








Identification of Proteins Associated with the Formation of Oral Biofilms

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ABSTRACT

Objective: To identify proteins associated with the formation of *Streptococcus gordonii* and *Fusobacterium nucleatum* biofilms. **Material and Methods:** Biofilms composed of two bacterial species, *S. gordonii* and *F. nucleatum*, were cultured for 1, 4, 7, and 10 days. The presence of both species was confirmed via amplification of the *srtA* and *radD* genes using real-time PCR. The concentrations of proteins associated with the biofilms and individual species were quantified using Western blotting. **Results:** The protein profiles of *S. gordonii* and *F. nucleatum* from individual cultures determined using one-dimensional electrophoresis revealed proteins found in *S. gordonii* and in *F. nucleatum*. Ct and reciprocal Ct values were determined for the exposed *S. gordonii* and *F. nucleatum* biofilms. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein was detected in biofilms and *F. nucleatum*, whereas HSP40 protein was present only in biofilms after 7 and 10 days of formation. **Conclusion:** HSP40 was detected only in the formed biofilms; thus, HSP40 is an essential proteins for adhesion.

Keywords: Dental Plaque; *Fusobacterium nucleatum*; *Streptococcus gordonii*; Genomics.

Introduction

The bacterial species of the human oral cavity depend on their ability to bind to surfaces or to each other for colonization. Therefore, proteins involved in adherence are important components that allow microorganisms to form and reside in complex oral biofilms, in which different groups of bacteria perform specific functions. Although microbial interactions within these biofilms trigger important physiological changes in the associated species, including expression of virulence characteristics, the physical interaction through specific adhesins is a key element for the successful initiation of surface colonization and biofilm integration [1,2].

Species of the genus *Fusobacterium* have been linked to a wide variety of microbial species and are considered important for biofilm formation and architecture. Fusobacteria integrate into biofilms by binding to early colonizers attached to the surface, such as streptococci and actinomycetes. In addition, fusobacteria recruit other bacterial species, including early colonizers and important periodontal pathogens that cannot directly attach to surfaces. This characteristic allows fusobacteria to promote changes in the microbial community and impact their pathogenesis [2].

Culturable oral fusobacteria are predominantly *Fusobacterium periodonticum* and *Fusobacterium nucleatum*. While *F. periodonticum* encompasses only one species, *F. nucleatum* includes five subspecies: *nucleatum*, *polymorphum*, *fusiforme*, *animalis*, and *vincentii*. This group of microorganisms thrives not only in subgingival environments [3,4] but also in the supragingival plaque [5]. Streptococci are the most common early colonizers and constitute the main binding partner for fusobacteria recruitment in oral biofilms [6].

The oral cavity is a great model system for studying polymicrobial interactions since it is home to more than 600 different recognized species of bacteria, most of which are considered commensal bacteria [7-10]. Microorganisms in oral biofilms have been categorized into early and late colonizers. The first colonizing species are mainly gram-positive bacteria capable of adhering directly to the tooth surface and forming the basal layers of the oral biofilm [11-13]. Late colonizers are mainly gram-negative bacteria, including certain periodontal pathogens, such as *Treponema denticola*, *Tannerella forsythia*, and *Porphyromonas gingivalis*, as well as other bacteria within the oral biofilm, forming a complex network of direct or indirect interactions. The spatial distribution of different bacterial species is important in the formation and architecture of oral biofilms. Many of the known oral bacterial species do not directly interact with each other; instead, they interact indirectly through their mutual association with *F. nucleatum* [14-18].

Recently, interactions of fusobacteria with streptococci have been described; however, the genes involved in the formation of biofilms of different species have not been identified in most of them [19-25]. This study aimed to identify the proteins associated with the adhesion and coaggregation of *F. nucleatum* and *S. gordonii*. In this way, possible targets for future therapies that block the incorporation of pathogenic bacteria can be found, and these targets can be used as the first biomarkers of oral diseases.

Material and Methods

Culture and Bacterial Strains

Strains of *S. gordonii* (ATCC 51656) and *F. nucleatum* (ATCC 10953) were used. The culture medium was 30 g/L tryptic soy broth (TSB) in distilled water that was supplemented with artificial saliva [26] (350 mL of distilled water with 3.15 g of NaCl), a solution of 4% carboxymethyl cellulose (4 g of carboxymethyl cellulose in 100 mL of distilled water), and 50 mL of glycerin. A final volume of 500 mL was obtained, autoclaved, and stored at 4°C.

Establishment of the Dual-Species Biofilm Model

Biofilms were formed on the surface of 25 mm x 75 mm transparent rectangular slides placed in 90 x 15 mm Petri dishes, with one Petri dish per slide. Biofilms were incubated under anaerobic conditions at 37°C using AnaeroGen packets in 2.5 L jars (12-Petri-dish capacity) for 24 hours (time 1), 5 days (time 2), 7 days (time 3), or 10 days (time 4).

A colony of each strain was inoculated in 15 mL of TSB at 37°C under anaerobiosis until reaching the exponential growth phase of each strain, i.e., an optical density at 550 nm of 0.125 (McFarland 0.5 scale), which equals 150×10^6 cells/mL, which took 4.5 hours for *S. gordonii* and 8 hours for *F. nucleatum*. Sterile slides were then incubated in Petri dishes with 16 mL of sterile artificial saliva and equilibrated for 4 hours at 37°C. The slides were removed with sterile tweezers and washed gently with 15 mL of phosphate-buffered saline (PBS) (pH 7.0, equilibrated) with a sterile 10 mL pipette, and the slides were placed in new sterile Petri dishes. Then, 100 µL of the *S. gordonii* culture was added to each slide and incubated for 1 hour at 37°C. Next, 100 µL of the *F. nucleatum* culture was added and incubated for 1 hour at 37°C. Sixteen milliliters of TSB (37°C) was added to the slides and incubated at 37 °C for 1, 5, 7, or 10 days [22]. The influence of HSP40 on aggregation was evaluated [27].

Quantification of *F. nucleatum* and *S. gordonii* cells

Genomic DNA was extracted from the cultured biofilms (2.5%) after 1 day, 4 days, 7 days, and 10 days. The DNA concentration was quantified, and for absolute quantification by real-time PCR, 100 ng/µL was used for all samples to determine the proportion of cells from both species. The oligonucleotides used were [22] srtA F: 5' TATTATGGTGCTGGTACGATGAAAGAGACTC 3' and srtA R: 5' TATAGATTTTCATACCAGCCTTAGCACGATC 3' for *S. gordonii* and radD F: 5' GGATTTATCTTTGCTAATTGGGGAAATTATAG 3' and radD R: 5' ACTATTCCATATTCTCCATAATATTTCCATTAGA 3' for *F. nucleatum*.

Isolation and Quantification of Proteins

The cells were detached from the glass surface of the Petri dishes by incubating them in trypsin at 37°C for 15 minutes and then were harvested by centrifugation at $5000 \times g$ for 10 minutes at 4°C. The cell pellet was washed with $1 \times$ PBS (pH 7.4). The pellet was resuspended in lysis buffer (50 mM HEPES, 8 M urea, and 1 mM dithiothreitol) and incubated at 95°C for 5 minutes. Immediately, the tubes were put on ice, and the cells were lysed by sonication (power of 0.6 W, three 30-second sonications; the samples were kept on ice for 3 minutes between sonications). Cellular debris was pelleted by centrifugation at $720 \times g$ for 7 minutes at 4°C, and then, the supernatant was collected and centrifuged at $10,000 \times g$ for 10 minutes at 4°C to pellet the membrane fraction. The supernatant was recovered, from which cytoplasmic proteins were precipitated with absolute ethanol. Five volumes of precooled absolute ethanol were added to the pellet, which was incubated at -70°C for 2 hours. Proteins were obtained by centrifugation at $17,000 \times g$ for 45 minutes at 4°C, and the pellet was resuspended with lysis buffer. Finally, the proteins were quantified by the Bradford method using a standard curve of known concentrations for bovine serum albumin [22].

Precipitation of Proteins

Two methods were used, one with acetone and one with ethanol. The acetone method consisted of adding five volumes of 100% acetone to 100 µL of sample and incubating at -20°C for 3 hours. The proteins

were obtained by centrifugation at 15,000×g for 20 minutes at 4°C. The supernatant was discarded, and the pellet was washed twice with 50% acetone, with centrifugation intervals at 15,000×g for 20 minutes at 4°C. The ethanol method consisted of adding five volumes of precooled absolute ethanol and incubating the sample at -70°C for 2 hours. The proteins were obtained by centrifugation at 17,000×g for 45 minutes at 4°C. The proteins were quantified by the Bradford method and separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1) [22].

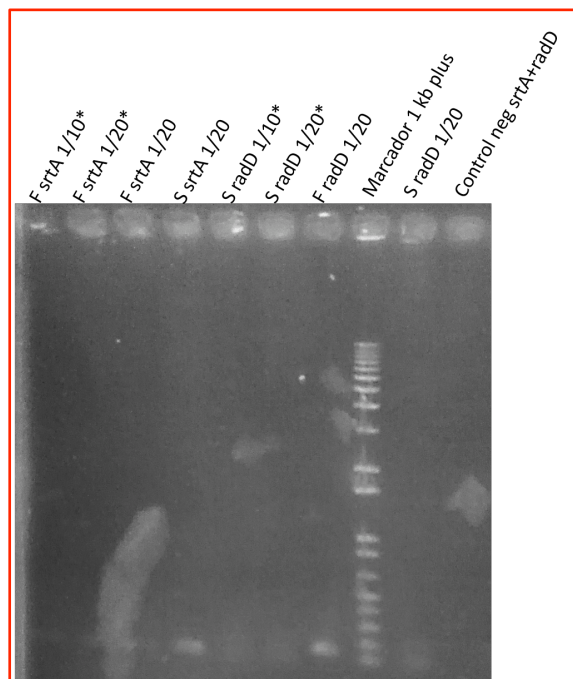


Figure 1. Electrophoresis for srtA and radD from *F. nucleatum* and *S. gordonii*.

Immunodetection of HSP40 Using Western Blotting

After separating the membrane or cytoplasmic proteins by 12% SDS-PAGE, the proteins were transferred to nitrocellulose membranes. The membranes were washed three times with 1× PBS (pH 7.4). Then, general protein blocking was performed with 5% milk with incubation at 37°C for 1 hour in constant motion. The membrane was washed three times with PBS plus 0.05% Tween 20 at room temperature. Immunodetection was performed by incubating the membrane with an anti-HSP40 antibody (SPA-087, Stressgen Biotechnologies, British Columbia, Canada) (1:1000 dilution in 5% milk) at 37°C for 1 hour. The antibody–antigen complex was detected by incubating the membrane with rabbit anti–mouse IgG antibody conjugated with horseradish peroxidase (1:2000 dilution in 5% milk) at 37°C for 1 hour. β -Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as loading controls. The membrane was washed again to remove excess antibodies and finally developed with diaminobenzidine plus H_2O_2 .

Results

The quantification of the number of copies of the srtA genes of *S. gordonii* and radD genes of *F. nucleatum* in the biofilm using log₁₀ Cells by PCR showed an elevated expression of the srtA gene at day 1; however, this increased expression decreased with time. In contrast, the radD gene reached its maximum expression on days 7 and 10 (Figure 2).

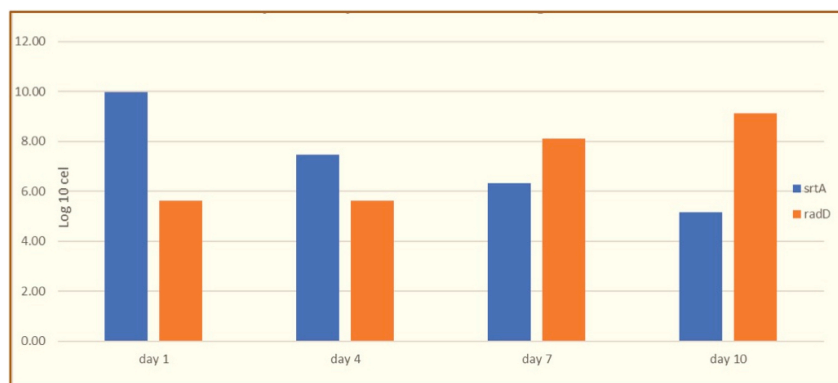


Figure 2. Quantification of the copy numbers of the *srtA* gene of *S. gordonii* and *radD* gene of *F. nucleatum*.

The protein profiles of *S. gordonii* and *F. nucleatum* from individual cultures determined using one-dimensional electrophoresis revealed proteins found in *S. gordonii* and in *F. nucleatum*, (Figure 3, red and yellow arrows). Ct and reciprocal Ct values were determined for the exposed *S. gordonii* and *F. nucleatum* biofilms (Table 1).

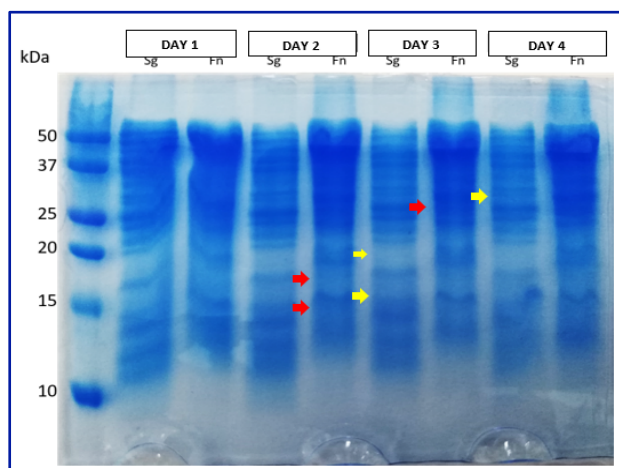


Figure 3. SDS-PAGE of proteins from the cytoplasmic fractions of individual cultures.

Table 1. Ct and reciprocal Ct values determined for the exposed *S. gordonii* and *F. nucleatum* biofilms.

Biofilms	Ct	1/Ct
<i>S. gordonii</i> srtA	19.757	0.051
	21.528	0.046
	21.714	0.046
	22.189	0.045
<i>F. nucleatum</i> radD	19.554	0.051
	24.739	0.040
	20.429	0.049
	27.758	0.036
F+S srtA+radD	27.133	0.037
	22.179	0.045
	21.647	0.046
	16.732	0.060
F+S srtA	36.687	0.027
	25.809	0.039

	22.192	0.045
	16.638	0.060
F+S radD	30.418	0.033
	32.55	0.031
	30.588	0.033
	0	nd

The cytoplasmic protein profile of the biofilms harvested after 1, 4, 7, or 10 days of culture remained constant over time and was similar to the profile of the individual culture of *S. gordonii*. However, a higher protein load was observed between 50 and 37 kDa (Figure 4), suggesting the presence of *F. nucleatum* proteins. On the other hand, no drastic changes were observed in the production of any particular protein from either *S. gordonii* or *F. nucleatum*.

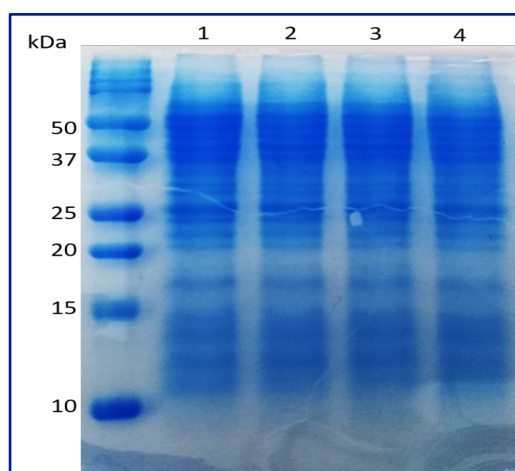


Figure 4. SDS-PAGE of proteins from the cytoplasmic fractions of *S. gordonii* and *F. nucleatum* biofilms.

Through the detection of GAPDH using a specific antibody, it was determined that the antibody detected only an epitope that is found in GAPDH of *F. nucleatum* and not in GAPDH of *S. gordonii* (Figure 5a). Its molecular weight ranged between 50 and 37 kDa. In biofilms, on days 1 and 4, the detection was quite faint compared to that at 7 and 10 days (Figure 5b), confirming that the adherence of *F. nucleatum* on *S. gordonii* was gradual and complete at 7 days of culture.

HSP40 protein was not detected in individual cultures (Figure 6a) but was detected in biofilms after 7 and 10 days of culture. In these biofilms, its molecular weight ranged from 50 to 37 kDa (Figure 6b), indicating that this protein is involved in coaggregation and, therefore, in biofilm formation.

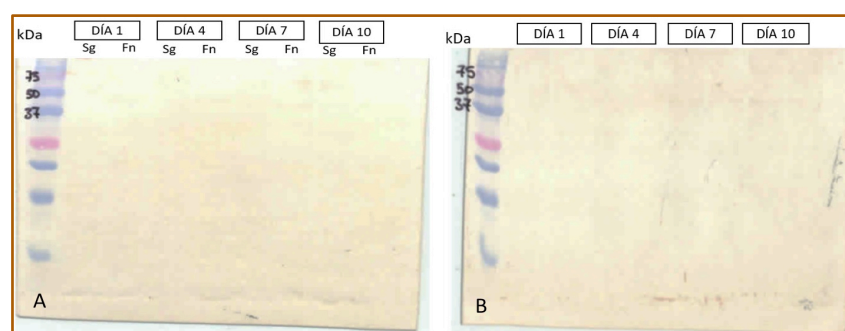


Figure 5. Immunodetection of GAPDH in the cytoplasmic fractions of individual cultures and biofilms.

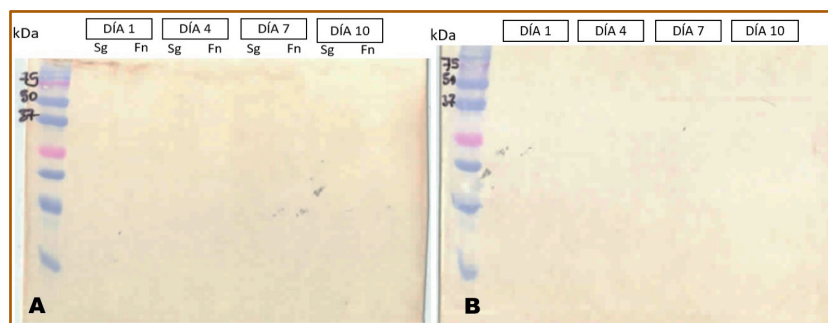


Figure 6. Immunodetection of HSP40 in the cytoplasmic fractions of individual cultures and biofilms.

Discussion

The development of oral multispecies biofilm implies competition and cooperation between different bacteria. Streptococci are dominant among the first colonizing bacteria, and the second colonizing *F. nucleatum* can bind and connect these bacteria with late colonization periodontal pathogens, including *P. gingivalis*, *T. denticola*, and *T. forsythia*. Therefore, *F. nucleatum* plays a crucial role as a bridge in the development of oral biofilms that are associated with periodontitis. Therefore, the identification of proteins that could inhibit the incorporation of *F. nucleatum* in oral biofilms could reduce its pathogenic potential [28-32].

Quantifying the number of copies of the *S. gordonii* *srtA* gene and *F. nucleatum* *radD* gene in the biofilm showed that the gene expression corresponds to the model established by Socransky, who demonstrated that for the development of subgingival biofilms, bacteria capable of adhering to oral surfaces must colonize first, with primary colonizers being initially much more abundant; however, with time, other groups of bacteria proliferate in greater quantity. After the first species die, they provide structural support for biofilm formation and the coaggregation of the other species [5].

Biofilms, which are one way in which such coexistence occurs [26,33], develop based on multiple mechanisms associated with protein expression [34-38]. However, the literature on this subject remains scarce [18,38,39]. In this study, we sought to identify proteins associated with the formation of biofilms and determine whether these proteins are found in membranes or the cytoplasm in *F. nucleatum* and *S. gordonii* biofilms.

In this investigation, the protein profiles of *S. gordonii* and *F. nucleatum* from single cultures determined using one-dimensional electrophoresis revealed that certain proteins were found only in *S. gordonii* but not in *F. nucleatum* (and vice versa). This finding has been demonstrated in previous studies that have reported the existence of proteins associated with metabolic, nutritional, adherence, and bacterial aggregation functions. The role of proteins in the adherence and coaggregation of microorganisms in biofilms has been demonstrated in two studies [27,40], showing the overexpression of biofilm growth-related proteins, such as the ATP-binding cassette [41,42].

The cytoplasmic protein profiles of the biofilms harvested after 1, 4, 7, and 10 days of cultivation remained unchanged over time, demonstrating that the examined proteins are at constant levels throughout different aspects of biofilm formation, such as cell division, control of cell volume, and control of which substances pass through the cell membrane. These results may be relevant because of the role of these proteins in bacterial resistance to antibiotics [42-46].

Another finding was that HSP40 protein was not detected in individual cultures but was detected in biofilms after 7 and 10 days of culture, as well as the GAPDH protein, indicating that both proteins are

involved in bacterial coaggregation. These proteins have been reported in previous studies as important elements for linking different bacterial species [47-49].








In summary, both the GAPDH and the HSP40 proteins regulate coaggregation with *S. gordonii*. Although the dental plaque biofilm is the result of multiple interactions among oral bacteria, our results indicate that the binding of the secondary colonizer *F. nucleatum* to the primary colonizers can be mediated by the proteins mentioned, which can ultimately influence periodontopathogen binding [5,6].

It is important to note that although the biofilm model used in this study is widely investigated [25,29,36], there are inherent limitations to the ability of these two microorganisms to mimic the complex biofilms that develop in the oral cavity. However, these results indicate that the behavior of biofilms is regulated by the expression of certain proteins. In the future, their identification and location could be potential targets for the application of molecules that can inhibit their expression and therefore the formation of these biofilms.

Conclusion

GAPDH protein was detected only in *F. nucleatum* samples. GAPDH and HSP40 proteins were detected only in biofilms evaluated after 7 and 10 days of culture and are, therefore, essential proteins for coaggregation.

Authors' Contributions

PAMG 	https://orcid.org/0000-0002-7105-0940	Conceptualization, Software, Validation, Resources, Writing - Original Draft, Writing - Review and Editing, Visualization and Supervision.
REBA 	https://orcid.org/0000-0003-3017-1540	Conceptualization, Formal Analysis, Investigation and Data Curation.
DJMT 	https://orcid.org/0000-0001-7198-3778	Resources, Visualization and Supervision.
RDCP 	https://orcid.org/0000-0002-5128-212X	Writing - Original Draft and Writing - Review and Editing.
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All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

Financial Support

None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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