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Streptococcus mutans and *Streptococcus dentisani* in dental biofilm of children with different caries status: a pilot study

Streptococcus dentisani e *Streptococcus mutans* no biofilme dental de crianças com diferentes estados de cárie: um estudo piloto

Claudia María BEDOYA-CORREA[1], María del Pilar ANGARITA-DÍAZ[2], Edwin Alonso ARISTIZABAL PADILLA[3], Paula Lucero LÓPEZ JIMÉNEZ[3], Julieth BUSTAMANTE ZAPATA[3]

1 - Universidad Cooperativa de Colombia, Facultad de Odontología, GIOM Group, Campus Medellín, Medellín, Antioquia, Colombia.
2 - Universidad Cooperativa de Colombia, Facultad de Odontología, GIOMET Group, Campus Villavicencio, Meta, Colombia.
3 - Universidad Cooperativa de Colombia, Facultad de Odontología, Campus Medellín, Medellín, Antioquia, Colombia.

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ABSTRACT

Objective: *Streptococcus mutans* is one of the etiological agents associated with caries due to its ability to metabolize carbohydrates and resist acidic environments. On the other hand, *Streptococcus dentisani*, shows characteristics associated with caries control due to its ability to inhibit growth of cariogenic bacteria. The aim of this work was to quantify the levels of *Streptococcus mutans* and *Streptococcus dentisani* from dental biofilm of children related to their caries situation. **Material and Methods:** After identification of morphologic characteristics of reference strains was performed, clinical isolates of biofilm compatible with these strains were selected and the Polymerase Chain Reaction technique was performed using species-specific primers. Biofilm samples from 25 children with caries and 21 without caries were collected to quantify the levels of *S. mutans* and *S. dentisani*. **Results:** There were statistically significant differences in the levels of *S. mutans* in the caries group and the levels increased as the severity of the carious lesion increased. By contrast, higher levels of *S. dentisani* were found in the caries-free group, although no statistically significant differences were found. In addition, the levels of *S. dentisani* decreased as the severity of the carious lesion increased. **Conclusion:** The increase in the frequency of *S. dentisani* in the caries-free group suggests the possibility of requiring minimum levels of this species in the dental biofilm to show an actual protective effect. It must also be considered that this effect might be related to intrinsic factors in children and the intraspecies genetic variability found in every individual.

KEYWORDS

Biofilm; Clinical isolates; Dental caries; *Streptococcus dentisani; Streptococcus mutans*.

RESUMO

Objetivo: *Streptococcus mutans* é um dos agentes etiológicos associados à cárie devido à sua habilidade de metabolizar carboidratos e resistir a ambientes ácidos. Já o *Streptococcus dentisani*, apresenta características associadas ao controle da cárie devido à sua capacidade de inibir o crescimento de bactérias cariogênicas. O objetivo deste trabalho foi quantificar os níveis de *Streptococcus mutans* e *Streptococcus dentisani* no biofilme dental de crianças em relação à situação de cárie destas. **Material e Métodos:** Após a identificação das características morfológicas das cepas de referência, foram selecionados do biofilme isolados clínicos compatíveis com essas cepas e realizada a técnica de Reação em Cadeia da Polimerase utilizando primers espécie-específicos. Amostras de biofilme de 25 crianças com cárie e 21 sem cárie foram coletadas para quantificar os níveis de S. mutans e S. dentisani. **Resultados:** Houve diferenças estatisticamente significativas nos níveis de S. mutans no grupo com cárie e os níveis aumentaram à medida que a gravidade da lesão cariosa aumentou. Por outro lado, foram encontrados níveis mais elevados de S. dentisani no grupo

sem cáries, embora não tenham sido encontradas diferenças estatisticamente significativas. Além disso, os níveis de S. dentisani diminuíram à medida que a gravidade da lesão cariosa aumentava. **Conclusão**: O aumento da frequência de S. dentisani no grupo livre de cárie sugere a possibilidade de exigir níveis mínimos desta espécie no biofilme dental para mostrar um efeito protetor real. Deve-se considerar também que esse efeito pode estar relacionado a fatores intrínsecos nas crianças e à variabilidade genética intraespécie encontrada em cada indivíduo.

PALAVRAS-CHAVE

Biofilme; Cárie dentária; *Streptococcus mutans*; *Streptococcus dentisani*; Isolados clínicos

INTRODUCTION

Dental caries is one of the most prevalent worldwide conditions that affects the general population from the early childhood [1]. It is defined as a dynamic, non-transmissible, multifactorial disease that is mediated by the dental biofilm and modulated by the diet that produces a net loss of minerals from the hard dental tissues due to the excessive synthesis of organic acids. It is determined by biological, behavioral, psychosocial and environmental factors [2-4].

The oral microbiota associated with dental caries is complex and exhibits a large number of acidogenic species [5]. When such microorganisms acquire a selective ecological advantage over other species, they alter the biofilm homeostasis and initiate the disease [6]. However, although dental caries is currently considered a disease of polymicrobial etiology, *S. mutans* is one of the most studied etiologic agents due to the ability to form a biofilm, produce organic acids (acidogenicity) and tolerate and survive in acid environments (aciduric) [7,8].

In 2014, a new streptococcal species from the mitis group, named *S. dentisani*, was isolated from the dental biofilm of healthy individuals who never developed caries. It is a Gram positive, immobile and facultative anaerobic coccus that grows at a pH of 7, even though it tolerates slightly acidic conditions [9,10]. Results from bioinformatics revealed that *S. dentisani* codifies multiple antimicrobial peptides, known as bacteriocins [11], and has the ability to express genes that activate the arginine metabolic pathway that buffers the acid in the biofilm when a reduction in the pH is detected [9,10,12,13]. Consequently, *S. dentisani* exhibits a double anticariogenic mechanism by inhibiting the growth of acidogenic bacteria and stimulating the formation of ammonium as a result of the arginine metabolism, thus controlling the pH to favor oral health. It might also act as a biomarker of beneficial oral bacteria [9-11].

S. mutans has been widely studied in children [14-16]. However, it has not been determined whether the proportion of this microorganism within the dental biofilm in children is conditioned by the presence of oral inhabitants associated to health conditions, specifically by *S. dentisani*. Therefore, the objective of this work was to quantify the levels of *S. mutans* and *S. dentisani* in the dental biofilm of children between 6 and 11 years of age with and without the presence of dental caries.

MATERIALS AND METHODS

Study design and population

A sample of 46 children selected by convenience was used in this transversal, descriptive study. Children were divided into two groups: 25 children were selected for the caries (C) group and 21 for the non-caries (NC) group. Children were selected from the dental clinic at School of Dentistry, Universidad Cooperativa de Colombia, La Inmaculada School and children from the DAMOS Protección from Divina Misericordia and Casa Verde Foundations in Envigado, Antioquia, Colombia. Children between 6 and 11 years of age was the inclusion criteria. Exclusion criteria included the presence of systemic conditions, orthodontic appliances, any other oral infectious condition different from dental caries and the use of antibiotic therapy during the previous three months before sample collection. Children from the NC group did not have a previous history of dental caries. Parents and/or legal representatives, as well as participating children, received detailed information on the study and signed an informed consent and an informed assent.

Clinical examination

In order to select the children, a clinical examination was performed, following a previous

calibration process, to identify the caries status using the ICDAS (International Caries Detection and Assessment System). According to ICDAS, children were grouped in a caries-free (NC) group for ICDAS 0; white spot lesions (WSL) group for ICDAS 1 and 2 and Cavity lesions (CL) group for ICDAS 3 to 6

Dental biofilm sample collection

Before sample collection, the visible index plaque was determined using the modified Silness and Löe index, and a classification of the oral hygiene index was performed according to the percentage of dental surfaces that showed oral biofilm as follows: good oral hygiene (0-15%), moderate (16-30%) and bad (31-100%) [17]. Biofilm samples of the caries and caries-free groups were collected from the buccal surface of the first permanent molar. In the caries group, samples were collected directly from the accumulated biofilm within the active carious lesion (white spot or cavity) only if such lesions were present at the first permanent molar. Samples were collected with a 1μL-calibrated and were transferred to 500μL of 0.9% saline with glass bids to disaggregate the biofilm and stored at 4°C for further processing within the following two hours.

Macroscopic characterization and identification of reference bacterial strains

Before sample collection, visual calibration was performed to recognize the morphology of *S. mutans* and *S. dentisani* colonies. *S. mutans* ATCC 25175 and *S. dentisani* CECT 7746 reference strains were used. *S. mutans* was grown in Mitis Salivarius (MS) agar (Difco Laboratories, Le pont de claix, France), supplemented with 1% potassium tellurite (Lab M Ltd, Lancashire, UK), 10% sucrose (Fisher Chemical, Waltham, USA) and 0.2 U mL^{-1} bacitracin (Sigma-Aldrich, Missouri, USA), and incubated in microaerophilic conditions at 37°C for 48 h in a 5% CO_2 atmosphere. *S. dentisani* was grown in Brain Heart Infusion (BHI) agar (Difco Laboratories, Le pont de claix, France) and incubated in aerophilic conditions at 37°C for 48 h. Colonies were macroscopically observed at 8-32X magnification and a highly detailed review and description of the phenotypic features of the colonies from each strain was performed.

Molecular identification of *S. mutans* and *S. dentisani*

In order to calibrate the macroscopic recognition of *S. mutans* and *S. dentisani* colonies, a molecular identification using the polymerase chain reaction (PCR) test was performed. 1μL of dental biofilm from NC and C children was obtained and serial dilutions in 0.9% saline solution were performed. For *S. mutans*, 100μL of 10^{-1}-10^{-3} dilutions were cultured in MS agar and incubated in microaerophilic conditions at 37°C for 48 h (5% CO_2). 100μL of 10^{-1}-10^{-3} dilutions were cultured in BHI agar and incubated in microaerophilic conditions at 37°C for 48 h to isolate colonies compatible with *S. dentisani*. After the incubation period, 35 clinical isolates phenotypically compatible with *S. mutans* and 35 with *S. dentisani* (70 isolates in total) were selected. For DNA extraction, each isolate was cultured in 5mL of BHI broth (Oxoid, Basingstoke, UK) supplemented with 5% sucrose and incubated under the aforementioned conditions.

Extraction and purification of genomic DNA

Pure cultures were centrifuged (Thermo Scientific Sorvall ST16, Waltham, USA) at 2800g for 3 min at 4°C. Cultures were washed two times with 500μL of ultra-pure water; the first was centrifuged at 2800g for 4 min at 4°C and the second at 21000g for 2 min at 4°C. The supernatant was discarded and the pellet was re-suspended in 150μL of Tris-EDTA buffer. Then, cell lysis by ebullition of the suspension at 95°C for 10 min was performed. Lastly, it was centrifuged at 21000g for 5 min at 4°C to recover the supernatant containing the genomic DNA and frozen at -20°C. DNA purification was performed by ethanol and 3M sodium acetate [15] precipitation. Spectrophotometric measures using a MicroDrop (Thermo Scientific Multiskan GO, Walthman, USA) were used to establish DNA concentration and purity.

Identification of *S. mutans* by PCR

Species-specific primers to amplify the glucosyltransferase B gene (*gtfB*) were used to confirm that the selected clinical isolates corresponded to *S. mutans* (Table I) [18]. Each 25μL of the PCR mix contained 1X Taq buffer with 20mM $(NH_4)_2SO_4$, 2.5mM of $MgCl_2$, 0.4μM of each

Table I - Primers and reference strains used for PCR identification of the clinical isolates from NC and C dental biofilm

Primers	Sequence (5' - 3')	Target	Size (bp)	Control strain
CkSdF	GTAAC CAACCGCCCAGAAGG	*arcC*	77	*S. dentisani*
CkSdR	CCGCTTTCGGA CTCGATCA			CECT 7746
GTFB-F	ACTACACTTTCGGGTGGCTTGG	*gtfB*	517	*S. mutans*
GTFB-R	CAGTATAAGCGCCAGTTTCATC			ATCC 25175

oligonucleotide primer, 0.2mM of each dNTP, 0.5U of Taq DNA polymerase and 2μL of DNA at a concentration of 50ng. Samples were amplified in a thermal cycler (MultiGene OptiMaxl, Edison, NJ, USA) under the following PCR conditions: initial denaturalization at 94°C for 1 min, followed by 34 denaturalization cycles at 94°C for 30 s, hybridization at 60°C for 35 s and extension at 72°C for 50 s. The final step was extension for 7 min. PCR amplified products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide (0.5 μg mL^{-1}) and photographed under UV light. A marker of DNA molecular weight of 1kb in each gel was used. A negative control (Master mix without DNA) and a positive control (*S. mutans* 25175) were included.

Identification of *S. dentisani* by PCR

Species-specific primers to amplify a segment of the carbamate kinase gene (*arc*) were used to confirm that the selected clinical isolates corresponded to *S. dentisani* (Table I) [10]. Each 25μL of the PCR mix contained 1X Taq buffer with 20mM $(NH_4)_2SO_4$, 2.5mM of $MgCl_2$, 0.4μM of each oligonucleotide primer, 0.2mM of each dNTP, 0.5U of Taq DNA polymerase and 2μL of DNA at a concentration of 50ng. Samples were amplified in a thermal cycler (MultiGene OptiMaxl, Edison, NJ, USA) under the following PCR conditions: initial denaturalization at 94°C for 1 min, followed by 34 denaturalization cycles at 94°C for 30 s, hybridization at 65°C for 40 s and extension at 72°C for 50 s. The final step was extension for 7 min. PCR amplified products were analyzed by 3.5% agarose gel electrophoresis, stained with ethidium bromide (0.5 μg mL^{-1}) and photographed under UV light. A marker of DNA molecular weight of 50pb in each gel was used. A negative control (Master mix without DNA) and a positive control (*S. dentisani* CECT 7746) were included.

Microbiologic processing of dental biofilm samples

After validation of the macroscopic recognition of the *S. mutans* and *S. dentisani* clinical isolates with the molecular identification, 1μL of dental biofilm from NC and C children was obtained. Serial dilutions were performed and inoculated into the aforementioned culture media. Samples were incubated for 48 h at 37°C. After incubation, *S. mutans* and *S. dentisani* colony count was performed and reported as colony-forming units per microliter of dental biofilm (UFC/μL).

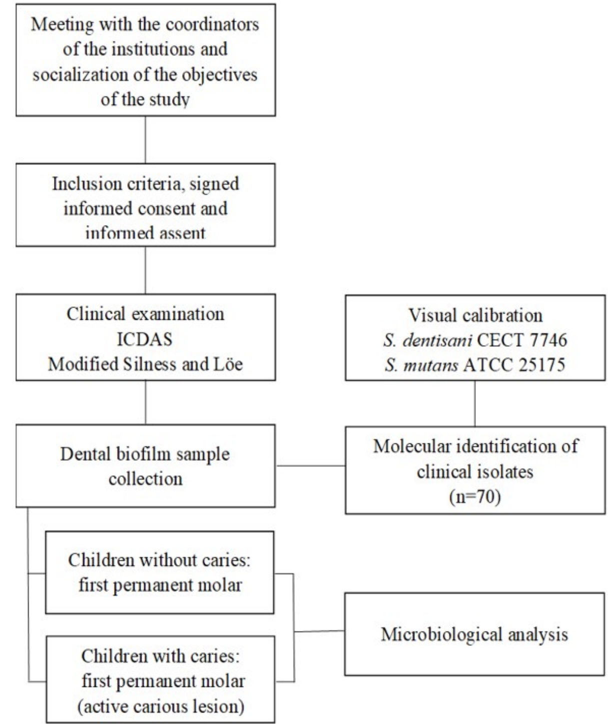

Figure 1 - Phases of the study carried out to quantify the levels of *S. mutans* and *S. dentisani* and in the dental biofilm of children with and without dental caries.

Figure 1 summarizes the phases of the study carried out to quantify the levels of *S. mutans* and *S. dentisani* in the dental biofilm of children with and without dental caries.

Statistical analysis

Statistical analysis was performed with the SPSS V.25 (IBM Corp, Armonk, NY, USA) software.

A descriptive analysis of the oral health status of the studied population by estimation

of proportions or summary measures according to the nature of the variables was carried out. In order to compare the number of *S. mutans* and *S. dentisani* viable cells, as well as the numbers obtained in the different phases of the carious lesion in the C group, a bivariate analysis using the Mann Whitney U test after verification of the normality using the Shapiro Wilk test was performed. $P<0.05$ values were considered statistically significant for all the analyses.

RESULTS

Out of the 46 samples of dental biofilm, 45.7% corresponded to the NC group and 54.3% to the C group. It was determined that all children showed poor oral hygiene after evaluation of the modified Silness y Löe plaque index, although 19% of the NC and 4% of the C children exhibited moderate oral hygiene. The C group showed a higher percentage of children with deficient oral hygiene compared to the NC group. After clinical evaluation, a recodification of the ICDAS system was performed and three categories were created: non-caries (NC), white spot lesion (WSL) and cavity lesion (CL). Table II shows the distribution of the evaluated characteristics.

After visual calibration and identification of the morphologic characteristics of the 70 clinical isolates of dental biofilm, PCR tests demonstrated that 35 (100%) corresponded to *S. mutans* and 35 (100%) to *S. dentisani*.

Table II - Characteristics of the evaluated children

	NC group (n=21)	C group (n=25)
Age in years (Mean ± SD)	8 ± 2	9 ± 2
Gender	**n (%)**	**n (%)**
Male	7 (33.3)	17 (68)
Female	14 (66.7)	8 (32)
Oral hygiene	**n (%)**	**n (%)**
Good	0 (0)	0 (0)
Moderate	4 (19)	1 (4)
Deficient	17 (81)	24 (96)
ICDAS		
Categories		**n (%)**
NC		21 (45.6)
WSL		13 (28.3)
CL		12 (26.1)

SD: Standard deviation. NC: Non-caries. WSL: White spot lesion. CL: Cavity lesion.

Quantification of *S. mutans* and *S. dentisani* in the dental biofilm from children with and without caries

Statistically significant differences were found in the number of *S. mutans* viable cells in both groups (p=0.007), and when a comparison between NC and CL groups was made (p=0.001). Even though the distribution frequency of *S. dentisani* in the NC group was a little higher, no statistically significant differences were found between groups (p=0.175), nor when a comparison between NC and WSL groups (p=0.38) and CL group (p=0.17) (Table III) was made.

Relation between the presence of *S. mutans* and *S. dentisani* in different types of carious lesions

Figure 2 shows the distribution of *S. mutans* was higher in children with more severe cavity

Table III - Number of *S. mutans* and *S. dentisani* viable cells from non-caries group (NC), caries group (C), white spot lesion (WSL) and cavity lesion (CL)

CFU μL^{-1}	Categories	*p*-value
***S. mutans* Median (IQR)**	**NC group**	0.007**
	1.5×10^2 (0.0 - 2.4×10^3)	
	C group	
	5.0×10^3 (3.5×10^2 - 3.7×10^4)	
	NC group	0.18
	1.5×10^2 (0.0 - 2.4×10^3)	
	WLS	
	7.0×10^2 (5.0×10^1 - 5.8×10^4)	
	NC group	0.001**
	1.5×10^2 (0.0 - 2.4×10^3)	
	CL	
	2.3×10^4 (4.1×10^3 - 6.0×10^4)	
CFU μL^{-1}	**Categories**	***p*-value**
***S. dentisani* Median (IQR)**	**NC group**	0.175
	5.4×10^4 (1.2×10^4 - 1.5×10^5)	
	C group	
	2.4×10^4 (9.5×10^3 - 5.8×10^4)	
	NC group	0.38
	5.4×10^4 (1.2×10^4 - 1.5×10^5)	
	WLS	
	3.8×10^4 (1.0×10^4- 6.4×10^4)	
	NC group	0.17
	5.4×10^4 (1.2×10^4 - 1.5×10^5)	
	CL	
	1.1×10^4 (5.0×10^3 - 1.0×10^5)	

**p<0.05. Mann–Whitney U test. IQR: Interquartile range. NC: Non-caries group, C: Caries group, WSL: White spot lesion, CL: Cavity lesion.

lesions and progressively reduced as the severity of the lesion decreased. In healthy patients, such number was very low. Despite finding significant differences and a higher number of *S. mutans* viable cells in group C, *S. mutans* was absent in three children with active carious lesions (WSL, n=2; CL, n=1). However, considerable amounts of *S. dentisani* were found in the same samples (Table IV). In contrast, the proportion of *S. dentisani* was higher in the dental biofilm of children from the NC group and gradually decreased as the severity of the lesion increased (Figure 3). It is relevant to highlight that *S. mutans* was absent in the biofilm of 9 NC children, but levels of *S. dentisani* were high (median 1.2×10^5 CFU μL^{-1}) (Table IV).

Table IV - Prevalence of *S. dentisani* in samples from the biofilm of children with undetectable levels of *S. mutans*

S. mutans	*S. dentisani*
CFU μL^{-1}	CFU μL^{-1}
NC	
0.0	1.1×10^4
0.0	2.7×10^5
0.0	1.3×10^4
0.0	2.8×10^5
0.0	5.6×10^4
0.0	1.6×10^5
0.0	4.5×10^2
0.0	2.9×10^5
0.0	1.2×10^5
WSL	
0.0	7.1×10^4
0.0	2.4×10^4
CL	
0.0	1.0×10^4

NC: Non-caries, WSL: White spot lesion, CL: Cavity lesion.

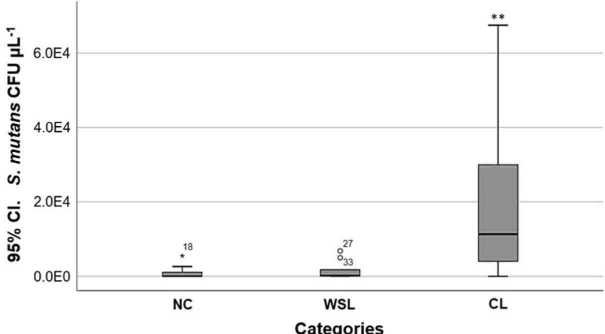

Figure 2 - Distribution of *S. mutans* according to caries status. Number of viable cells. ICDAS recodification: NC, without caries; WSL, white spot lesion; CL, cavity lesion. **$p<0.05$. Mann–Whitney U test. Significant differences between NC and CL groups.

DISCUSSION

Conventional microbiological cultures constitute an adequate tool to quantify and characterize the isolated microorganisms in the oral cavity. However, it is necessary to implement additional molecular techniques, such as PCR, to validate the identification of bacterial species [19]. Microbiological and molecular methods employed in the current investigation allowed for a correct macroscopic characterization and visual calibration of the colonies from the studied bacterial strains before determining the prevalence of *S. mutans* and *S. dentisani* in the biofilm of children with and without caries.

S. mutans was detected in most samples from dental biofilm of children irrespective of their caries status. However, statistically significant differences have been found in the levels of *S. mutans* in children with and without dental caries ($p<0.05$). This species was found in higher numbers in the dental biofilm from children with cavity carious lesions and such numbers decreases considerably in children without caries. These results are in agreement with published results by other authors who found a positive correlation between the levels of *S. mutans* and the onset and progression of dental caries [20-22]. Likewise, other investigations have demonstrated a higher genetic variability in clinical isolates from cavity lesions, which may be related to the physiopathology of *S. mutans* [15,23].

The results of the present work suggest that *S. mutans* plays a significant role in the progression of dental caries by participating in the destruction of the hard dental tissues. Therefore, it is mandatory to develop treatment and preventive strategies directed at maintaining the homeostasis

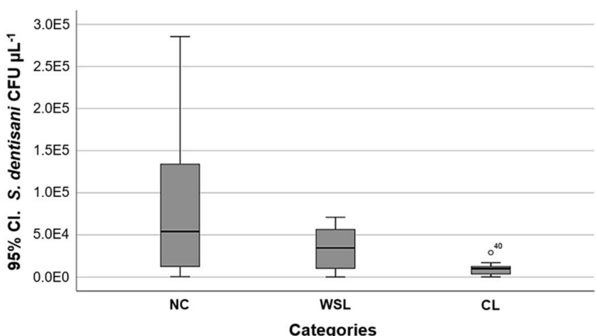

Figure 3 - Distribution of *S. dentisani* according to caries status. Number of viable cells. ICDAS recodification: NC, without caries; WSL, white spot lesion; CL, cavity lesion.

of the oral cavity, such as probiotics, and reducing the levels of *S. mutans* and other acidogenic species that coexist within the dental biofilm.

S. dentisani has exhibited beneficial effects that may assist in promoting oral health, including antibacterial activity against oral pathogens by producing bacteriocins, increasing the pH by producing ammonium and anti-inflammatory activities that favor the immune response thus exhibiting probiotic properties [10,12,24]. 100% of the samples exhibited the presence of *S. dentisani*, despite the absence of significant differences between groups, it was possible to find *S. dentisani* in biofilm samples of children with undetectable levels of *S. mutans.* In addition, a higher proportion of *S. dentisani* was found in the samples from the NC group and such numbers decreased as the severity of the carious lesion increased. This finding may indicate that this bacterial species probably plays a protective role against acidogenic species that promote the onset of carious lesions. But, it is possible that the beneficial effect of *S. dentisani* requires minimum levels of this species to be effective, although the fact that this protective effect responding to intra-species genotypic variability and the presence of *S. dentisani* specific genotypes cannot be ruled out. It is also important to consider intrinsic factors in children, such as sugar consumption, physico-chemical properties of saliva and use of fluoride-containing products. Further epidemiological and molecular studies are essential to understand the protective role of this species in the control of cariogenic microorganisms.

Similar results were found in a study that assessed the biofilm from 100 Colombian children with and without dental caries. The presence of *S. dentisani* was identified and quantified by qPCR in all the patients, but higher numbers were found in the children without caries, despite that the differences were not statistically significant [25]. López-Santacruz et al. [26] quantified by qPCR the presence of *S. dentisani* in supragingival biofilm samples collected from different teeth of Mexican children (25 without and 29 with caries) and found higher numbers of this species in the group without caries and a negative correlation between the severity of the carious lesion and the presence of *S. dentisani*. These authors concluded that this species seems to be present in higher numbers in the dental biofilm of children without dental caries.

In vitro studies have demonstrated a significant inhibitory effect by *S. dentisani* in the growth of several oral pathogens. However, such experiments were performed using reference strains, planktonic cultures, and controlled laboratory conditions [10,12,13]. Nonetheless, the evidence shows that multiple microbial and metabolic interactions occur under *in vivo* conditions that may promote eubiosis status or alter the balance within the oral cavity [27,28]. Therefore, the results of this investigation are not consistent with published literature since the processed samples were obtained directly from dental biofilm and the oral cavity exhibits a high heterogeneity. In addition, bacterial adaptation and colonization to specific microsites may vary considerably among individuals [27]. The dental biofilm is composed by a multispecies complex that may interact synergistically or antagonistically, thus interrupting the antibacterial effect exhibited by *S. dentisani.*

Limitations of the current investigation include the low sample size and intrinsic variability of the children within each group. The lack of statistical significance may be related to the fact that biofilm was collected from only one tooth, which does not represent the complete oral cavity. Therefore, it is recommended to continue this work in the future using pooled samples from different teeth to ensure that the assessed bacterial composition is representative of the whole dental plaque microbial community.

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Author’s Contributions

CMBC, MDPAD: Conceptualization, Methodology, Resources, Data Curation, Writing – review and editing, Supervision, Project administration and Funding Acquisition. CMBC, MDPAD, EAAP, PLLJ, JBZ: Investigation, Writing – original draft preparation.

Conflict of interest

The authors declare that they have no conflict of interest.

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Regulatory Statement

The study was approved by the ethics committee from Universidad Cooperativa de Colombia, Medellín campus (004/2018).

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Claudia María Bedoya-Correa
(Corresponding address)
Universidad Cooperativa de Colombia, Facultad de Odontología, Medellín, Antioquia, Colombia
Email: claudia.bedoyac@campusucc.edu.co

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