ANTAGONISTIC ACTION OF INDIGENOUS STREPTOCOCCUS MUTANS STRAINS

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ABSTRACT
Dental caries is an infectious process which ultimately destroys the tooth. Streptococcus mutans is considered to be the main agent causing this disease. If microorganisms with antagonistic action on S. mutans were found, they might provide a way of avoiding or controlling the disease. Within the framework of the Oral Microbial Ecology approach, the aim of this project was to identify S. mutans strains with antagonistic effect upon S. mutans. Saliva samples were taken from 66 children and cultured on Blood agar and Mitis Salivarius Bacitracin agar. They were incubated at 37°C in anaerobic atmosphere for 48 hours, after which bacteria were counted and biochemical tests performed on colonies compatible with S. mutans. Antagonistic effect was determined using the double layer in agar technique. In children without and with caries, the frequency of S. mutans was 91.7% and 96.7%, respectively. In the group of patients without caries, only two strains had no antagonistic action, and three strains had full antagonistic action (100%), while the rest showed different kinds of inhibitory action. In the group of patients with caries, only 5 strains had no antagonistic action, 32 strains had full antagonistic action (100%) and the rest had variable inhibitory action. To conclude, 112 S. mutans strains with high antagonistic potential were identified, which, after other requirements are fulfilled, could be used in caries prevention or control strategies.

Key words: dental caries, S. mutans, antagonism, probiotics.
The recognition of S. mutans as the most important microorganism initiating caries has led to the design of preventive measures aimed at eliminating or reducing it in the oral cavity6. Different strategies have been proposed for preventing and controlling dental caries8. In Colombia, the actions carried out by institutions and health guidelines for caries prevention and control have not sufficed to achieve a significant reduction in the disease, which still persists in over 90% of the Colombian population7. This situation calls for further study and review of all the specific dental caries prevention techniques or measures8.

The search for microorganisms with antagonistic action on caries-causing S. mutans strains, as well as the application of these microorganisms, belong to an increasingly researched strategy, which might allow the disease to be prevented or controlled8-10. Microbiological control (replacement therapy), more recently known as “probiotic therapy”, may be an alternative for controlling the microbial species involved in dental caries, without any negative effect on the other species that make up the oral microbiota8-10. This study is part of the Oral Microbial Ecology line of research, and the aim was to identify indigenous S. mutans strains with antagonistic effect.

MATERIALS AND METHODS

1. S. mutans isolation, count and identification

Study population

After obtaining informed consent from parents or tutors, and in compliance with bioethical standards for sampling and handling, 66 children aged 3 to 5 years from a pre-school in Bogotá were included in the study. Each child was examined clinically for caries experience by an examiner who determined the dmft index (decayed, missing and filled teeth) according to the World Health Organization criteria11. No X-rays were taken of any of the children. Of the 66 children, 36 had no dental caries, while 30 had caries with an average dmft index of 3.3 (range 2-5). The 66 children included in the study had no infectious systemic disease and had not been under antimicrobial treatment during at least 7 days prior to the sampling. A sample of unstimulated saliva was collected from each child using gentle aspiration with a plastic pipette.

Sample processing

The saliva samples were vortexed for 15 seconds and serially diluted (1/10, 1/100 y 1/1000) with 0.05 M phosphate buffer. 50 ul of each dilution was plated on Lamb’s Blood agar and Mitis Salivarius Bacitracin agar (MSB, Difco Laboratories; Detroit, MI). The MSB agar was used to count S. mutans and carry out its selective isolation. For its definitive use, MSB agar contains digested pancreatic casein, proteose peptone N° 3, proteose peptone, dextrose, 20% sucrose, dipotassium phosphate, trypan blue, crystal blue, agar, Chapman tellurite and bacitracin 0.2 U/ml. The Petri dishes containing the agars (Lamb’s Blood Agar and MSB Agar) were incubated at 37°C for 48 hours in anaerobic atmosphere (H2:CO2:N2 10:10:80). After bacterial growth on MSB Agar, colonies with morphological characteristics of S. mutans were counted12 and 5 colonies per sample were collected for Gram stain, examination of catalase activity and biochemical tests. The samples were plated on Lamb’s Blood Agar to observe total growth of bacteria present and correlate it with the growth on MSB agar. On Lamb’s Blood Agar, the characteristics of colonies compatible with S. mutans can be observed. They are small, translucent, creamy, shiny and with type α or γ hemolysis. The number of S. mutans colonies on MSB Agar was expressed in colony-forming units (CFU) per ml of unstimulated saliva. The following biochemical tests were run to identify S. mutans: fermentation of raffinose, mannitol, melibiose, trehalose and inulin; esculin hydrolysis in presence and absence of bile; urease; arginine hydrolysis and bacitracin resistance. S. mutans has the following biochemical profile: positive fermentation of raffinose, mannitol, melibiose, trehalose and inulin; negative esculin hydrolysis in presence of bile and positive esculin hydrolysis in absence of bile; negative urease; negative arginine hydrolysis, and resistance to 2 U of bacitracin. The commercial Api 20S system (bioMerieux, Marcy-létoile, France) was also used for identifying strains.

2. Strain biotyping

All isolated S. mutans were biotyped using the api-ZYM (bioMérieux, Marcy- létoile, France) system according to the manufacturer’s instructions. The api-Zym system is a semi-quantitative micro-method for research that enables 19 enzymatic activities to be detected rapidly and simultaneously from small amounts of bacterial inoculum. It consists of a strip with 20 microtubes or wells (1 control and 19 tests), the bottoms of which contain the substrates and
buffer. Microtube 1 is the control for the test, and substrates 2 to 20 correspond respectively to 2-naphthyl phosphate, 2-naphthyl butyrate, 2-naphthyl caprylate, 2-naphthyl myristate, L-leucyl-2-naphthylamide, L-valyl-2-naphthylamide, L-cystyl-2-naphthylamide, N-benzoyl- DL-arginine- 2-naphthylamide, N-glutaryl-phenylalanine-2-naphthylamide, 2-naphthyl phosphate, Naphthol-AS-BI-phosphate, 6-Br-2 naphthyl-Alfa D-galactopyranoside, 2-naphthyl-Beta D-galactopyranoside, Naphthol-AS-BI- Beta D-glucuronide, 2-naphthyl-Alfa D-glucopyranoside, 6-Br-2naphthyl-Beta D-glucopyranoside, 1-naphthyl-N-acetyl-BD-glucosaminide, 6-Br-2naphthyl-Alfa D-mannopyranoside, 2-naphthyl-Alfa L-fucopyranoside. The base of the system allows contact between the microorganism’s enzyme and the usually insoluble substrate. Substrates are inoculated with a dense bacterial suspension (turbidity equivalent to a McFarland number 5 or 6), which rehydrates the substrates and produces enzymatic action on them. The end-products formed during a 4-hour incubation period are detected by colour reactions after adding reagents. The tests are read by comparing the colours they produce to a colour code provided by the manufacturer. Biotyping was duplicated and the biotypes were assigned according to the action of the S. mutans strains on the system’s 19 substrates.

3. Determination of antagonistic effect in the isolated S. mutans strains

The antagonistic effect was determined by means of the double layer test in BHI (Brain Heart Infusion) Agar, on which strains that act as effectors and strains that act as indicators were plated. Effector strains are those that will have antagonistic action on indicator strains. To this end, two or three colonies of each S. mutans strain from the BHI Agar were re-suspended in BHI broth and incubated at 37°C in anaerobic atmosphere (H₂:CO₂:N₂ 10: 10:80) for 48 hours. This suspension was plated on BHI Agar (1.5% agar and 2% yeast extract) using a micro-pipette (2 ul) and incubated at 37°C in anaerobic atmosphere (H₂:CO₂: N₂ 10:10:80) for 48 hours. The indicator strains were subsequently placed upon the effector strains. Indicator strains are those that will be acted upon by the effector strains, and they were selected according to the frequency of biotypes present. To this end, two or three colonies of each S. mutans strain from the BHI Agar were re-suspended in BHI broth and incubated at 37°C in anaerobic atmosphere (H₂:CO₂:N₂ 10: 10:80) for 48 hours. Subsequently, 0.5 ml of this suspension was mixed with 5 ml of BHI Agar (0.75% agar and 2% yeast extract) and immediately added to the BHI agar (1.5% agar and 2 % yeast extract) on which the strains prepared in the previous step had grown. These Petri dishes with double layer BHI Agar, in which both the effector strains and the indicator strains are plated, were incubated at 37°C in anaerobic atmosphere (H₂:CO₂:N₂ 10: 10:80) for 48 hours. After these final 48 hours, antagonistic action is reflected by the presence of an inhibition halo produced by the effector strain on the indicator strain. Inhibition halos larger than 4 mm are considered in order to determine antagonistic effect.

RESULTS

S. mutans frequency and count

In children without caries and with caries, the frequency of S. mutans was, respectively, 91.7% (33/36) and 96.7% (29/30). Children with caries had a higher S. mutans count than children without caries and the differences in counts between the two populations were statistically significant (Two-sample Wilconson rank-sum (Mann-Whitney) test, Prob > / z / = 0.0000). Of the 62 children (33 in the group without caries and 29 in the group with caries) from whom S. mutans was isolated, 119 colonies were identified as S. mutans: 51 in children without caries and 68 in children with caries.

S. mutans biotypes

Tables 1 and 2 show enzymatic behaviour on the substrates in the api-ZYM system, of the 119 S. mutans strains isolated from children with and without caries. Biotypes were assigned according to a previous standardization. The wide range of activity of these microorganisms allowed 85 biotypes to be formed from the 119 S. mutans strains isolated: 33 biotypes in the strains isolated from children without caries and 52 biotypes in the strains isolated from children with caries. The two groups of strains had 4 biotypes in common (biotypes 5, 6, 9 and 12). The most frequent biotypes in children without caries were 6, 9, 5 and 3, with 5, 4, 3 and 3 strains respectively; and in patients with caries the most frequent biotypes were 37, 39, 6 and 9, with
Tabla 1: Biotipos en las 51 cepas de <i>Streptococcus mutans</i> aisladas de niños sin caries dentales.

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* - Reacción negativa
+ - Reacción positiva
* - Ver corresponentes substratos en materiales y métodos

5, 3, 3 y 3 cepas respectivamente (Tabla 1 y 2). A number of biotypes represented by a single strain was found in both groups of patients.

**Antagonistic effect in S. mutans strains**

The antagonistic effect was determined in all 119 S. mutans strains isolated in this study (Tabla 3 and 4). It is important to specify, as mentioned in materials and methods, that the strains that have antagonistic effect are taken as effectors, and those that suffer the antagonistic effect are taken as indicators. In each group of patients, 12 indicator isolates were selected, including representatives of the most frequent biotypes, in order to find out the antagonistic effect of the 119 strains isolated from patients with and without caries (Tabla 3 and 4).

In the group of patients without caries, only two strains (28FS1 and 35FS3) had no antagonistic action on the 12 indicator strains (Tabla 3). In the same group, only three strains (22FS4, 23FS1 and 24FS1) had full antagonistic action (100%) on the 12 indicator strains. The rest of the strains (n=46)
Table 2: Biotypes in the 68 *Streptococcus mutans* strains isolated from children with dental caries.

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- : Negative reaction; + : Positive reaction
* See corresponding substrates in materials and method
Table 3: Antagonistic effect of the 51 *Streptococcus mutans* strains isolated from patients without dental caries.

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*: No antagonistic effect

*: The values provided correspond to inhibition in millimetres
Table 4: Antagonistic effect of the 68 *Streptococcus mutans* strains isolated from patients with dental caries.

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- : No antagonistic effect
* : The values provided correspond to inhibition in millimetres
had different kinds of antagonistic action ranging from inhibition of 2 to 11 indicator strains. In the group of patients with caries, only 5 strains (1FC2, 4FC1, 6FC1, 8FC3 and 9FC3) had no antagonistic action on the 12 indicator strains (Table 4). In the same group, 32 strains (47%) had full antagonistic action (100%) on the 12 indicator strains. The rest of the strains (n=31) inhibited 3 to 11 indicator strains.

DISCUSSION

A microorganism may survive and multiply if it manages to eliminate or displace an organism from an ecological niche that has a range of microbial species\textsuperscript{14}. Studies on antagonism in dental caries began in 1972 with experiments with \textit{S. mutans} and \textit{Veillonella alcalescens}\textsuperscript{3}; which showed that the growth of \textit{V. alcalescens} in dental plaque was influenced by the anaerobic atmosphere of plaque and the amount of lactic acid produced by the plaque-forming organisms. The search for \textit{S. mutans} strains with antagonistic capacity and their application in microbiological control to displace virulent native \textit{S. mutans} strains has been ongoing for many years\textsuperscript{8-10}.

Different studies show that the antagonistic capacity of \textit{S. mutans} is due to the bacteriocins it produces, which could provide great capacity for displacing indigenous (native) strains of the same species\textsuperscript{15-17}. The double layer test is often used to show the action of bacteriocins or bacteriocin-like inhibitory substances (BLIS) produced by effector strains on the selected indicator strains, therefore the antagonistic or inhibitory capacity of the strains is due to the action of these substances\textsuperscript{15,18}.

In this study, antagonistic effect was determined in 119 \textit{S. mutans} strains isolated: 51 strains from children without caries and 68 strains from children with caries. Biotyping with the Api-ZYM system has been valuable in several studies for typing and relating \textit{S. mutans} strains\textsuperscript{19,20}. Due to the wide range of biotypes found in this study and in order to have a more realistic approach to the effect of antagonistic strains on the most commonly found strains, the most frequent biotypes in the study population were used as indicator strains. In order to assess the antagonistic action of the strains, two groups were formed: the group of strains from patients without caries and the group from patients with caries. In order to assess the strains from the group of patients without caries, 12 indicator strains were selected, representing the most frequent biotypes from the same group of

<table>
<thead>
<tr>
<th>Strains evaluated</th>
<th>Antagonistic effect on indicator strains (halos in millimetres)*</th>
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<td>52 22FC3</td>
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<td>67 29FC5</td>
<td>6 7 10 - 8 9 7 8 9 - 10 -</td>
</tr>
<tr>
<td>68 30FC4</td>
<td>6 6 7 6 8 8 6 7 9 7 10 -</td>
</tr>
</tbody>
</table>

- : No antagonistic effect
* : The values provided correspond to inhibition in millimetres

Table 4: (Continued).
strains; and to assess the strains from the group of patients with caries, 12 indicator strains were selected, representing the most frequent biotypes of the same group of strains. One hundred and twelve (94%) of the 119 *S. mutans* strains isolated showed high antagonistic action on the strains used as indicators. In contrast, the study by Balakrishnan et al.\textsuperscript{9}, in which strains of different species were used as indicators, reported finding 39 strains belonging to the genera *Streptococcus, Enterococcus* and *Staphylococcus* with antagonistic capacity, representing 14.3% of the 272 strains evaluated. Kamiya et al.\textsuperscript{13} evaluated bacteriocin production in 319 *S. mutans* strains isolated from 8 patients with caries and 8 patients without caries against only 12 *S. mutans* strains, and found antagonistic effect in 254 strains (79.62%). These differences in antagonistic effect were probably caused by different conditions in the tests and the use of different indicator strains.

There has been much debate regarding the association between *S. mutans* and the onset of dental caries\textsuperscript{1,5}. Many studies have shown that there is a direct relationship between *S. mutans* count in the oral cavity and the incidence and prevalence of dental caries\textsuperscript{1,5,13}. In this study, even when the frequency of *S. mutans* in children with and without caries was high and very similar (91.7% vs. 96.7%), there were statistically significant differences in the *S. mutans* count between the two populations studied. These findings show the high *S. mutans* colonization rate in children with dental caries.

Ecologically, dental caries is considered to be a consequence of an imbalance in the oral ecosystem leading to the prevalence of flora, before normal and then transformed in pathogenic\textsuperscript{1}. Any replacement therapy or microbiological control must take into account the ecological aspects in the oral cavity, since replacing one bacterium with another might lead to more imbalance than balance\textsuperscript{1,3,8}. There are currently different effector strains that come from oral bacteria for use in replacement therapy for caries\textsuperscript{8,10,21}. The main problem that arises when applying this strategy in the oral cavity, specifically in prevention of dental caries, is due to the fact that in addition to being a normal inhabitant of the human oral cavity, *S. mutans* is also a pathogen there. Studies on humans need to find an effector strain that can colonize effectively as well as displace indigenous *S. mutans* strains that live naturally in the oral cavity\textsuperscript{21}. Another important fact is that the local ecological implications that the absence of the indigenous *S. mutans* strain and the presence of other(s) could have on the oral cavity cannot be clearly predicted. Moreover, it is not known which species are best able to replace it (if it becomes absent) in its ecological niche. However, replacing it with a strain of the same genus and species with known genetic and phenotypic characteristics, which maintains the balance of the oral ecosystem, is an option worth considering in the prevention of caries\textsuperscript{10,21}.

As a result of this project, there are biotyped *S. mutans* strains with high antagonistic potential which can be used in further studies on replacement therapy strategies. Subsequent action should be aimed at learning the characteristics of the antagonistic strains in their interaction with other microorganisms that are important in forming the oral biofilm and in models that are closer to the real model\textsuperscript{22}.

To conclude, (1) in this study, 119 *S. mutans* strains were isolated and grouped in 85 biotypes; (2) the most frequent biotypes in patients without and with caries were, respectively 6, 9, 5 and 3, and 37, 39, 6 and 9; and (3) 112 *S. mutans* strains were found with high antagonistic potential and inhibition range of 3 to 12 indicator strains.

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