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Research Article

Human Serum Modulates Biofilm Formation By Strains Of Viridans Group *Streptococcus* Associated With Infective Endocarditis.

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Abstract

Oral *Streptococcus* spp. are among the most frequent bacteria associated with infective endocarditis (IE), forming biofilms on cardiac tissues. This study aimed to evaluate the contribution of serum components to the capacity of strains of oral streptococci associated with IE to form biofilms. To this end, biofilm phenotypes were determined in eight bacterial strains isolated from the bloodstream of patients with IE, which were taxonomically identified using PCR with species-specific primers as *Streptococcus sanguinis* (n=2), *Streptococcus gordonii* (n=3) or *Streptococcus salivarius* (n=2). Reference strains of each species (SK36, Challis, and NCTC 8618) were also tested. The biomass of biofilms formed during 18h was measured in 96-well plates with BHI medium with 1% sucrose (BHIS), supplemented with 10% saliva or 20% human serum. Initial stages of biofilm formation (4 h) in these culture media were also analyzed by scanning electron microcopy (SEM). Supplementation of BHIS with serum significantly increased the biofilm biomass as compared to non-supplemented BHIS in strains of *S. sanguinis* (2/3, 2.69 versus 1.85), *S. gordonii* (3/4, 1.41 versus 1.12) and *S. salivarius* (2/3; 3.36 versus 2.64) ($p<0.05$). Differently, no significant changes in biofilm formation was promote by BHIS supplementation with saliva. SEM analyzes confirmed the contributing effects of serum in biofilm formation. These data highlights the significant effect of serum in biofilm formation capacity by oral streptococci, further indicating that bacterial interactions with serum components likely contribute to cardiovascular virulence of oral streptococci.

Keywords : *Streptococcus viridans, biofilm, serum, endocarditis.*

Highlight : *Serum induces biofilm formation of Viridans group Streptococcus.*

INTRODUCTION

Infective endocarditis (IE) is an infection of the cardiac or adjacent vascular endothelium by microorganisms, mostly endogenous, accessing the bloodstream [1,2].

Staphylococcus spp. and *Streptococcus* spp. represent 80% of cases of bacterial endocarditis [3]. Viridans group *Streptococcus* [VGS], which includes species of oral streptococci, are associated with 30-50% of all cases of bacterial endocarditis [4,5].

Oral streptococci are abundant in multiple sites of the oral microbiome, accounting for up to 50% of the cultivable microbiota of the tongue and saliva and representing 80% of cultivable microorganisms of dental biofilms forming during the first 6 h of colonization [6,7]. Dental biofilms provide privileged sites of microbial access to the blood circulation due to their association with the highly vascularized periodontal tissues [7–9]. Common species of dental biofilms include *S. sanguinis* and *S. gordonii* biofilm, while other species, e.g. *S. salivarius*, are abundant not in multiple mucosal sites including the tongue surface as well as the pharynx and tonsils [10–15]. The high frequency of oral *Streptococcus* spp. in cardiac tissues and IE lesions [4,16– 18], suggests that these species have a predilection for colonizing these tissues. Strains of

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these species can express multiple mechanisms of evasion to immune surveillance in the bloodstream and cardiovascular tissues [16,19,20], but the influence of blood (or serum) components on their capacities to form biofilms remains to be investigated, a function likely associated with their capacity to adhere and to infect cardiovascular tissues contributing to the formation of vegetations [15,21–24]. Heart vegetations primarily consist of platelets, fibrin, and microorganisms, often embedded in a biofilm matrix and resembling biofilm-like formations and showing increased antibiotic tolerance compared to planktonic bacteria [25,26]. The extracellular matrix of the biofilms provides protection to bacteria against external factors, such as antimicrobial agents and the host immune responses, making biofilm control a significant clinical challenge [27,28]. In addition, transition from planktonic to biofilm lifestyle provided multiple physiological changes associated with persistence in host niches [29–32], strengthening the need of investigating bacterial interactions with serum components promoting biofilm formation.

Thus, the aim of this study was to evaluate the effect of human serum in biofilm formation capacity of strains of *S. sanguinis*, *S. gordonii* and *S. salivarius* isolated from bloodstream of patients with bacterial endocarditis. To this purpose, we applied comparative in vitro analysis of biofilms on surfaces bathed or not with human serum and saliva, a major fluid bathing oral surfaces.

MATERIAL AND METHODS

Samples of blood, serum and saliva

Blood, serum and saliva samples were collected from study approved by the Research Ethics Committee of the Dante Pazzanese Institute of Cardiology (CAAE: 67892223.9.0000.5462) according to Resolution 466/2012 of the Ministry of Health regarding research involving human. Strains were isolated from blood cultures of patients with IE treated at the Dante Pazzanese Institute of Cardiology – DPIC (São Paulo-SP, Brazil) between 2022 and 2024. Serum and saliva collected from a healthy volunteer and were used in the biofilm assays.

Culture conditions and growth curves

The strains used in this study are listed in **Table 1**. The cultures obtained were frozen at -70°C in 20% glycerol to obtain stocks. These strains were routinely grown from frozen stocks on BHI (Brain heart infusion) agar (BD Difco, USA). About six colonies of BHI agar cultures of these strains were used to inoculate BHI broth and incubated (18h at 37°C) under aerobic atmosphere containing 10% CO_2. Growth curves of the strains were in BHI, under 10% CO_2, as described below. Briefly, the strains were inoculated from adjusted inoculums of 18 h culture (for $A550_{nm}$ 0.05) in 25 mL of BHI and the cultures incubated at 37°C under an atmosphere of 10% CO_2. Aliquots of 500 µL of these cultures were removed each 1 h for determination of the absorbances ($A550_{nm}$) (Spectrophotometer, Kasvi) during the period of 8 h. Three independent growth curves were determined in duplicate for each strain.

Table 1. *Streptococcus* spp. strains isolated and used in this study.

Strain	Isolation site	Source
Streptococcus sanguinis		
S. sanguinis SK36	oral cavity	Reference strain
S. sanguinis Ss8648	blood	Dante Pazzanese Institute of Cardiology
S. sanguinis Ss13197	blood	Dante Pazzanese Institute of Cardiology
Streptococcus gordonii		
S. gordonii Challis	oral cavity	Reference strain/ ATCC 35105
S. gordonii Sg12745	blood	Dante Pazzanese Institute of Cardiology
S. gordonii Sg8774	blood	Dante Pazzanese Institute of Cardiology
S. gordonii Sg5769	blood	Dante Pazzanese Institute of Cardiology
Streptococcus salivarius		
S. salivarius NCTC 8618	oral cavity	Reference strain/ NCTC 8618
S. salivarius Sv11312	blood	Dante Pazzanese Institute of Cardiology
S. salivarius Sv13331	blood	Dante Pazzanese Institute of Cardiology

Bacterial DNA extraction

The MasterPure Gram Positive DNA Purification Kit (LGC Biosearch Technologies, England, UK) was used to extract genomic DNAs of the studied strains, following the manufacturer's recommendations. The quality and concentration of the extracted DNA assessed by determining the absorbance ratios ($A260_{nm}/A260_{nm}$) using a NanoDrop One spectrophotometer (Thermo Scientific). The integrity of the DNA samples was monitored in 1% agarose gels stained with Blue Green Loading Dye (LGC Biosearch Technologies, England, UK). The DNAs were stored at -20ºC for later use in PCR reactions.

Identification of *Streptococcus* spp. by PCR with species-specific primers

The taxonomic identification of the strains was confirmed using the classification system developed by Garnier [33]. Briefly, PCR with species-specific primers targeting the ddl gene, which encodes the enzyme involved in the biosynthesis of the cell wall D-alanine:D-alanine ligase, was applied to identify the species *S. sanguinis* (Primer I), *S. gordonii* (Primer H) and *S. salivarius* (Primer C). The primers used for each species and the expected amplicon sizes are described in **Table 2**. The strains *S. sanguinis* SK36, *S. gordonii* Challis substr CH1 and *S. salivarius* NCTC8618 were used as positive controls of the PCR reactions. The products were electrophoretically resolved in 1% agarose gels stained with Blue Green Loading Dye (LGC Biosearch Technologies, England, UK), and species identification was based on the size of the amplicons (*S. sanguinis* (374 bp), *S. gordonii* (260 bp) and *S. salivarius* (331 bp).

Table 2. Primers used in this study for the identification of clinical isolates.

Strain or target gene	Oligonucleotide	Primer Forward/Reverse (5' – 3')	Product size (bp)	Reference
S. salivarius	C	GCAGCAGTAGCAGAGACGCT/ GTCATGACTTCTGCAGGCAC	331	[33]
S. gordonii	H	GTCGATGGCGAGGATCTAGAGC/ AACAACTTCCCCTGGAAGAC	260	[33]
S. sanguinis	I	GTCGATGGCGAGGATCTAGAGC CTCTGCATTTTGACGCATGAG	374	[33]

Analysis of biofilm formation in the presence of human saliva and serum

The analysis of biofilm formation was performed according to a previous study [34] with some modifications. Briefly, in 96-well flat-bottomed polystyrene plates (Cralplast, Brazil) were added (180 µl/well) BHI medium supplemented with 1% sucrose in three conditions: BHI medium only, BHISA (BHI supplemented with 10% sterilized human saliva) or BHISO (BHI supplemented with 20% human serum). Each condition was inoculated with 20 µL/well of each strain grown in the exponential phase ($A550_{nm}$ = 0.3). The plates with final volumes of 200 µL/well in triplicate were then incubated for 18 h (37°C, 10% CO_2). The absorbance measurements were expressed as indirect measures of biofilm biomass in a microplate reader (Bio-Tek, PowerWave XS2) for determination of absorbance ($A575_{nm}$).

Analysis of biofilm formation by scanning electron microscopy (SEM)

The initial stages of biofilm formation on serum- and saliva-coated glass slides were analyzed by scanning electron microscopy (SEM), as previously described [35] with some modifications. Briefly, the biofilms of *Streptococcus* spp. strains were formed on sterilized glass coverslips (10 x 10 mm) placed in wells of 24-well microplates (Corning, USA). In each well with coverslips, 1 mL of BHI with 1% sucrose (BHIS) was added under the conditions: without treatment
(BHIS), with 10% saliva (BHIS-SA; plus 100 µL of clarified sterile saliva) and 20% serum (BHIS-SE; plus 200 µL of sterile human serum). The media were then inoculated with cultures adjusted to the same absorbance ($A550_{nm}$ ~ 0.3) and the plates incubated at 37°C for 4 h in an atmosphere of 10% CO_2. Representative images of the specimens were obtained at 2,000X magnification.

Data analysis

GraphPad Prism 8 software was used to perform statistical analyses for all data. The growth curves were compared for using ANOVA with Dunnett's post-hoc test. Measures of biofilm biomass were compared using parametric analysis by Two-way ANOVA with Tukey's post-hoc test.

RESULTS

Taxonomic identification and patterns of planktonic growth

PCR reactions for ddl confirmed that all the *Streptococcus* isolates were *S. sanguinis*, *S. gordonii* or *S. salivarius*. The integrity of genomic DNAs and PCR products obtained with each primer set are shown in **Figure 1**.

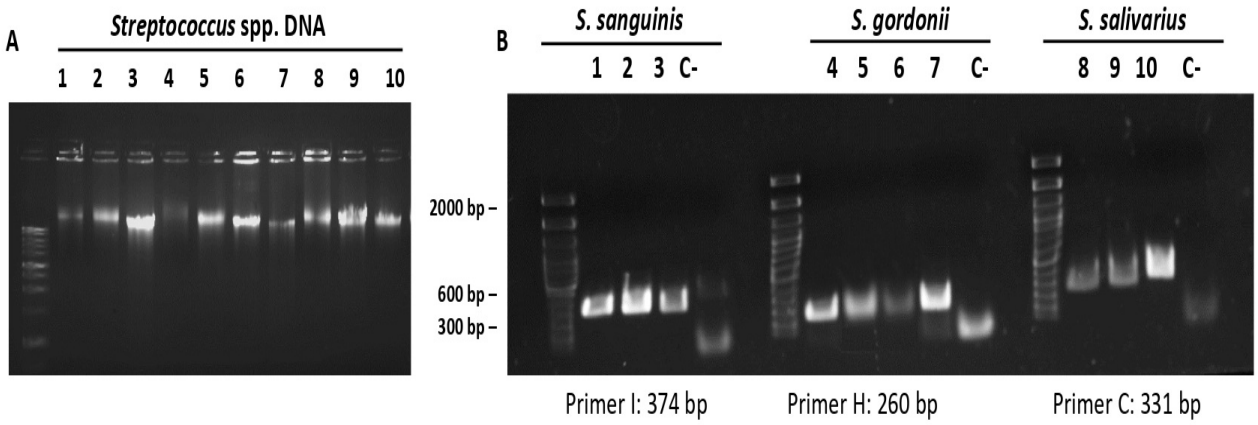

Figure 1. PCR products with arbitrary primers for *S. sanguinis* (Ss), *S. gordonii* (Sg), and *S. salivarius* (Sv) using genomic DNA from clinical isolates and reference strains of each species. Product sizes for PCR with primers I: 374 bp, H: 260 bp, and C: 331 bp on a 1% agarose gel. Legend: 1: *S. sanguinis* Sk36 (reference strain); 2: Ss8648; 3: Ss13197; 4: *S. gordonii* Challis (reference strain); 5: Sg12745; 6: Sg8774; 7: Sg5769; 8: *S. salivarius* NCTC 8618 (reference strain); 9: Sv11312; 10: Sv13331; C-: negative control (no DNA).

Comparisons of the growth curves of the *S. sanguinis* isolates (Ss8648 and Ss13177) with the reference strain SK36 **(Figure 2A)**, revealed similar growth profiles, although the growth yield of blood isolates were lower when compared to SK36 ($p<0.05$). The reduced growth rate and/or yield were more evident in the blood isolates of S. gordonii when compared to the oral reference *S. gordonii* strain Challis **(Figure 2B)**.

Larger variations in planktonic profiles were detected among *S. salivarius* strains **(Figure 2C)**. Whereas the blood isolate Sv13331 reached the log phase 3h earlier than the reference strain NCTC8618, this strain still showed a lower growth yield as compared to NCTC8618. The blood isolate Sv11313 showed the lowest growth rate and yield as compared to the other two *S. salivarius* strains. Thus, the blood strains of all the strains analyzed showed a lower growth yield in complex medium when compared to oral reference strains, although differences between blood versus reference oral strain were more evident for the *S. gordonii* species.

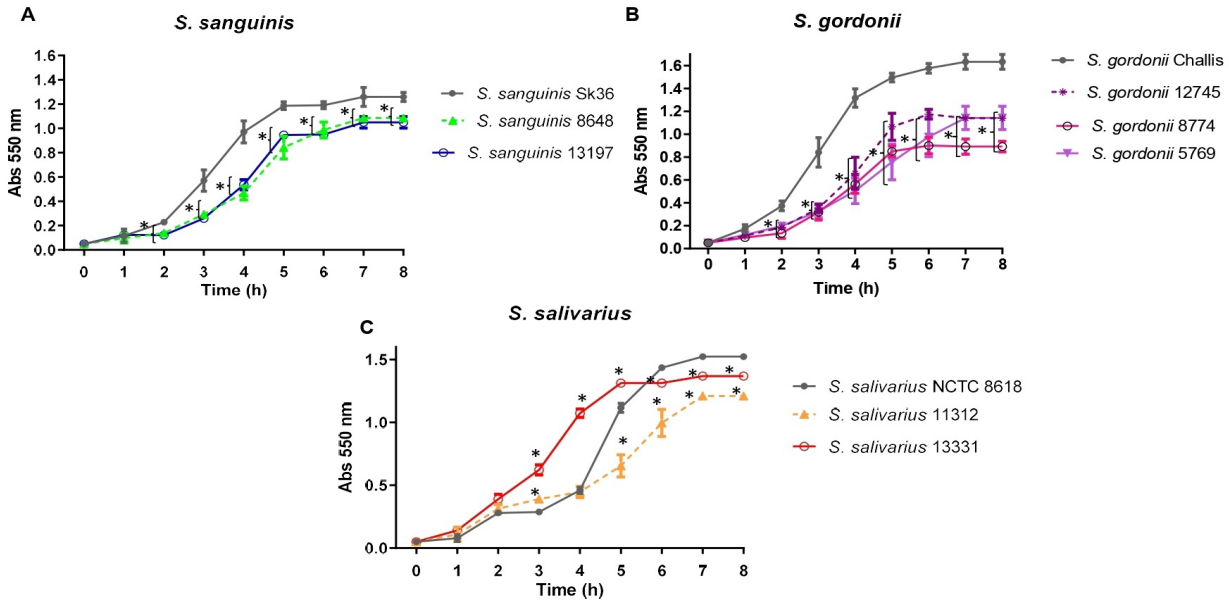

Figure 2. Comparison of planktonic growth of *Streptococcus* spp. strains under 10% CO_2 atmosphere. A) *S. sanguinis* (Ss) strains, including two endocarditis isolates (Ss8648 and Ss13197) and one reference oral isolate (Sk36); B) *S. gordonii* (Sg) strains, including three endocarditis isolates (Sg12745, Sg8774, Sg5769) and one reference oral isolate (Challis); C) *S. salivarius* (Sv) strains, including two endocarditis isolates (Sv13331 and Sv11312) and one reference oral isolate (NCTC 8618). The values represent the means of an independent experiment performed in triplicate; bars represent standard deviations. Symbols (*) indicate statistically significant differences in absorbance values ($A550_{nm}$) compared to the reference strain of each species at the same incubation time. Two-way ANOVA with Dunnett's post-test, $p<0.05$.

Serum increases biofilm formation in oral species of streptococci in a strain-specific fashion

As expected, profiles of biofilm formation of the studied strains were not associated with growth rate/yield observed in planktonic cultures **(Figure 2)**. More important, for all the studied species, medium supplementation with serum significantly enhanced biofilm formation by the stains, with few exceptions (*S. gordonii* 12745 and *S. salivarius* 13331 isolates). The stimulating effect of serum on biofilm formation was also observed in all reference strains isolated from oral sites **(Figure 3)**.

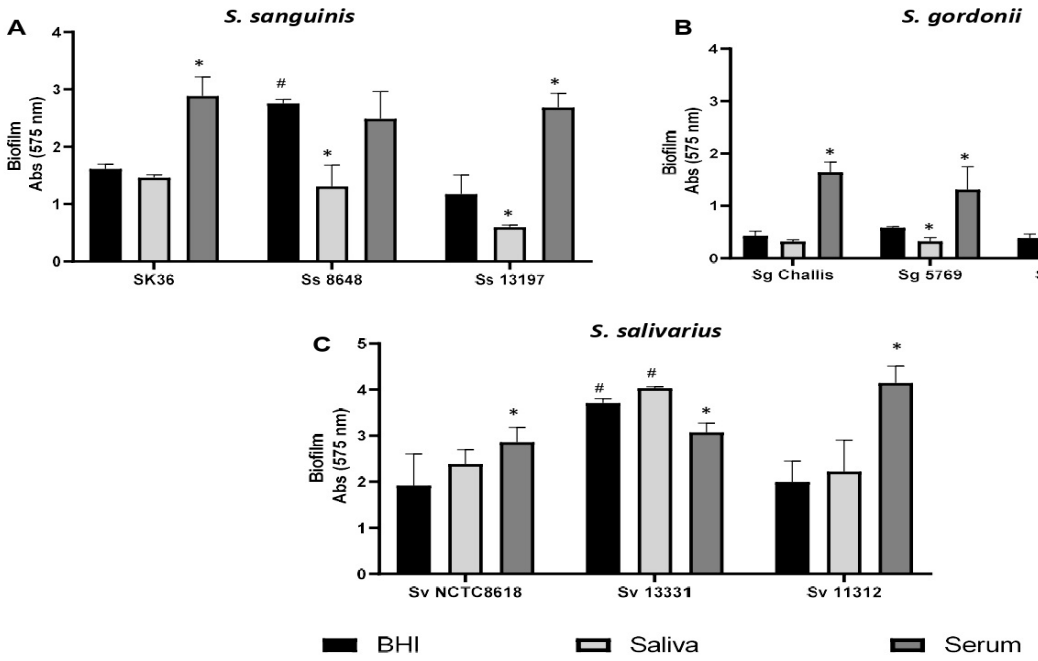

Figure 3. Comparative analyses of biofilm biomass formed after 18 hours by *S. sanguinis*, *S. gordonii*, and *S. salivarius* strains. Biofilms were formed in 96-well plates in BHI medium with 1% sucrose under the conditions of BHI alone (black), BHI supplemented with 10% human saliva (light gray), and BHI supplemented with 20% human serum (dark gray). Columns represent the mean of a triplicate experiment, representative of four independent experiments. Bars indicate standard deviations. Symbols indicate significant differences; Two-way ANOVA with Tukey's post-test ($p<0.05$). Asterisk (*) indicates significant difference between conditions for the same strain, using the BHI condition as the control. Hash mark (#) indicates significant difference between strains for the same condition, using the reference strain as the control.

The biofilm biomass of *S. sanguinis* strains **(Figure 3A)** increase 1.79 to 2.28-fold in BHIS supplemented with serum when compared to biofilm formed in BHIS only. Differently, saliva supplementation did not promote significant changes in biofilm formation for refence strain SK36, whereas it reduced biofilm biomass in blood isolates (Ss8648 and Ss13197).

As shown in **Figure 3B**, all *S. gordonii* strains (Challis and the IE isolates Sg5769 and Sg 8774) increase 2.25 to 3.83-fold biofilm formation in presence of human serum compared to the BHI condition, except for isolate Sg12745, which decreased biofilm formation in BHIS supplemented with serum. The presence of saliva did not promote significant changes in biofilm formation for *S. gordonii* strains, except for strain Sg5769.

For *S. salivarius* **(Figure 3C)**, biofilm biomass increases 1.48 to 2.07-fold in BHIS supplemented with serum when compared to biofilm formed in BHIS only. In the same way as for other species, saliva supplementation did not promote significant changes in biofilm formation, except for isolate Sv13331, which showed greater biofilm formation in the presence of saliva when

compared to the reference strain NCTC 8618.

In general, among the 10 strains analyzed across the species *S. sanguinis*, *S. gordonii*, and *S. salivarius*, 7 strains showed increased biofilm formation in the presence of human serum. However, in the presence of saliva, only 2 blood isolates increased biofilm formation compared reference strains.

Serum increases the thickness and formation of extracellular matrix in *Streptococcus* **spp. by SEM**

The analysis of the formation and structure of early biofilms (4h) formed on glass slides in BHIS supplemented or not with saliva or serum indicates differences in the capacity to initiate biofilm formation among the strains. **Figure 4** illustrates the structural variations of these biofilms observed by scanning electron microscopy under the analyzed conditions.

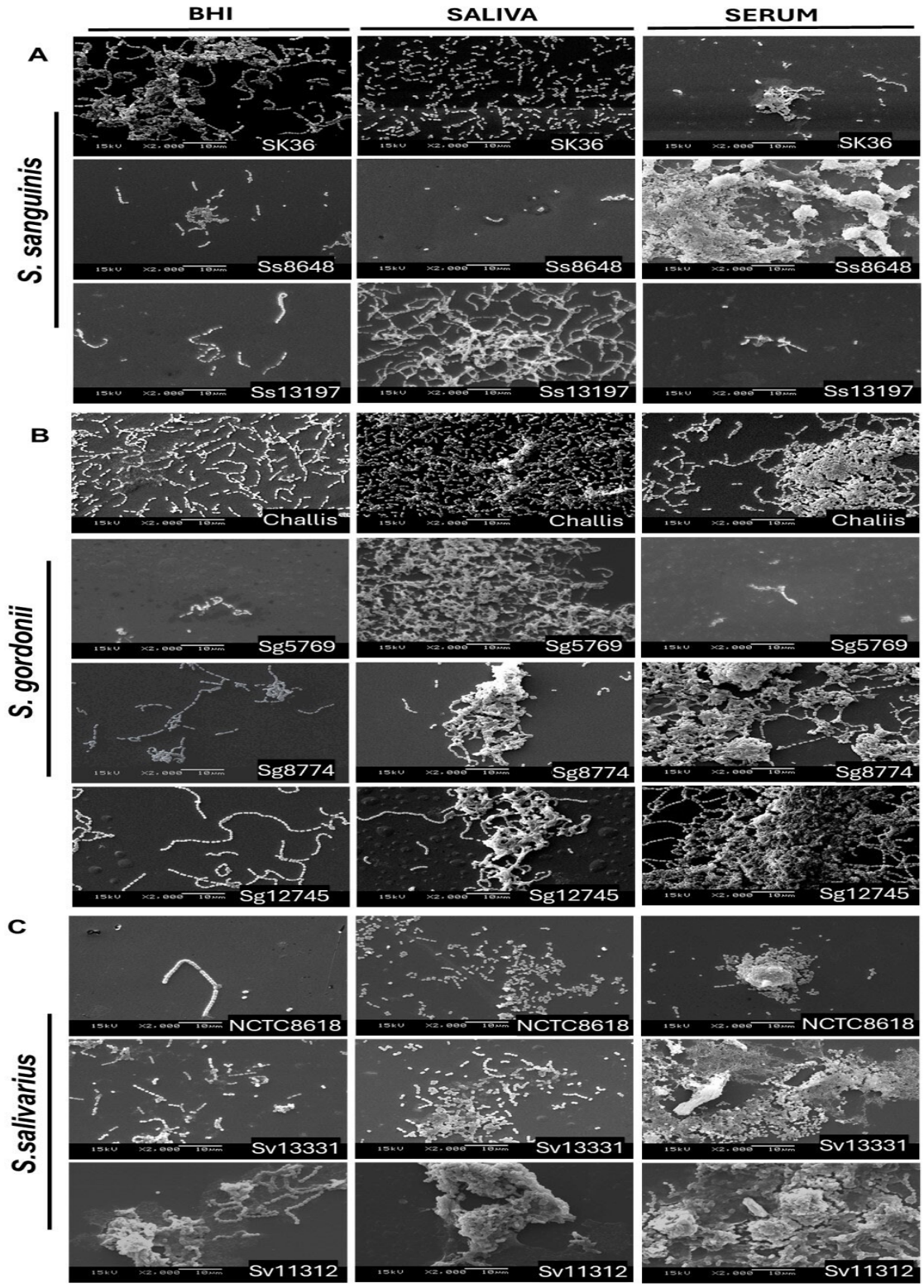

Figure 4. Early stages of biofilm formation by clinical isolates and reference strains analyzed by Scanning Electron Microscopy (SEM). A) *S. sanguinis*; B) *S. gordonii*; and C) *S. salivarius*. Glass slides were incubated with bacterial cultures under three conditions: BHI, 10% saliva, and 20% human serum for 4 hours with 1% sucrose in a 10% CO_2 atmosphere and then processed for scanning electron microscopy (SEM) analysis. Representative images were taken at 2,000x magnification.

The *S. sanguinis* SK36 strain and the IE isolate Ss8648 exhibited biofilms with microcolonies surrounded by extracellular matrix in the presence of serum, while under BHI and saliva conditions, a homogeneous layer of long chains was observed on the glass slides. The Ss13197 strain formed more biofilm in the presence of saliva, with more isolated chains in BHI and serum during the early stages of biofilm formation **(Figure 4A)**. When analyzing the biofilm formation of *S. gordonii* strains **(Figure 4B)**, robust biofilm formation was observed in the presence of saliva and serum for the reference strain (Challis) as well as for isolates Sg8774 and Sg12745. The Sg5769 isolate formed a more homogeneous biofilm in the presence of saliva but did not form significant quantities of microcolonies in the presence of BHI and serum. The Sg12745 isolate formed a more robust biofilm in the presence of serum, aggregated chains in saliva, and more isolated chains in BHI.

In **Figure 4C**, it can be observed that the initial biofilms analyzed by SEM for all *S. salivarius* strains exhibited dense biomass formation, particularly in the presence of human serum. The reference strain (NCTC 8618) displayed more isolated chains in BHI, whereas in the presence of saliva, more aggregated microcolonies were observed. The clinical IE isolate Sv13331 showed a more homogeneous biomass formation on the coverslips, with a progressive increase in biomass under BHI, saliva, and serum conditions. In the presence of serum, colonies surrounded by extensive extracellular matrix formation were observed in the 13331 isolate. Meanwhile, the Sv11312 isolate exhibited aggregated chain formation, with lower biofilm formation in the BHI condition and higher biomass and aggregates in the saliva and serum conditions. These initial biofilm formation results on glass coverslips were consistent with the high quantifications of mature biofilms formed in microplates in the presence of human serum in the analyzed strains.

DISCUSSION

In this study, we aim to characterize strains of *Streptococcus* spp. from the Viridans group isolated from patients with bacterial endocarditis at a reference cardiology center in Brazil (São Paulo/SP). Additionally, we evaluated the ability of these clinical isolates to form biofilms in presence of saliva or human serum), as these strains are often found organized in biofilm form in the oral cavity [32] and in endocarditis lesions [36] [31]. A relevant aspect observed was the significantly slower planktonic growth of the blood-isolated strains compared to the oral reference strains under microaerophilic conditions (10% CO_2). Bacterial growth, represented by the bacterial growth curve, is influenced by a variety of factors such as pH levels, nutrient availability, temperature, and oxygen levels. These factors determine the growth rate and population dynamics of the bacteria [37]. The acidity or alkalinity of the environment affects bacterial metabolism and growth rates. The pH in the oral environment ranges from approximately 6.8 to 7.2, whereas in blood, the pH is slightly basic at around 7.4 [37,38]. The lower growth rate observed in the endocarditis isolates may suggest a metabolic adaptation, reflecting a response to the systemic environment, such as the interior of cardiac vegetations, where oxygen is limited.

In this study, we evaluated the influence of systemic components (saliva and human serum) on the biofilm formation of reference strains (oral isolate) and clinical IE isolates (blood isolates). Among the 10 strains analyzed from *S. sanguinis, S. gordonii*, and *S. salivarius* species, 7 showed increased biofilm formation in the presence of human serum. These data suggest that serum components modulate biofilm formation in the analyzed *Streptococcus* spp. strains.

Some studies report the impact of human serum on biofilm-forming microorganisms, which can either enhance or inhibit biofilm formation depending on the environment and the microorganisms involved [39–41]. In *Staphylococcus aureus* and *Pseudomonas aeruginosa*, human serum significantly increased biofilm formation due to interaction with surface amino acids such as N-acetylcysteine (NAC). This interaction increased the gene expression related to biofilm development in these species [40]. Studies show that blood serum increases intercellular polysaccharide adhesions in *S. aureus,* and furthermore, serum exposed (from burns in an in vivo rabbit model) increases *S. aureus* biofilm formation through the enhancement of oxidative stress [39].

RT-qPCR analysis comparing gene expression in the *S. mutans* UA159 strain and the VicK two-component system mutant revealed that the genes encoding murinases (SmaA and Smu.2146c) were affected by the presence of serum [42]. Furthermore, it was demonstrated that the deletion of smaA and smu.2146 in *S. mutans* significantly reduces biofilm production [43]. We analyzed in silico by BLAST (Basic Local Alignment Search Tool) the similarity of the smaA gene of *S. mutans* with the reference strains included in this study, and found identity percentages of 72.73% for *S. sanguinis* SK36, 84.13% for *S. gordonii* str. Challis, and 79.55% for *S. salivarius*. Thus, although there are no studies in the literature to date comparing the influence of serum on biofilm formation in oral *Streptococcus* spp. strains, these findings reveal that surface

and cell wall proteins may be related to biofilm formation in this species.

It has been demonstrated that for some microorganisms, serum inhibits biofilm formation [44,45]. In *Candida albicans*, human serum inhibited biofilm formation by reducing adhesion and negatively regulating genes related to adhesion, demonstrating a protective role against biofilm development [44]. In *Staphylococcus epidermidis*, human serum exhibited significant antibiofilm activity against *S. epidermidis*, particularly through its protein components, although it did not affect already established biofilms [45].

Saliva-derived biofilms exhibit varied compositions based on the substrate and growth medium, with biotic surfaces supporting more diverse microbiomes compared to abiotic ones [50]. Thus, the in vitro biofilm performed in our experiments includes abiotic surfaces of polystyrene (biofilm in microplates) and glass slides (for SEM), which may explain the lower biofilm formation in the presence of saliva on these surfaces, as they were often weakly adhered and detached during biofilm processing. Additionally, we performed biofilm assays using mechanically stimulated saliva. The study by Inui et al. (2019) demonstrates that mechanically stimulated saliva influences initial bacterial colonization, with differences in binding activity observed between unstimulated and stimulated saliva, showing less biofilm formation when saliva is stimulated [51]. Our findings corroborate those showing that saliva reduces biofilm biomass accumulation and alters the spatial arrangements of the biofilm [48,49].

SEM analyses showed extensive biofilm and extracellular matrix formation when the strains were grown in the presence of human serum. Components present in the serum may contribute to the formation of a denser and richer extracellular matrix. Serum can provide an additional source of proteins and glycoproteins. This can be observed in studies showing that when dental plaque grows below the gum line, it becomes isolated from saliva and, instead, is exposed to gingival crevicular fluid, a serum exudate, which is rich in proteins that can be released and bound by bacteria, serving as a source of molecules in the subgingival dental plaque matrix [52].

It is important to highlight that part of the success of streptococci as colonizers and biofilm formers is attributable to the spectrum of proteins expressed on their surfaces.

Adhesins enable interactions with salivary, serum, and extracellular matrix components. This is the first essential step for colonization, the development of complex communities, and potential host tissue invasion [53]. Thus, it is suggested to conduct more detailed molecular evaluations on the strains analyzed in this study, such as assessing the expression of genes that regulate surface proteins, in order to better understand what influences biofilm formation behavior in the presence of saliva or human serum in these species.

CONCLUSIONS

In conclusion, the results obtained in this study indicate that:

- The IE isolates of *S. sanguinis, S. gordonii* and one strain of *S. salivarius* showed slower growth when compared to the reference strains.
- Most strains of *S. sanguinis*, *S. gordonii* and *S. salivarius* showed a significant increase in biofilm formation in the presence of human serum.
- The initial biofilm formation observed by SEM showed greater biofilm formation in the presence of serum.

These results suggest that components of human serum can modulate biofilm formation among the *Streptococcus* spp. strains analyzed, which may contribute to the formation of vegetations in endocarditis lesions. Thus, understanding the mechanisms of biofilm formation of these species may offer new perspectives for therapeutic strategies in the management of infective endocarditis.

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Ethical statement

This study was conducted in strict accordance with the National Commission on Ethics in Experimentation (CONEP), Brazil.

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