Identification and localization of proteins associated with the formation of *Streptococcus gordonii* and *Fusobacterium nucleatum* biofilms

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Abstract

Background:To successfully colonize the oral cavity, bacteria must adhere directly or indirectly to the oral surfaces available. *Fusobacterium nucleatum* plays an important role in the development of the oral biofilm community due to its broad adhesion capabilities, serving as a bridge between the members of the oral biofilm community that cannot be directly joined together. The purpose of this study was to identify and localize the proteins associated with the formation of biofilms of *Streptococcus gordonii* and *F. nucleatum*.

Methods: Multispecies biofilms were identified by amplification of the *srtA* and *radD* genes by real-time PCR. Biofilm cells cultured with sucrose were counted. The protein concentrations in the membrane and cytoplasmic fractions were quantified by western blot. Results: The proteins HSP40 and GAPDH were detected in the cytoplasmic fraction of biofilm and *F. nucleatum*, respectively. The available anti-GAPDH antibody is specific for GAPDH produced by *F. nucleatum*, which indicated the coaggregation of *F. nucleatum* on *S. gordonii*.

Conclusions: HSP40 was only detected in the cytoplasmic fraction of the biofilms, making it one of the essential proteins for adherence. This complex set of interactions could have critical implications for the formation and maturation of oral biofilms in vivo and could provide clues to the mechanism behind the distribution of organisms within the human oral cavity.

Background

The bacterial species of the human oral cavity depend on their ability to bind to surfaces or to each other for colonization and persistence in this nutrient ecological niche. 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 virulence characteristics, the physical union 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 the formation and architecture of biofilms. Fusobacteria integrate into biofilms by binding to early colonists 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 them to promote changes in the microbial community and impacts their pathogenesis [2].

Culturable oral fusobacteria are predominantly *F. periodonticum* and *F. 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 the recruitment of fusobacteria in oral biofilms [6].

Bacterial interspecies interactions mediated by adherence are important elements in the formation of oral biofilms. These interactions often occur at a species-specific level, which could determine the health or disease association of a biofilm community. Among the key actors involved in these processes are the fusobacteria that have been recognized for their ability to interact with numerous bacterial groups.

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 to be commensal bacteria [7–10]. Microorganisms in oral biofilms have been categorized into early and late colonizers. The first colonizing species are mainly gram-positive, capable of adhering directly to the surface of the tooth 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 union with *F. nucleatum [*14].

F. nucleatum is a gram-negative fusiform anaerobic bacterium that has been associated with periodontal disease and a number of systemic diseases. It is considered a “bridge organism” due to its ability to form a colonization bridge between species that do not interact directly, thus playing an integral role in the formation of a mature dental plaque. The physical attachment between the interacting species is mediated by specific cellular adhesion proteins in their outer membranes [15]. For example, proteins of the SrtA family that anchor to the cell wall have been identified, such as SspA/SspB of *S. gordonii,* which allow interactions with other streptococci and other oral species, including *Porphyromonas gingivalis, Actinomyces,* and *Candida [*16–19]. Egland et al. [20] showed that the SspA/SspB homologue of *S. mutans* binds to the host’s saliva proteins; the host’s matrix proteins, including type I collagen, fibronectin, laminin, and keratin; and serum components such as fibrinogen. Certain strains of actinomycetes can be recognized by the expression of the SpaP protein in *S. gordonii* along with CshA, a protein typically not present in *S. mutans [*21].

Recently, fusobacterial interactions with *Streptococcus,* an important oral carcinogenic pathogen, have been described, but most of the studies focussed on binding to non-mutant streptococci, and paired specific adhesins have not yet been identified [22–25]. The purpose of this study was to identify the proteins linked to the adhesion and coaggregation of microorganisms of the species *F. nucleatum* and *S. gordonii.* In this way, possible targets for future therapies that block the incorporation of pathogenic bacteria can be found and can be used as the first biomarkers of oral diseases.

Methods

Culture and bacterial strains

Strains of *S. gordonii* (ATCC 51656) and *F. nucleatum* (ATCC 10953) were used, characterized by biofilm formation in addition to playing an important role in periodontal disease. Tryptic soy broth (TSB), 30 g/L of distilled water, was used as culture medium, to which artificial saliva (350 mL of distilled water with 3.15 g of NaCl), a solution of 4% carboxymethyl cellulose (4 grams of carboxymethyl cellulose in 100 mL of distilled water), and 50 mL of glycerine were added. A final volume of 500 mL was obtained, autoclaved, and stored at 4 °C.

Formation of multispecies biofilm

The biofilm was formed on the surface of 25 mm x 75 mm transparent rectangular slides placed in 90 x 15 mm Petri dishes, one Petri dish per slide. Biofilms were incubated under anaerobic conditions at 37 °C using AnaeroGen sachets 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 in anaerobiosis until reaching the exponential growth phase of each strain: 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.* The sterile slides were then incubated in Petri dishes with 16 mL of sterile artificial saliva and tempered 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, tempered) with a sterile 10 mL pipette, and the slides were placed in new sterile Petri dishes. Then, 100 µL of *S. gordonii* culture was added to each slide and incubated for 1 hour at 37 °C. Next, 100 µL of the culture of *F. nucleatum* was added and incubated for 1 hour at 37 °C. Sixteen millilitres of TSB (37 °C) was added to the slides and incubated at 37 °C for 1, 5, 7, or 10 days.

Verification of the presence of both bacteria

Genomic DNA was extracted from *S. gordonii* and *F. nucleatum* biofilms, pooled, and separately cultivated. To verify the specificity of the srtA and radD primers, conventional PCR was first performed, adding the *F. nucleatum* genome to the reaction with the srtA primers and the *S. gordonii* genome to the reaction with the radD primers. (Figure 1) Once the specificity was verified, real-time PCR was run, performing reactions with the srtA primers and *S. gordonii* genome, radD primers and the *F. nucleatum* genome, srtA+radD primers and the biofilm (*S. gordonii* and *F. nucleatum)* genome, and srtA and radD primers separately to amplify these genes in the biofilm genome.(figure 2,3,4)

Quantification of F. nucleatum and S. gordonii cells

Genomic DNA was extracted from the cultured biofilms at 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 srtA F: 5' TATTATGGTGCTGGTACGATGAAAGAGACTC 3' and srtA R: 5' TATAGATTTTCATACCAGCCTTAGCACGATC 3' for *S. gordonii* and radD F: 5' GGATTTATCTTTGCTAATTGGGGAAATTATAG 3' and radD R: 5' ACTATTCCATATTCTCCATAATATTTCCCATTAGA 3' for *F. nucleatum.*

Separation and quantification of proteins of the membrane and cytoplasmic fractions

The cells were detached from the glass surface of the Petri dishes with by incubating them in trypsin at 37 °C for 15 minutes and were harvested by centrifugation at 5000×g for 10 minutes at 4 °C. The cell pellet was washed with 1× 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, kept on ice for 3 minutes between sonications). Cellular debris was pelleted by centrifugation at 720×g for 7 minutes at 4 °C, and then the supernatant was collected and centrifuged at 10,000×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 absolute ethanol, previously cooled, was added to the pellet and left at −70 °C for 2 hours. Proteins were obtained by centrifugation at 17,000×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.

Precipitation of cytoplasmic proteins

Two methods were used, with acetone and ethanol. The acetone method consisted of adding 5 volumes of 100% acetone in 100 µL of sample and leaving 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 with 50% acetone twice with centrifugation intervals at 15,000×g for 20 minutes at 4 °C. The ethanol method consisted of adding volumes of previously cooled absolute ethanol and leaving it 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).

Immunodetection of HSP60 by western blot

After separating the membrane or cytoplasmic proteins by 12% SDS-PAGE, the proteins were transferred to nitrocellulose membranes. The membranes were washed with 1× PBS pH 7.4 three times. Then, a

general protein block was performed with 5% milk with incubation at 37 °C for 1 hour in constant motion. The membrane was washed with PBS plus 0.05% Tween 20 at room temperature three times. Immunodetection was performed with the anti-HSP60 antibody generated in mice (1:1000 dilution, in 5% milk) and incubated 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. The membrane was washed again to remove excess antibodies and finally developed with diaminobenzidine plus H_2O_2.

Results

The protein profile of S. gordonii and F. nucleatum from individual cultures run in one-dimensional electrophoresis revealed that some proteins were only found in S. gordonii and not in F. nucleatum, and vice versa (Figure 5, red and yellow arrows). Ct and inverse-proportional Ct values determined for the exposed S. gordonii and F. nucleatum biofilms.(table 1)

The cytoplasmic protein profile of the biofilms harvested after 1, 4, 7, and 10 days of culture kept 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 6), 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.

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

The protein HSP40 was not detected in individual cultures (Figure 8a), but it was detected in biofilms after 7 and 10 days of culture, its molecular weight ranging from 50 to 37 kDa (Figure 8b), indicating that protein is involved in coaggregation and therefore in biofilm formation.

Discussion

The oral cavity is a complex bacterial ecosystem composed of multiple microorganisms that intimately coexist [26–30]. Biofilms are one way they coexist [31, 32], and they develop thanks to multiple mechanisms associated with protein expression [33–37]. However, the literature on this subject is still scarce [18, 37, 38]. In this study, we sought to identify proteins associated with the formation of biofilms and whether these proteins are found in the membranes or in the cytoplasm of *F. nucleatum* and *S. gordonii* biofilms.

The role of proteins in the adherence and coaggregation of microorganisms in biofilms has been demonstrated in two studies [39, 40] showing the overexpression of biofilm growth-related proteins such

as the ATP-binding cassette [41, 42]. These membrane proteins are fundamental to many biological processes, 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 the bacterial resistance to antibiotics [42–46].

A group of proteins that were mostly overexpressed in the biofilm were proteins of unknown function called hypothetical proteins [47]. These data are in line with what has been described by other authors [48, 49]. The analysis of these proteins and of their roles in biofilm can provide information on the role of this bacterium in this form of growth [50]. Another protein that is overexpressed in biofilm and may be of interest is NusG [51]. This protein can be used as a marker of *F. nucleatum [*52] and may be relevant for the development of new diagnostic tools [52, 53] not only for periodontitis but also for other diseases associated with the presence of the two microorganisms studied in this paper.

Conclusions

The proteins HSP40 and GAPDH were detected in the cytoplasmic fraction of the biofilm and in *F. nucleatum,* respectively. The available anti-GAPDH antibody is specific for GAPDH produced by *F. nucleatum,* so our findings indicate the coaggregation of *F. nucleatum* on *S. gordonii.* The HSP40 protein was only detected in the cytoplasmic fraction of the biofilms, being one of the essential proteins for adherence.

List Of Abbreviations

BHI: Brain heart infusion; DNA: deoxyribonucleic acid; PCR: polymerase chain reaction; S. gordonii: Streptococcus gordonii; F. nucleatum. Fusobacterium nucleatum; SDS-PAGE: sodium dodecylsulphate–polyacrylamide gel electrophoresis.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors read and approved the final version of this manuscript.

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Not applicable.

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Table

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

	Ct	1/Ct
S. gordonii srtA	19.757	0.051
	21.528	0.046
	21.714	0.046
	22.189	0.045
F. nucleatum 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

Figures

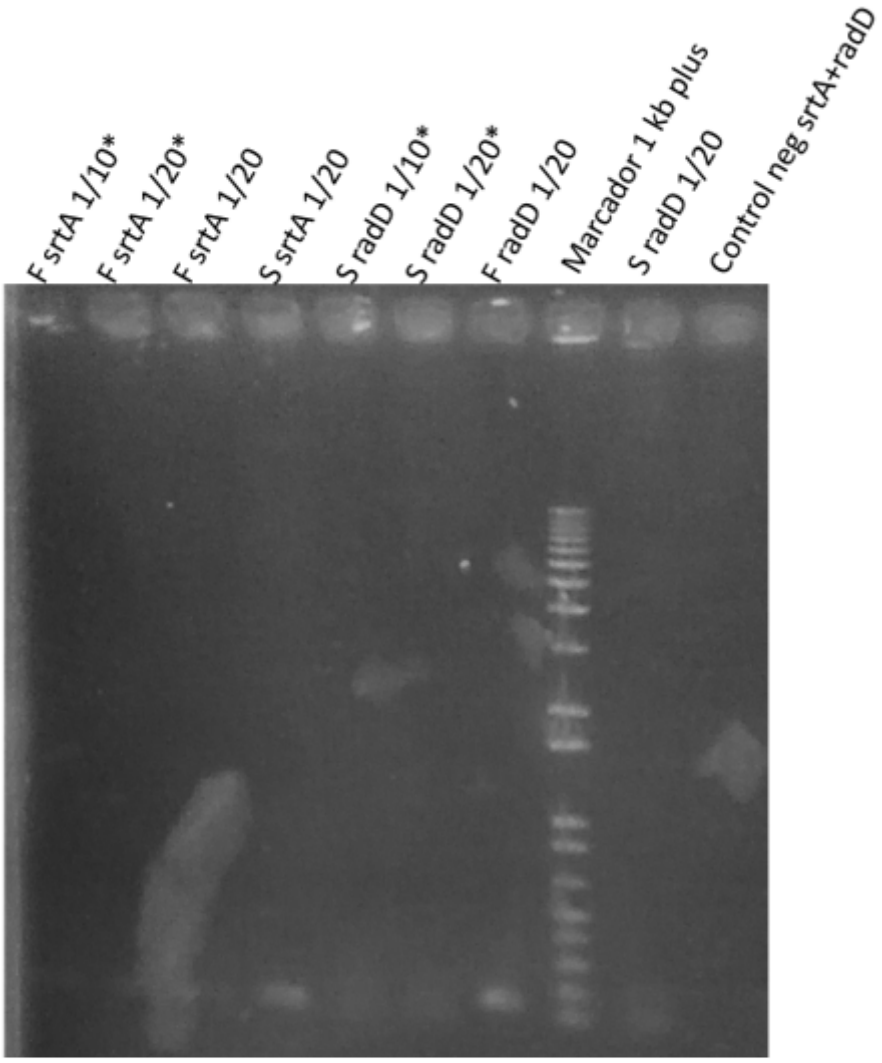

Figure 1

Figure 1

Electrophoresis using srtA and radD from F. nucleatum and S. gordonii.

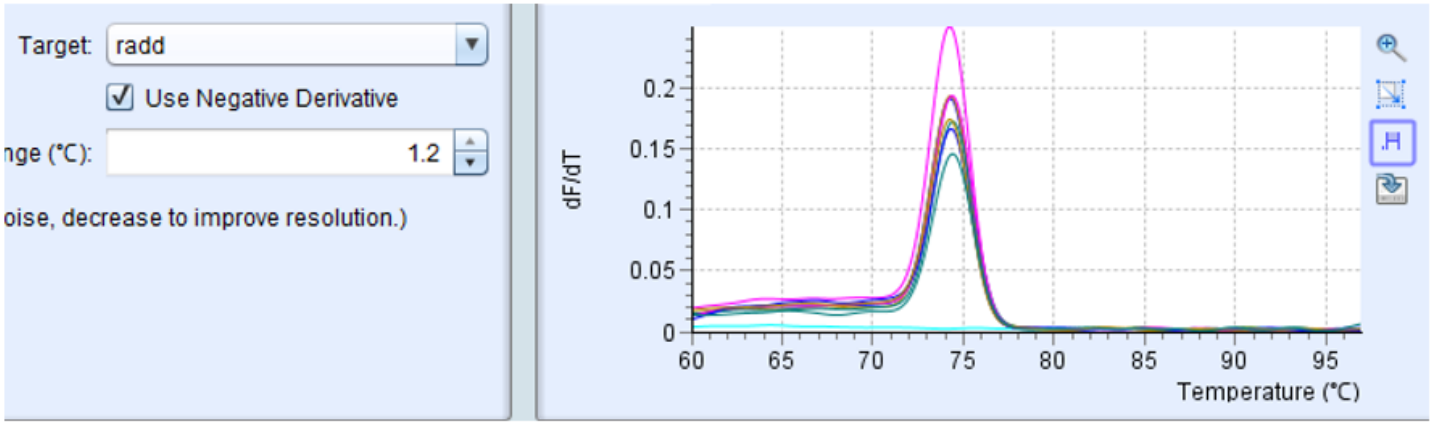

Figure 2

Figure 2

Tm curve of radD

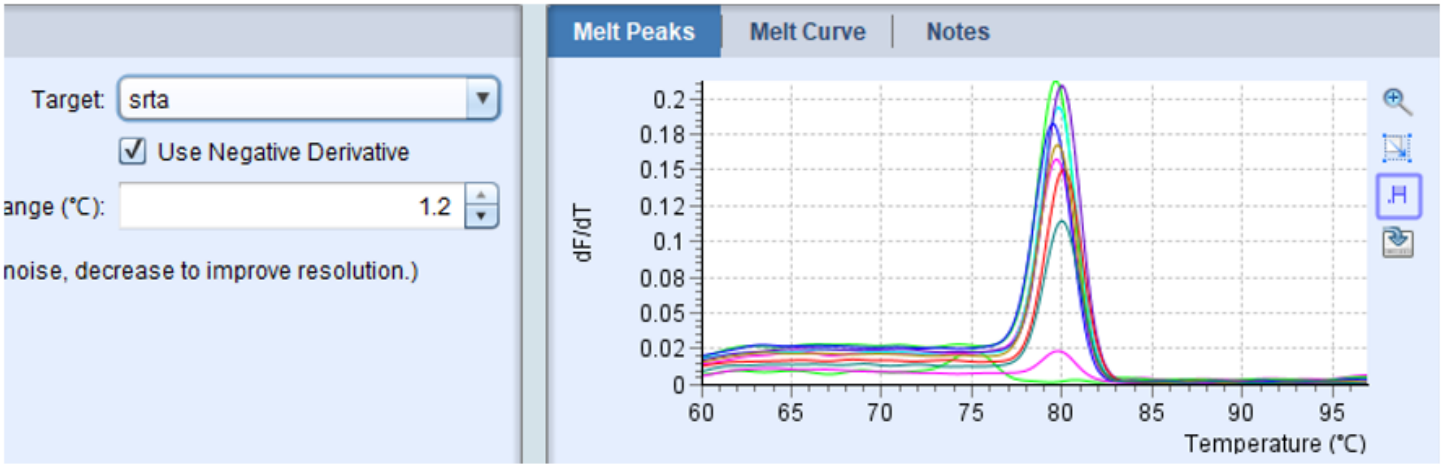

Figure 3

Figure 3

Tm curve of srtA

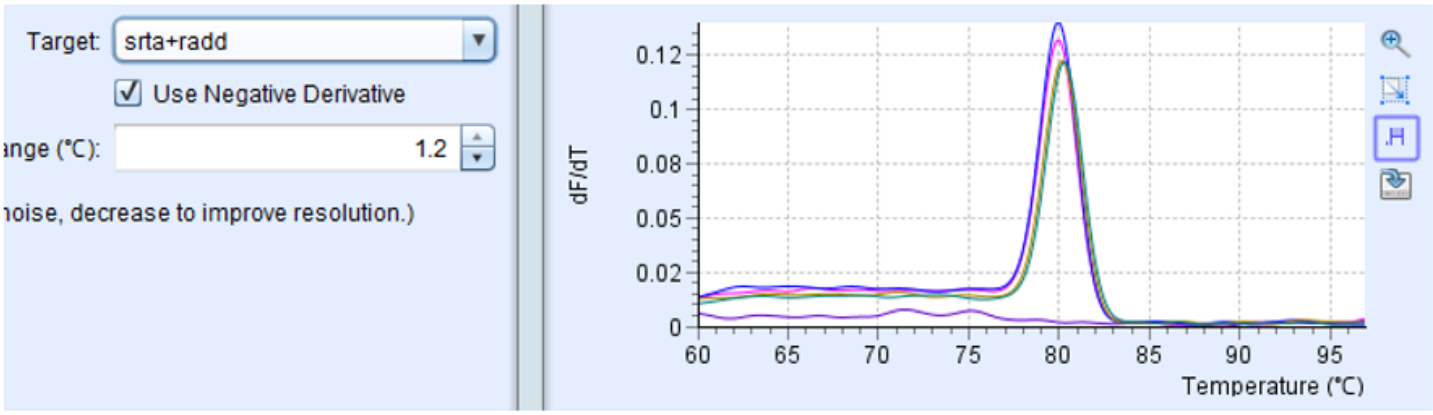

Figure 4

Figure 4

Tm curve of srtA+radD

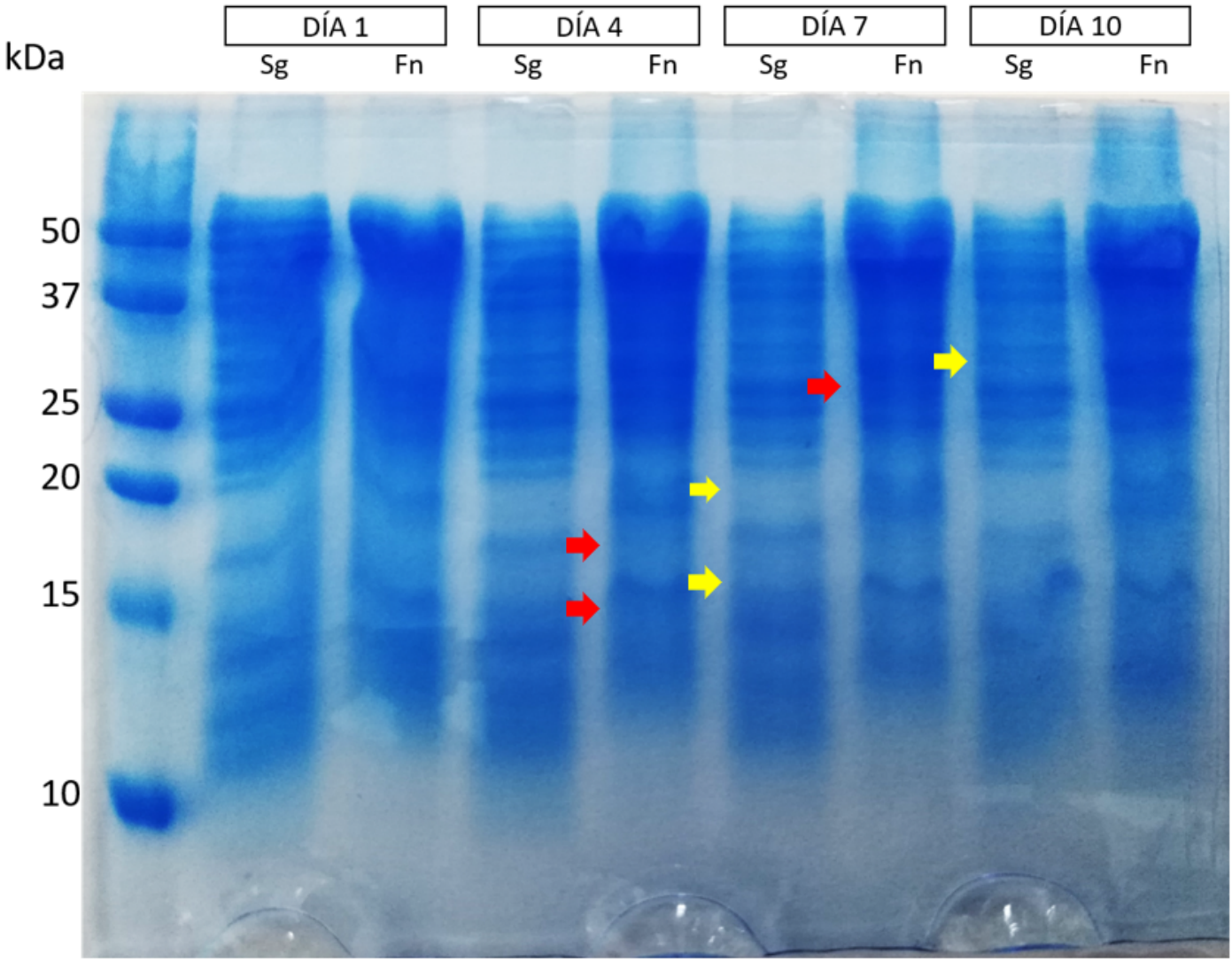

Figure 5

Figure 5

SDS-PAGE of proteins of the cytoplasmic fraction of individual cultures

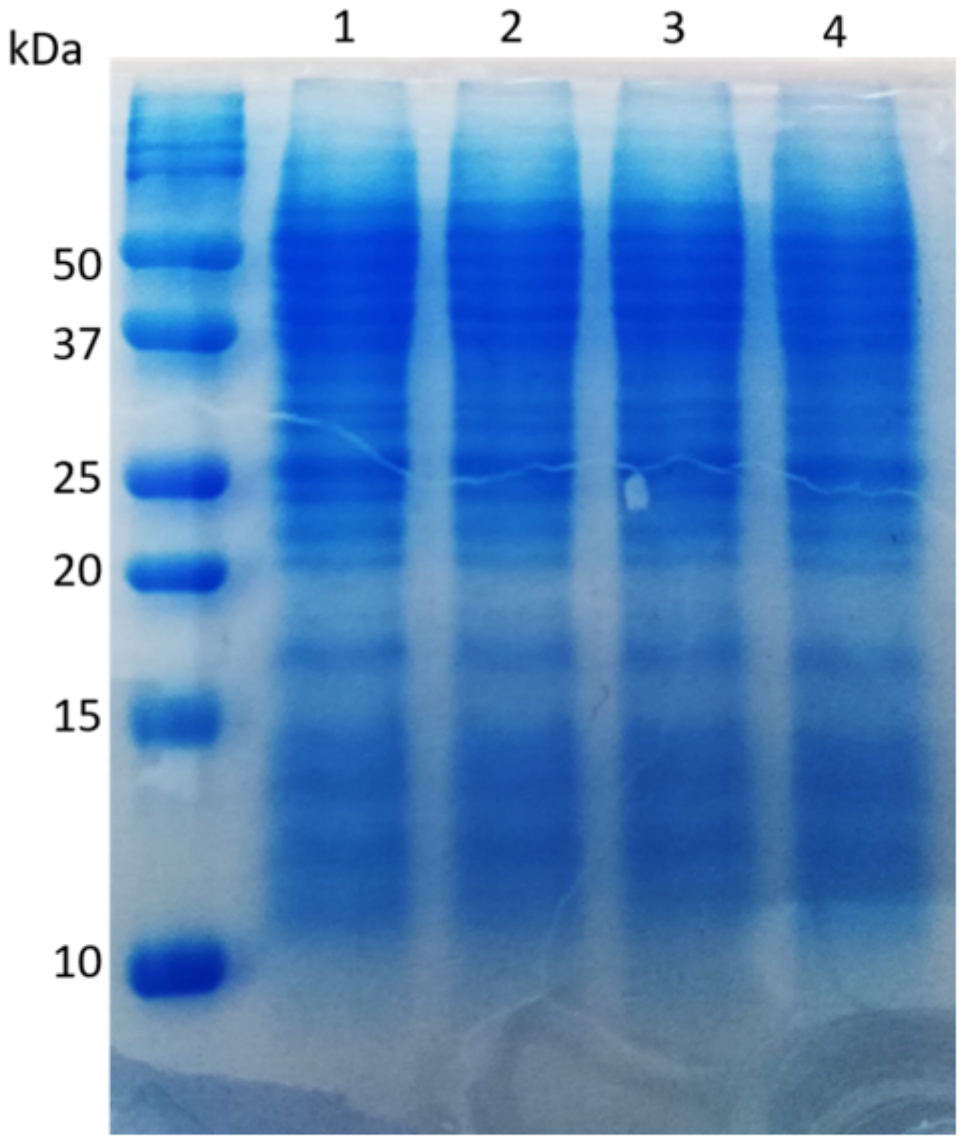

Figure 6

Figure 6

SDS-PAGE of proteins of the cytoplasmic fraction of the S. gordonii and F. nucleatum biofilm

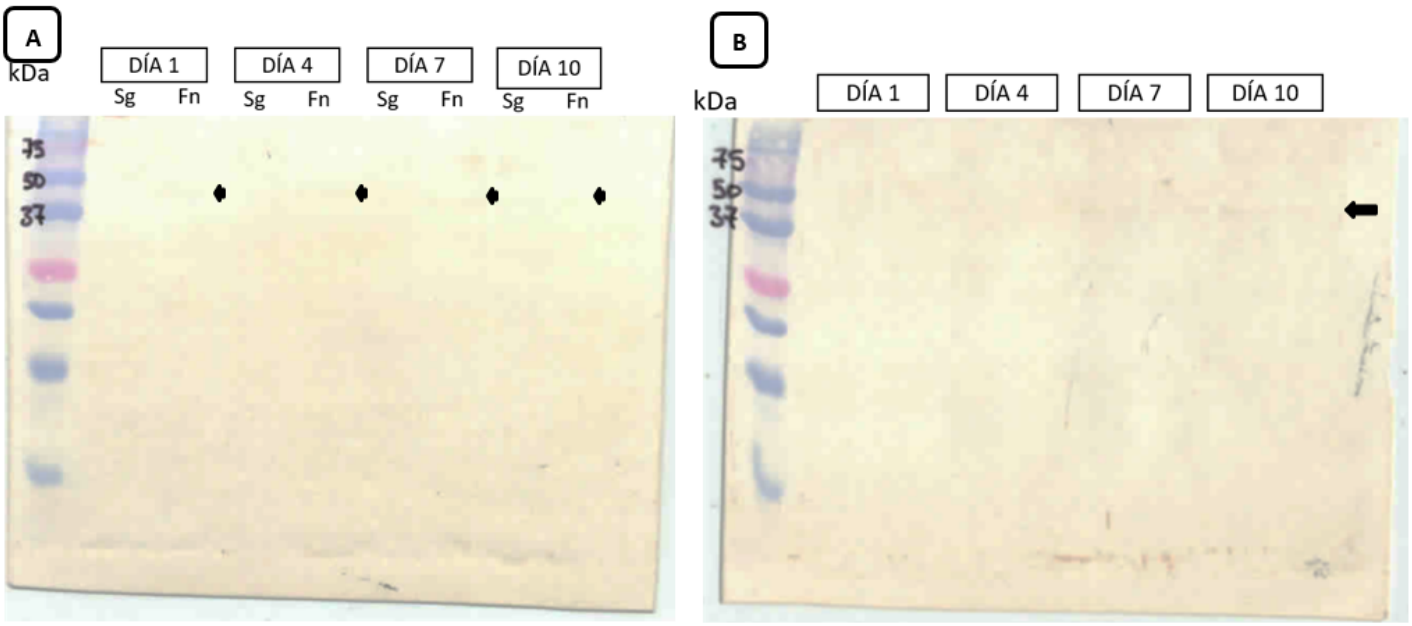

Figure 7

Figure 7

Immunodetection of GAPDH in the cytoplasmic fraction of individual cultures and biofilm

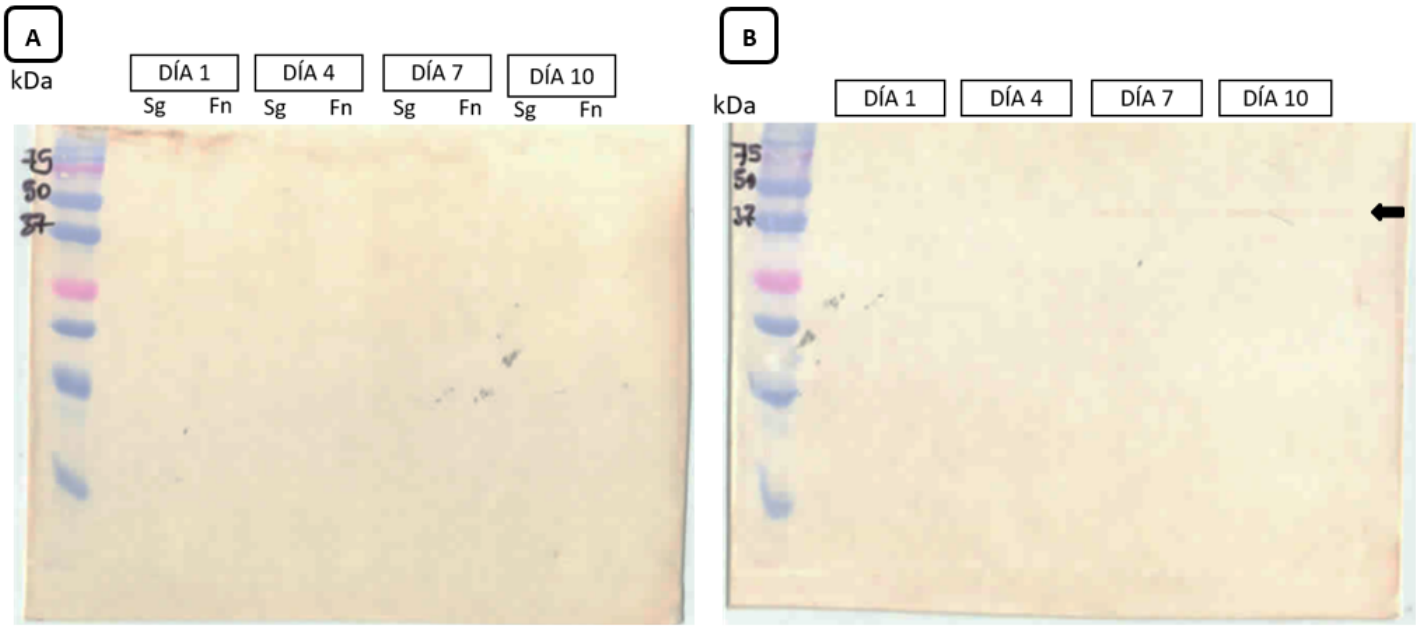

Figure 8

Figure 8

Immunodetection of HSP40 in the cytoplasmic fraction of individual cultures and the biofilm