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ANTAGONISTIC POTENTIAL OF *S. SALIVARIUS*, *S. ORALIS* AND *S. DENTISANI* ON DIFFERENT BIOTYPES OF *S. MUTANS*

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Abstract

Dental caries is an infectious process that results in tooth decay, where *Streptococcus mutans* is considered the main causing agent. An alternative which might prevent or control this disease is to identify microorganisms with *S. mutans* antagonistic potential.

This study, encompassed within an Oral Microbial Ecology approach had as an objective to identify *Streptococcus salivarius, Streptococcus oralis* and *Streptococcus dentisani* strains with *S. mutans* antagonistic activity.

Saliva samples were collected from 36 children without tooth decay and cultured on Lamb's Blood Agar and Mitis Salivarius Agar. After incubation at 37 °C under aerobic atmosphere for 48 hours, *S. salivarius*, *S. oralis* and *S. dentisani* confirmed colonies were subjected to biochemical tests. To determine the antagonistic effect the double layer in agar technique was employed.

Sixteen, 17 and 14 strains of *S. salivarius, S. oralis* and *S. dentisani* were identified representing a frequency of 44.4% (16/36), 47.2% (17/36) and 38.9% (14 / 36), respectively. *S. salivarius*, *S. oralis* and *S. dentisani* presented different modes of antagonistic action on *S. mutans* with inhibition halos from 6 to 15 mm. Only two strains, one from *S. oralis* and the other from *S. dentisani*, demonstrated complete antagonistic activity (100%) on the 12 *S. mutans* indicator strains.

In conclusion, in the present study, 16, 17 and 14 strains of *S. salivarius*, *S. oralis* and *S. dentisani*, respectively with great antagonistic potential on different biotypes of *S. mutans* strains were identified, which after further characterization could be employed in strategies for the prevention or control of dental caries and other infections.

Keywords: Antagonism, *S. salivarius, S. oralis, S. dentisani, S. mutans.*

Introduction

Dental caries is an infectious, multifactorial, localized, post-eruptive and transmissible pathological process that leads to the destruction of hard dental tissue (1). The main microorganism associated with caries is *Streptococcus mutans*, an acidic and aciduric microorganism that usually colonizes the oral cavity (1–3). Furthermore, different investigations have demonstrated a strong correlation between *S. mutans* quantity in the oral cavity and caries prevalence and incidence of (1–3).

The recognition of *S. mutans* as the most significant contributor in initiating the caries process has led to the design of prevention measures aimed at eliminating or reducing this microorganism in the oral cavity (2–4). Different strategies have been proposed for dental caries prevention and control (3–5), where the measures by themselves are effective. However, if they are not implemented and properly organized, the expected results will not be achieved (4).

Health institutions actions in dental caries prevention and control have not been sufficient to achieve a significant reduction of this disease. In Colombian children with the ages of three and five years old, this disease was found in 47.1% and 62.1% of this population, respectively (4). This fact highlights the importance of studying all the possible techniques or specific prevention measures against this disease (6–8).

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An alternative, which is gaining more acceptance is the search and application of microorganisms with antagonistic action on *S. mutans* strains. Surely this strategy can prevent or control the disease, without any negative effects on other the oral microbiota species (5,7,8).

In oral health *Streptococcus salivarius* and *Streptococcus oralis* are very important commensal bacterial species. Moreover, *Streptococcus dentisani*, has been recently described as a bacterium that belongs to the Streptococcus mitis group (9). At present, these species of the Streptococcus genus are being evaluated as potent probiotics for use in the control of dental caries and other infections(10–14).

The aims of this work were twofold: 1. To isolate and identify *S. salivarius*, *S. oralis* and *S. dentisani* from the oral cavity in children without dental caries; and 2. To determine the antagonistic action of these three bacterial species on different biotypes of *S. mutans*. This work was encompassed within "Oral Microbial Ecology" research.

Methods

Isolation and identification of *S. salivarius*, *S. oralis* and *S. dentisani*

a. Study population

This was a pilot exploratory study that included a population selected by convenience, which included 36 children without dental caries from a preschool in Bogota, between the ages of three and five years old. Prior to the study parents or guardians signed the informed consent that complied with the bioethical requirements for sample collection and handling. The absence or presence of dental caries was determined by a dental clinician who established the dmft index (decayed, missing and filled teeth) according to the World Health Organization criteria. The 36 children did not present systemic infectious diseases and were not on antimicrobial treatment for at least the last seven days prior to sample collection. Saliva samples were collected between 8:00 and 10:00 am, with prior commitment by the children and parents not to eat in the morning and not to brush their teeth before sample collection. Through gentle suction with a plastic pipette, a saliva sample (0.2 - 1 ml) was collected from each child. Sample was placed on ice and transported to the laboratory for processing. Saliva sample collection was under the responsibility of the same researcher who determined the dmft index.

b. Sample processing

For *S. salivarius*, *S. oralis* and *S. dentisani* isolation and identification, collected saliva samples were vortexed for 15 seconds and serially diluted (1/10, 1/100 and 1/1000) with 0.05 M phosphate buffer. Subsequently, for selective isolation 50 ul of the respective dilutions were seeded in duplicate in

Lamb's Blood Agar and Mitis Salivarius Agar (MSA). The culture media was incubated at 37 °C for 48 hours under aerobic atmosphere conditions. After performing an alpha hemolytic colony selection, Gram stain, catalase test, and biochemical tests were carried out. To screen for the *S. mitis* group, including *S. mitis*, *S. oralis* and *S. dentisani* and the *S. salivarius* group (*S. salivarius* and *S. vestibularis*) the following biochemical tests were performed: hydrolysis of arginine and esculin, VP test, mannitol and sorbitol fermentation, urease production and growth in 6.5% NaCl. To verify the bacterial species under study, the following biochemical tests were completed: inulin acid production, lactose, melibiose, n-acetylglucosamine, raffinose, salicin, sucrose, fructose, glucose, maltose, production of alpha arabinosidase and alkaline phosphatase. Strain identification was also corroborated by the commercial Api 20S system (bioMerieux, Marcy-létoile, France), from which reactions on key substrates were obtained according to the study by Camelo-Castillo A et al (9).

Determination of the antagonistic activity of *S. salivarius*, *S. oralis* and *S. dentisani* on biotypes of *S. mutans*

Determination of the antagonistic activity was carried out with the double layer test in Brain Heart Infusion (BHI) agar, in which the producing strains (*S. salivarius, S. oralis* and *S. dentisani*) acted on the indicator strains (susceptible to antagonistic action), and corresponded in previous projects to the most frequent biotypes of *S. mutans* isolated and biotyped by the api-ZYM system (bioMérieux, Marcylétoile, France).

a. Preparation of producer strains

To this end, two to three colonies of each strain of *S. salivarius*, *S. oralis* and *S. dentisani* previously cultivated on BHI Agar were suspended in BHI broth and incubated under aerobiosis conditions at 37 °C for 48 hours. From this suspension, 2 ul were seeded with a micropipette on the BHI Agar (1.5% agar and 2% yeast extract) and incubated at 37 °C under aerobiosis conditions (H2: CO2: N2 10:10:80) for 48 hours. After this incubation the indicator strains were placed on the producer strains.

b. Preparation of indicator strains

The indicator strains corresponded to the most frequent *S. mutans* biotypes from previous investigations from our research group. To this end, 2 to 3 colonies of each *S. mutans* strain from a previous culture on BHI Agar were resuspended in BHI broth and incubated under aerobiosis at 37 ° C for 48 hours. Subsequently, 0.5 ml of this suspension was taken and mixed with 5 ml BHI Agar (0.75% agar and 2% yeast extract) and immediately placed on the BHI agar (1.5% agar and 2% yeast extract), where the producer strains previously prepared (Preparation of producer strains) were grown. Last, petri dishes were incubated at 37 °C under aerobiosis conditions (H2: CO2: N2 10:10:80) for 48 hours. The antagonistic action was evidenced by an inhibition halo (greater than 4 mm) generated by the producer strain on the indicator strain (15).

Statistical analysis of the antagonistic effect

A Student's-*t* test was applied for independent paired samples by means of the complement -Data Analysis- from the Microsoft Excel office automation tool. Statistically significant differences between the paired samples (*S. salivarius* - *S. oralis*; *S. oralis* - *S. dentisani*; *S. salivarius* - *S. dentisani*) were determined on the 12 biotypes of the selected *S. mutans* biotypes. In total, 36 paired analyzes were obtained. Before determining Student's *t* test statistical significance, data distribution was verified by a Fisher's F test and the degrees of freedom of each binomial were calculated. Last, to validate the statistical procedure performed in Microsoft Excel, R Studio software was employed.

Results

Identification of *S. salivarius, S. oralis and S. dentisani*

In total, 47 isolates belonging to these three species were identified. Of these, 16, 17 and 14 isolates corresponded respectively to *S. salivarius*, *S. oralis* and *S. dentisani*, which represented a frequency of 44.4% (16/36), 47.2% (17/36) and 38.9% (14/36), respectively, (Table 1). Microorganism's presence was observed as follows: in five children the three microorganisms were presented simultaneously, in six children *S. salivarius* and *S. oralis* were presented, and in five children *S.*

salivarius and *S. dentisani* were present. On the other hand, five and three children presented *S. oralis* and *S. dentisani*, respectively, and one child presented *S. oralis* and *S. dentisani*.

Selected *S. mutans* Biotypes (indicator strains) to perform *S. salivarius*, *S. oralis* and *S. dentisani* bacterial antagonism tests

To this end, biotypes XV, XI and XII, and biotypes XXVI, XX and XXXVI were included, from children with and without dental caries, respectively (16). The biotypes were selected, due to their frequent presence in a previously studied Colombian population (16). Two isolates from each of the six selected biotypes were used for antagonism tests, representing a total of 12 strains.

Antagonistic effect of *S. salivarius*, *S. oralis* and *S. dentisani*

The antagonistic effect was determined in all the strains of *S. salivarius*, *S. oralis* and *S. dentisani* isolated in this study (Table 2). *S. salivarius*, *S. oralis* and *S. dentisani* strains presented different modes of antagonistic action ranging from 2 to 12 indicator strains of *S. mutans* and inhibition halos from 6 to 15 mm (Table 2). In detail, *S. salivarius* presented antagonism from 5 to 10 indicator strains and inhibition halos from 6 to 12 mm; *S. oralis* presented antagonism from 3 to 12 indicator strains and inhibition halos from 6 to 15 mm. Last, *S. dentisani* presented antagonism from 3 to 12 indicator strains and inhibition halos from 6 to 14 mm. Only two strains, one from *S. oralis* (strain 14) and one from *S. dentisani* (strain 10), demonstrated complete antagonistic activity (100%) on the 12 *S. mutans* indicator strains (Table 2).

Statistically significant differences were identified only in six paired samples with a 95% confidence interval,: *S. salivarius* - *S. oralis* ($p = 0.006$) in the inhibition of the XXXVI-2 biotype of *S. mutans*, *S. oralis* - *S. dentisani* ($p = 0.02$) in the inhibition of biotype XXXVI-2 of *S. mutans*, *S. salivarius* - *S. dentisani* ($p = 0.02$) in the inhibition of biotype XII-2 of *S. mutans*, *S. salivarius* - *S. dentisani* ($p = 0.02$) in the inhibition of the XX-1 biotype of *S. mutans*; and *S. salivarius* - *S. dentisani* ($p = 0.04$) in the inhibition of the XX-2 biotype of *S. mutans*.

Discussion

Survival and proliferation of a certain microorganism takes place when it eliminates or displaces another competent organism from its ecological niche, with very intense competition due to species diversity (17). Studies on antagonism in dental caries began in 1972 with tests carried out with *S. mutans* and *Veillonella alcalescens* (18). It was demonstrated *V. alcalescens* growth was influenced by plaque's anaerobic environment and by the amount of lactic acid produced by plaque-forming organisms (18). The search for bacteria with antagonistic capacity and their application in microbiological control to displace virulent native strains of *S. mutans* has progressed through the years. Different studies suggest the antagonistic capability is due to bacteriocin production, which could confer a great potential to displace *S. mutans* strains causing dental caries (5,19–21).

In the present study, by means of bacterial phenotypic identification and culture techniques, *S. salivarius*, *S. oralis* and *S. dentisani* were identified at frequencies, of 44.4% (16/36), 47.2% (17/36) and 38.9% (14/36), respectively. Likewise, in the study by Conrads Georg et al (14), in 35 healthy caries-free volunteers, *S. dentisani* was also identified with culture techniques from saliva samples with a frequency of 20%. In contrast, in the study by Angarita-Díaz et al (22), in patients with and without dental caries *S. dentisani* was observed in a frequency of 100%. The difference in the Angarita-Díaz et al study (22), with the present one and that of Conrads Georg et al (14), was the use of quantitative RT-PCR technique, which provides greater sensitivity when amplifying and quantifying bacterial DNA. However, it has the limitation of not having live bacteria to subsequently carry out other phenotypic characterization tests, which are performed with bacterial culture. Nevertheless, the use of these two techniques together could lead to an improved recognition of *S. dentisani* in children with and without dental caries to establish the actual role of this microorganism in healthy states in oral microbial ecology.

The double layer test is commonly used to demonstrate bacteriocins or similar substances action produced by certain bacteria on the chosen

indicator strains, suggesting the antagonistic or inhibitory capacity was due to the action of bacteriocins or similar substances (7,15). In this study, the antagonistic effect of *S. salivarius* (n = 16), *S. oralis* (n = 17) and *S. dentisani* (n = 14) on different biotypes of *S. mutans* was determined. With the use of 12 *S. mutans* indicator strains the antagonistic capacity of the strains evaluated was high (100%) and presented a varied profile. In contrast, in the work by Balakrishnan M et al (7), where strains of various species were used as indicators, 39 strains belonging to the Streptococcus, Enterococcus and Staphylococcus genera were identified, revealing an antagonistic capacity of 14.3%. Kamiya RU et al (23) evaluated against 12 *S. mutans* indicator strains. They described bacteriocins production in 319 *S. mutans* strains isolated from eight patients with caries and from eight patients without caries, and established an inhibitory effect in 254 strains (79.62%). On the other hand, in the study by Conrads Georg et al (14), the bacterial antagonism was mediated by bacteriocins of the *S. dentisani* 7746 (AB-Dentisanium) reference strain. This strain inhibited five of six (83%) *S. mutans* strains and other species of the genus Streptococcus; however, did not have an effect on *S. sanguinis* OMI 332, *S. salivarius* OMI 315, *S. parasanguinis* OMI 335, *S. vestibularis* OMI 238 and the strain of intestinal origin *S. dysgalactiae* OMI 339. The differences found in investigations on the production of the antagonistic effect by bacteriocins are most likely accounted by diverse test conditions (solid medium, liquid medium, bacterial inoculum, temperature) and the bacteria number and diversity used as an indicator in the tests (7,14,23).

S. salivarius, *S. oralis* and *S. dentisani* are very important commensal bacterial species in oral health. At present, these three bacterial species are being explored as powerful probiotics for use in the oral cavity, control of dental caries and other infections in the medical and dental field (10,12,13,24). *S. dentisani* is being studied for its particular characteristics as a probiotic in the oral cavity (24). Henceforth, the study by María D Ferrer et al (24) demonstrated the clinical efficacy of *S. dentisani* administration in volunteers, as 58% of the participants on day 30 of the study presented increased levels of this microorganism and 71% on day 45. Essentially the colonization and permanence potential of this microorganism in dental plaque was due to bacteriocin production (24). The studies by Cantarruti Anna et al (13) and Manti Sara et al (12) confirmed *S. salivarius* 24SMB and *S. oralis* 89a usefulness in acute otitis media (AOM) and upper respiratory tract infections (URTIs) in children. In children with AOM, it was concluded that intermittent treatment with these two bacteria in the form of a nasal spray could be effective in preventing antibiotic use associated with recurrent episodes of this disease. Use of these two bacteria in URTIs exhibited great safety and tolerability. Furthermore, in the work by Ghalia Kaci et al (11) in a mouse model, it was shown the *S. salivarius* JIM8772 strain significantly inhibited inflammation in moderate and severe colitis.

Herein, we describe *S. salivarius*, *S. oralis* and *S. dentisani* strains of Colombian origin, with great antagonistic potential to be used in other areas of medical and dental research. Subsequent studies should be aimed at understanding the chemical, biochemical and molecular bacteriocin characteristics that confer these three bacteria their antagonistic effect. All directed at performing oral cavity in vivo studies in animals and humans to determine the antagonistic and probiotic effect on native *S. mutans* strains and other bacteria of infectious importance.

In conclusion, in the present study, 16, 17 and 14 strains of *S. salivarius*, *S. oralis* and *S. dentisani, respectively* with great antagonistic potential on different biotypes of *S. mutans* strains were identified.

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Table 1. *S. salivarius*, *S. oralis* and *S. dentisani* identification in saliva samples of 36 children included in the study

Sample number	*S. salivarius*	*S. oralis*	*S. dentisani*
1	+	+	-
2	+	+	-
3	-	+	-
4	+	-	+
5	-	-	+
6	+	-	+
7	-	-	+
8	-	-	+
9	-	-	-
10	+	+	+
11	+	-	+
12	+	+	-
13	+	+	+
14	-	-	-
15	-	+	-
16	-	-	-
17	+	-	+
18	+	+	+
19	-	-	-
20	+	+	-
21	-	+	-
22	-	-	-
23	-	-	-
24	+	+	-
25	-	-	-
26	-	-	-
27	+	+	-
28	+	+	+
29	-	-	-
30	+	-	+
31	+	+	+
32	-	+	-
33	-	-	-
34	-	+	-
35	-	-	-
36	-	+	+

+: Presence of microorganism -: Absence of microorganism

Table 2. Antagonistic effect of *S. salivarius*, *S. oralis* and *S. dentisani* isolated from children without dental caries on different biotypes of *S. mutans*

Strains evaluated		Biotypes of *S. mutans* indicating the antagonistic effect (halos in millimeters) *											
		XV-1	XV-2	XI-1	XI-2	XII-1	XII-2	XXVI-1	XXVI-2	XX-1	XX-2	XXXVI-1	XXXVI-2
1	*S. salivarius*	11	-	-	11	-	-	-	8	9	-	-	8
2	*S. salivarius*	-	10	11	-	9	-	8	-	7	-	-	9
3	*S. salivarius*	-	11	-	-	-	11	-	10	-	11	-	9
4	*S. salivarius*	-	10	-	10	-	-	9	12	9	-	10	9
5	*S. salivarius*	10	-	-	-	9	10	-	6	7	-	9	8
6	*S. salivarius*	-	11	-	-	-	-	-	11	7	11	10	8
7	*S. salivarius*	-	9	-	10	-	-	11	-	7	10	10	9
8	*S. salivarius*	12	-	10	9	8	-	12	11	9	10	9	8
9	*S. salivarius*	8	-	10	8	9	-	10	-	12	10	9	8
10	*S. salivarius*	-	-	8	9	9	-	11	12	7	10	10	7
11	*S. salivarius*	1	-	-	9	-	-	10	7	7	7	8	7
12	*S. salivarius*	10	-	-	9	-	-	10	7	8	6	9	-
13	*S. salivarius*	-	12	-	9	-	-	11	7	11	8	8	-
14	*S. salivarius*	12	-	7	-	9	-	10	7	6	9	9	-
15	*S. salivarius*	-	-	12	-	-	-	11	7	9	8	7	-
16	*S. salivarius*	-	-	-	-	10	-	-	6	11	10	8	-
1	*S. oralis*	9	12	-	11	9	-	12	6	12	9	7	-
2	*S. oralis*	11	-	-	10	8	-	9	8	11	10	7	-
3	*S. oralis*	10	-	9	10	8	11	8	7	10	10	9	7
4	*S. oralis*	-	14	-	-	10	10	7	9	8	-	-	-
5	*S. oralis*	9	-	12	-	7	10	10	6	8	-	8	-
6	*S. oralis*	-	-	-	-	9	11	9	7	7	-	8	-
7	*S. oralis*	10	12	-	13	8	-	14	11	11	10	8	-
8	*S. oralis*	-	-	-	11	7	8	-	-	8	-	-	-
9	*S. oralis*	-	11	-	10	-	-	-	5	9	7	9	-
10	*S. oralis*	-	-	-	9	-	7	-	6	-	-	-	-
11	*S. oralis*	7	13	12	10	9	-	15	10	10	9	10	-
12	*S. oralis*	-	-	-	10	-	9	-	-	-	10	9	-
13	*S. oralis*	-	-	-	-	-	-	14	-	-	-	7	-
14	*S. oralis*	13	12	12	14	14	14	14	8	14	14	14	7
15	*S. oralis*	-	10	7	-	7	7	6	7	-	-	7	-
16	*S. oralis*	-	8	7	-	7	7	7	6	-	-	7	6
17	*S. oralis*	8	6	-	-	-	7	-	-	-	-	-	-
1	*S. dentisani*	8	9	-	8	8	10	7	6	-	-	10	6
2	*S. dentisani*	7	8	-	8	7	8	6	6	-	-	7	-
3	*S. dentisani*	-	-	-	-	7	7	-	7	7	6	7	-
4	*S. dentisani*	-	-	-	8	-	-	-	9	-	7	-	-
5	*S. dentisani*	-	8	8	-	7	7	-	-	-	-	10	6
6	*S. dentisani*	10	10	10	7	-	9	9	7	7	8	9	7
7	*S. dentisani*	9	7	9	7	7	9	7	7	-	-	-	7
8	*S. dentisani*	10	7	10	-	-	7	7	7	10	10	-	10
9	*S. dentisani*	-	-	-	-	-	-	-	-	-	-	-	-

10	*S. dentisani*	14	11	14	14	14	14	12	9	14	14	14	7
11	*S. dentisani*	10	9	10	8	7	9	8	6	-	-	-	-
12	*S. dentisani*	11	10	10	-	9	-	9	7	-	-	7	7
13	*S. dentisani*	10	8	8	7	7	-	8	6	-	-	7	7
14	*S. dentisani*	9	8	8	-	7	9	7	6	-	-	9	-

-: No antagonistic effect *: The valuesgiven correspond to inhibition in millimeters