarditis causation, particularly in the earliest stages. Ge *et al.* [58] examined biofilm formation in a library of 800 signature-tagged mutants of *S. sanguinis* strain SK36 that had been used previously for an endocarditis virulence screen [182]. The later study identified eight mutants that were defective for biofilm formation in a standard crystal violet assay. Four of these mutants appeared to have reduced endocarditis virulence in the original screen, and the other four did not. Interestingly, five of the biofilm-defective mutants had insertions in genes related to purine or pyrimidine synthesis; the reduced-virulence mutants included two with insertions in the *purB* gene and the normal-virulence mutants included two with an insertion in the *purL* gene and one with an insertion in *pyrE*. The retention of virulence in one of the *purL* mutants and the *pyrE* mutant was confirmed, indicating that *in vitro* biofilm formation could be severely reduced without affecting endocarditis virulence in an animal model. Similar results have been obtained previously by another group working with the closely related oral species, *S. gordonii* [183]. Later studies employing isogenic mutants of *Enterococcus faecalis* [184] or analysis of biofilm formation in clinical strains isolated from patients with or without endocarditis also failed to uncover a correlation between biofilm-forming ability and endocarditis causation in *E. faecalis* [185], *S. epidermidis* [186] and *S. aureus* [187]. Finally, Ge *et al.* [58] showed previously that *in vitro* biofilm formation by *S. sanguinis* was far more efficient in the presence of sucrose because sucrose is converted into

extracellular glucan [62]. The near absence of sucrose in the blood of normal people [188,189] would be expected to preclude glucan formation on infected heart valves.

The results presented above suggest that *S. sanguinis* endocarditis causation is not dependent upon biofilm formation. There are, to be sure, some caveats to this conclusion. Leuck *et al.* [190] reported that biofilm formation in a standard *in vitro* assay did not correlate with the ability of *E. faecalis* strains to form biofilms on porcine heart valve explants. It is possible that a similar situation exists with *S. sanguinis*. Biofilm formation in the cardiac environment could conceivably occur through mechanisms distinct from biofilm formation in the oral cavity or *in vitro*. Nevertheless, at present, there is no evidence for a role of biofilm formation in *S. sanguinis*-mediated endocarditis.

Conclusion & future perspective

There are several reasons why *S. sanguinis* is an ideal model organism for research on the interaction between commensal bacteria and pathogens in biofilms. First, *S. sanguinis* is highly abundant in a broad range of habitats in the oral cavity [15,20,29–31]. Second, the genome of *S. sanguinis* strain SK36 has been sequenced and this strain is highly amenable to genetic manipulation [25,191]. Third, many studies suggest that *S. sanguinis* is significantly related with oral health [13–16,89].

Current studies suggest that *S. sanguinis* competes with *S. mutans*, which may lessen or prevent dental caries. However, as a pioneering colonizer, *S. sanguinis* may also facilitate the attachment of succeeding pathogens. In addition, certain environmental conditions seem to affect the ability of *S. sanguinis* to maintain an ecologically balanced biofilm in the oral cavity. To more clearly define the role of *S. sanguinis* in oral health, the first requirement is to establish reasonable models of interaction between commensal bacteria and pathogens for these diseases. A suitable visualization technology is essential to distinguish different bacteria in dual- or multispecies biofilms. It has been reported that codon-optimized fluorescent proteins are available for continuous visualization of *S. sanguinis* and *S. mutans* in microaerobic conditions [62,192]. Luciferase enzymes will facilitate *in vivo* studies [193]. FISH can be used for multispecies biofilm observation [170]. Furthermore, there is significant variation in phenotypes within species of oral *S. sanguinis* [120,194]. Analysis of phenotypic and genotypic variations of *S. sanguinis* in different oral samples should be an effective method of exploration of the mechanisms by which *S. sanguinis* survives in different environmental conditions.

Both dental caries and periodontitis are caused by microbial dysbiosis in the oral cavity. It has been well established that the homeostasis of microbial communities is tightly related with health. However, much more research is aimed at examination of pathogenesis than commensalism. Our inattention to commensal microorganisms may cause many serious problems. For example, the abuse of broad-spectrum antibiotic therapy leads to dysbiosis in the oral cavity and constitutes the major risk factor for invasive candidiasis [195]. We have very limited knowledge concerning the mechanisms leading to microbial dysbiosis.

Future studies on beneficial commensal microorganisms should focus on two things – mechanisms by which commensal microbiota interact with pathogens and the factors leading to microbial dysbiosis. It is necessary to clarify the relationship between environment, beneficial commensal microorganisms, pathogens and diseases. A further understanding of commensal microbiomes will afford new strategies for not only management but prevention of oral diseases.

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Author contributions

All authors wrote, reviewed and discussed the manuscript.

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Executive summary

- Dental caries and periodontitis are two most prevalent diseases in the oral cavity. They are caused by the dysbiosis of oral microbiomes.
- *Streptococcus sanguinis* is a pioneering colonizer and a key player in oral biofilm development.
- The initial attachment of *S. sanguinis* is facilitated by its fimbriae and adhesins. The production of glucans and eDNA promotes the maturation of *S. sanguinis* biofilm.
- Epidemiological studies suggested that *S. sanguinis* may suppress the generation of dental caries. *In vitro* studies showed the competition between *S. sanguinis* and *S. mutans*, a most common cariogenic species.
- The results from 16S rRNA sequencing indicated that *S. sanguinis* might be associated with periodontal health. However, *in vitro* studies exhibited that *S. sanguinis* may also facilitate the attachment of succeeding pathogens associated with periodontitis.
- In contrast to the situation in the oral cavity, there is as yet no evidence that biofilm formation is important for *S. sanguinis* in the cardiac environment in relation to infective endocarditis.
- Future studies should focus on mechanisms by which commensal microbiota interact with pathogens and the factors leading to microbial dysbiosis.

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