Abstract: The present study aimed to test the antimicrobial activity of *Streptococcus dentisani* (*S. dentisani*) supernatant against a collection of microorganisms implicated in dental root infections, and to analyze morphological changes induced in a selection of the tested microorganisms. A total of 22 microbial species were selected, and their growth was monitored by spectrophotometry in the presence and absence of the supernatant of *S. dentisani* at different assay concentrations (0.2×, 1×, 2×). The generation time and maximum growth rates were evaluated under every tested condition. Scanning electron microscope (SEM) images were obtained to assess the effect on the cell surface following incubation of the pathogens with the concentrated (2×) supernatant of *S. dentisani*. The supernatant of *S. dentisani* was found to exert effective inhibitory activity against most of the studied microorganisms implicated in dental root infections (20 out of 22). Total growth inhibition was observed in the case of *Streptococcus oralis*, *Streptococcus sobrinus*, *Streptococcus salivarius*, *Prevotella intermedia*, and *Streptococcus mutans*, while the rest of the microorganisms showed an increase in the generation time (between 30 min and 4 h). SEM images revealed structural changes in the membrane consistent with bacteriocin activity, although the effects were heterogeneous among the different species tested.

Keywords: root infection; *S. dentisani*; microbiology.

Introduction

The application of molecular techniques to assess the microbial diversity present in dental root canal system infections has demonstrated that the implicated microorganisms are highly diverse (1). Primary infections are characterized by a mixed anaerobic microbiota with a predominance of gram-negative bacteria, while gram-positive anaerobic bacteria predominate in secondary infections (2). *Fusobacterium nucleatum* (*F. nucleatum*), *Prevotella* spp., and *Campylobacter rectus* are species often found after canal instrumentation (3,4). While one to five different microbial species can be found in well-treated root canals, inadequately instrumented canals have been reported to contain over 30 species, very similar to those found in primary infections (1,5). The microbiological profile of acute apical abscesses comprises a mix of species, with a strong predominance of gram-negative anaerobes (6), more diverse than those identified in teeth with asymptomatic apical periodontitis, and contain many subgingival and periodontopathogenic species (7).

Endodontic infections are mediated by biofilms located in the root canal system, the formation of which likely begins following invasion of the first planktonic microorganisms after tissue decomposition (8). *Enterococcus faecalis* (*E. faecalis*) is a gram-positive facultative anaerobe strongly associated with endodontic treatment.
failure (8,9), with the reported prevalence ranging from 24% to 90% (1,6,7). According to Siqueira et al., teeth subjected to canal treatment are 9-fold more susceptible to colonization by E. faecalis than teeth with primary infections (1,4), due to the capacity of this species to invade the dentinal tubules and its resistance to the chemo-mechanical preparatory treatment. For this reason, E. faecalis is related to secondary infections that become persistent (7). Nevertheless, it has been questioned whether E. faecalis is the main cause of endodontic treatment failure, since it has not been isolated in all cases, is rarely the dominant species in bacterial communities, and is not more prevalent in treated canals with apical lesions than in those without apical lesions (4). A pH equal to or higher than 11.5 can destroy E. faecalis cells, and chlorhexidine is less effective against this bacterium than sodium hypochlorite (9). Moreover, recent investigations have demonstrated that E. faecalis is able to form aggregates with F. nucleatum, and when both bacteria form a biofilm in the apical region, it becomes highly resistant to antimicrobial agents and removal through chemical-mechanical preparation (8).

Streptococcus dentisani (S. dentisani) is a bacterium belonging to the mitis group, which was isolated from dental plaque samples of individuals that had never suffered dental caries (10,11). This species has been demonstrated to produce small hydrophobic peptides with antimicrobial activity, called bacteriocins, which have a net positive charge (12), suggesting that their bactericidal effect is due to the induction of bacterial cell envelope stress and destabilization. In some cases, the bacteriocins form vesicles on the bacterial surface, while in other cases, the bacterium undergoes total lysis, or pores are formed in the cell membranes (13). Bacteriocins have been shown to be released extracellularly by a wide range of gram-negative and -positive bacteria, and the term BLIS, i.e., bacteriocin-like inhibitory substance, was coined to describe a variety of incompletely characterized proteinaceous inhibitors produced by gram-positive bacteria, which have been proposed as therapeutic agents against dental caries (14). In the case of S. dentisani, at least five of these bacteriocins have been identified and classified, although genomic analyses suggest that the microorganism may be able to produce and secrete up to eight of these molecules. When acting jointly, they are known to exert potent effects upon mutans group streptococi (15), but their effects upon endodontic pathogens have not been established to date. The bacteriocins of S. dentisani have a molecular weight of 2-10 kDa and are accumulated in the extracellular medium during growth, which facilitates their large-scale synthesis and production. The various S. dentisani strains differ in their inhibitory activity, and in this regard, strain CECT 7746 appears to have the greatest activity (11).

Different concentrations of sodium hypochlorite and chlorhexidine are not totally effective in eliminating all the microorganisms present in root canal infections (16,17). Therefore, the development of a novel therapy involving the use of antimicrobial peptides may represent an effective adjunct therapy for the microbiological control of endodontic infections, since these molecules have been shown to be effective at low concentrations and offer the advantage of a lack of toxicity (15).

The present study was designed to quantitate the antimicrobial effectiveness of the supernatant of S. dentisani against a single strain of several microorganisms implicated in root canal infection, and to assess the morphological changes induced in a selection of the studied microorganisms.

Materials and Methods
Selected microorganisms
The most often cited microorganisms in the literature as being responsible for pulp-periodontal disease were selected (1,2,4,18). The selection of these species was made based on their association with root canal infection in different pulp-periapical pathological processes. The strains included in the present study, are listed in Table 1 according oxygen requirements of each one, culture medium used for growth, and identification. In addition, Corynebacterium matruchotii (C. matruchotii) (DSM 20635) was included due to its relevance in dental plaque biofilm formation (19,20). Type strains of the selected species were used, except when they were not isolated from an oral sample, in which case a clinical strain of oral origin was used.

Inhibition assays with S. dentisani supernatant
Inhibition experiments against the selected microorganisms were carried out using concentrated supernatants of well-grown cultures of S. dentisani 7746 (stationary phase). To obtain 5 mL concentrated supernatant, 50 mL brain heart infusion broth (BHI), (Biolife Milan, Italy) was inoculated with single colonies of S. dentisani and incubated aerobically at 37°C without agitation for 48 h, or until reaching an optical density (OD) of approximately 1.5 at 610 nm (OD610). After the incubation period, the cultures were centrifuged at 4,000 rpm for 10 min, and the pellets were discarded. The resulting supernatants were 10-fold concentrated by rotary evaporation using an RV 10 digital device (VWR, Chicago, IL, USA) with the following settings: heating bath at 40°C,
70 rpm rotation, 10 kPa pressure, and 30 min operating time. The resulting 5 mL concentrated supernatant was filtered through a 0.2-μm pore filter (Merck Millipore, Darmstadt, Germany) and stored at −20°C until use. To obtain the 5-fold concentrated supernatant, a dilution of the 10-fold supernatant with sterile water was performed. An aliquot of the non-concentrated supernatant was also sterilized by filtration prior to concentration. As controls, 10-fold concentrated BHI, a 5-fold BHI dilution in sterile water, and non-diluted medium were used.

Pre-inocula of the tested microorganisms (Table 1) were obtained by inoculation of 10 mL appropriate culture medium with a single colony of each strain, followed by incubation under the optimal oxygenation and temperature conditions. The optical densities of the pre-inocula were measured at 600 nm (OD 610) in a spectrophotometer (BioPhotometer, Eppendorf) (VWR, Chicago, IL, USA), and diluted with the culture medium to obtain a final OD of 0.03-0.05. To assess the inhibitory effect of *S. dentisani* supernatant, 160 μL of each microbial suspension was mixed with different variations of 40 μL supernatant (non-concentrated, concentrated 5×, and concentrated 10×), resulting in final assay concentrations of 0.2×, 1×, and 2×, respectively. These solutions were loaded in duplicate into a Nunc Microwell 96-well microplate (Thermo Fisher Scientific, Waltham, MA, USA). For anaerobic microorganisms, a drop of mineral oil was added on top of the medium to avoid oxygen diffusion into the cultures.

As negative controls, 160 μL each suspension was mixed with 40 μL different concentrations of BHI, and loaded in duplicate into the 96-well microplate. A positive control was not included because there is not a known bacteriocin with inhibitory activity against all the tested strains, since they normally have a narrow range of activity.

The microplate was placed into a microplate reader, ( Infinite 200 PRO, Tecan) (Geneve, Switzerland) and incubated at 37°C for 24-48 h under aerobic or anaerobic conditions, depending on the species. The OD 610 of each inoculated well was automatically measured every 30 min during the incubation time.

**Table 1** Strains included in the present study

| Microorganism                  | Oxygen requirements | Culture medium                         | Identification |
|--------------------------------|---------------------|----------------------------------------|----------------|
| *Porvimonas micra*             | Strict anaerobe     | BHI + glutamate (0.25%)                | DSM 20468T     |
| *Olsenella uli*                | Strict anaerobe     | BHI + 1 mL/L Vit K + 5 mL/L Hemin     | DSM 7084T      |
| *Actinomyces naeslundii*       | Strict anaerobe     | BHI + 1 mL/L Vit K + 5 mL/L Hemin     | DSM 17233      |
| *Lactobacillus casei*          | Facultative anaerobe| BHI                                    | ATCC 393       |
| *Enterococcus faecalis*        | Facultative anaerobe| BHI                                    | ATCC 29212     |
| *Candida albicans*             | Facultative anaerobe| BHI                                    | ATCC 14053     |
| *Streptococcus oralis*         | Facultative anaerobe| BHI                                    | CECT 907       |
| *Streptococcus mutans*         | Facultative anaerobe| BHI                                    | CECT 479       |
| *Streptococcus sobrinus*       | Facultative anaerobe| BHI                                    | CECT 4034      |
| *Streptococcus salivarius*     | Facultative anaerobe| BHI                                    | DSM 20560      |
| *Streptococcus gordonii*       | Strict anaerobe     | BHI                                    | AM19           |
| *Streptococcus anginosus*      | Strict anaerobe     | BHI                                    | AM43a          |
| *Corynebacterium malruchotii*  | Strict anaerobe     | TSB + 3g/L Ye                          | DSM 20635      |
| *Fusobacterium nucleatum*      | Strict anaerobe     | BHI + Glutamate 0.25%                  | DSM 20482      |
| *Tannerella forsythia*         | Strict anaerobe     | BHI + 1 mL/L Vit K + 5 mL/L Hemin     | ATCC 43037     |
| *Porphyromonas gingivalis*     | Strict anaerobe     | BHI + 1 mL/L Vit K + 5 mL/L Hemin     | DSM 20709T     |
| *Porphyromonas endodontalis*   | Strict anaerobe     | BHI                                    | DSM 24491T     |
| *Eikenella corrudens*          | Strict anaerobe     | BHI                                    | DSM 8340T      |
| *Prevotella intermedia*        | Strict anaerobe     | BHI + 1 mL/L Vit K + 5 mL/L Hemin     | DSM 20706T     |
| *Actinomyces odontolyticus*    | Strict anaerobe     | BHI + 1 mL/L Vit K + 5 mL/L Hemin O BHI| AM98a          |
| *Dialister invisus*            | Strict anaerobe     | BHI                                    | DSM 15470      |
| *Pseudoramibacter alactolyticus*| Strict anaerobe     | BHI                                    | DSM 3980       |

1)The numbers indicate the registry reference in the Spanish (CECT) or German type culture collection (DSMZ). The symbol T indicates type strain for that species.

**Calculation of growth parameters**

To assess the inhibitory effect of the supernatant at different concentrations, the growth rates (μ) and generation times (g) of each tested microorganism grown under the different conditions were calculated, as detailed in “Brock biology of microorganisms” (14th ed., Madigan et al., 117-131, Pearson, 2015).

When microorganisms are grown in a closed system, the population of cells exhibits the following growth dynamics: cells initially adjust to the new medium (lag phase) until they can start dividing regularly by the
process of binary fission (exponential phase). When their growth becomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Balanced microbial growth mimics a first-order chemical reaction: \( \frac{dN}{dt} = \mu N \), where \( N \) is the concentration of cells, \( t \) is the time, and \( \mu \) is the growth rate constant. Integration of the previous equation gives the following: \( \ln\frac{N_2}{N_1} = \mu (t_2 - t_1) \); and conversion to logarithms to the base of 10 gives \( \log_{10}N_2 - \log_{10}N_1 = \mu (t_2 - t_1) / 2.303 \). Determining the number of microbial cells present at various times during the exponential growth (here estimated through the OD measurements) and plotting the log of that number obtains a straight line. From the slope of this line, the specific growth rate \( \mu \) of the culture can be calculated, which is the most encompassing parameter of how fast a particular microorganism grows under particular conditions. Moreover, the rate of growth of a microbial culture can be described by the time required for the number of cells to increase by a factor of 2 (generation time “g”). The relationship between \( g \) and \( \mu \) can be established using the following equation: \( \ln\frac{N_2}{N_1} = \mu (t_2 - t_1) \), where \( N_2 \) is the cell number at \( t_2 \) and \( N_1 \) is the cell number at \( t_1 \).

Scanning electron microscopy (SEM)

SEM was used to directly observe the effect of \( S.\ dentisani \) supernatant on the cell surface of a selection of microorganisms (\( F.\ nucleatum,\ Prevotella.\ intermedia [P.\ intermedia],\ Eikenella\ corrodens [E.\ corrodens],\ E.\ faecalis,\ Parvimonas\ micra [P.\ micra],\ and\ Streptococcus\ mutans [S.\ mutans] \)). Briefly, 160 \( \mu \)L each bacterial culture in the exponential phase (OD\(_{610} = 0.8-1\)) was mixed with 40 \( \mu \)L supernatant (assay concentration 2\( \times \)) and incubated for 60 min at 37°C. After the incubation period, the suspensions were centrifuged at 4,000 rpm for 10 min, and the supernatant was discarded. The pellets were fixed in Karnovsky solution, washed twice with phosphate-buffered saline (PBS), and exposed to 1% osmium tetroxide in PBS buffer for 1 h. The samples were rinsed with PBS a further three times and subsequently moved through a gradual process of dehydration, starting with 30% ethanol and ending with absolute ethanol (multiple rinse steps at 30%, 50%, 70%, 80%, 90%, and 100% ethanol). Finally, the samples were mounted on scanning electron micrograph stubs, sputter-coated with gold, and viewed under a SEM Hitachi S-4800 (Hitachi, Tokyo, Japan).

Results

Evaluation of microbial growth inhibition

The addition of the supernatant of \( S.\ dentisani \) to the pure cultures resulted in the slowing or inhibition of the growth of most of the tested microorganisms. Regarding the growth dynamics in the presence and absence of the supernatant, the tested species could be divided into four major groups: i) species completely inhibited by the presence of the supernatant at some of the evaluated concentrations (group 1); ii) species showing slowed growth, generally associated with an increase in latency (group 2); iii) species without substantial changes in their growth rates but reaching lower OD values than the controls (lower bacterial load) (group 3); and iv) species unaffected by the presence of the supernatant of \( S.\ dentisani \) (group 4).

Species completely inhibited by the presence of the supernatant (group 1)

As can be seen in Table 2 and Fig. 1, the growth of \( S.\ mutans,\ S.\ salivarius,\ Streptococcus\ sobrinus (S.\ sobrinus),\ Pseudoramibacter\ alactolyticus (P.\ alacto-\ lyticus),\ P.\ intermedia,\ and\ S.\ oralis \) was completely inhibited by the supernatant at an assay concentration of 2\( \times \), with the total absence of growth. The same effect was observed in the case of \( P.\ alactolyticus,\ P.\ intermedia,\ and\ S.\ oralis \) in the presence of the non-concentrated supernatant (1\( \times \)), and in the case of \( S.\ oralis \) in the presence of the diluted supernatant (0.2\( \times \)). However, \( S.\ mutans,\ S.\ salivarius,\ and\ S.\ sobrinus \) were seen to grow in the presence of 1\( \times \) supernatant, although with decreased growth rates and longer generation times. On the other hand, it must be mentioned that the concentrated (2\( \times \)) BHI medium was toxic when applied to \( P.\ intermedia \) and \( S.\ oralis \), both of which showed no growth upon exposure. In the case of \( P.\ alactolyticus \), the concentrated medium (2\( \times \)) resulted in total growth inhibition, although growth was seen to be stimulated upon exposure to the non-concentrated medium.

Species showing slowed growth (group 2)

This group comprised most of the tested species, in which the presence of the supernatant of \( S.\ dentisani \) did not induce total inhibition of growth but a slowing of growth (Table 3), as evidenced by the curves (Fig. 2). With the sole exception of \( Porphyromonas\ endodontalis (P.\ endodontalis) \), all the species exposed to 2\( \times \) supernatant were seen to grow until reaching a lesser final microbial load. In the case of \( Candida\ albicans (C.\ albicans) \) and \( Streptococcus\ anginosus (S.\ anginosus) \), even the diluted supernatant (0.2\( \times \)) induced the same decrease in final microorganisms load as both 1\( \times \) and 2\( \times \) supernatant.

A special mention must be made regarding the behavior of \( E.\ faecalis \), since its maximum growth rate in
the presence of 2× supernatant decreased by 97% versus the controls. The generation time (i.e., the time taken for the number of cells to double) in turn increased to 2,409.2% versus the controls. Furthermore, the latency period was considerably longer in the presence of both 1× and 2× supernatant.

Species reaching a lower OD than the controls (group 3) The growth rates of the species that showed no slowing of growth but exhibited a decrease in the final microorganisms load in the presence of 2× supernatant are reported in Table 4, and the growth curves of each microorganism are shown in Fig. 3. Dialister invisus (D. invisus) exhibited the same growth dynamics in the presence of the

### Table 2 Maximum growth rate, generation time, and latency period of the species in group 1

| Microorganism                  | Maximum growth rate (μ) | Effect on μ (%) | Generation time (min) | Effect on GT (%) | Latency period (h) | Effect on LP (%) |
|-------------------------------|-------------------------|-----------------|------------------------|------------------|-------------------|------------------|
|                               | Control 2×              | 0.004           | 197.15                 |                | 6.37              |                  |
|                               | Sb 2×                   |                 |                        |                  |                   |                  |
|                               | Control 1×              | 0.005           | 126.36                 |                | 3.93              |                  |
|                               | Sb 1×                   | 0.003           | 259.57                 | 205.4           | 6.87              | 174.8            |
|                               | Control 2×              | 0.009           | 76.94                  |                | 0.49              |                  |
|                               | Sb 2×                   |                 |                        |                  |                   |                  |
| Streptococcus salivarius      | Control 1×              | 0.02            | 3.237                  |                | 0.49              |                  |
|                               | Sb 1×                   | 0.01            | 50.93                  | 157.3           | 0.49              | 100              |
|                               | Control 2×              | 0.008           | 89.96                  |                | 0.49              |                  |
|                               | Sb 2×                   |                 |                        |                  |                   |                  |
| Streptococcus sobrinus        | Control 1×              | 0.02            | 38.51                  |                | 0.49              |                  |
|                               | Sb 1×                   | 0.01            | 50.30                  | 130.6           | 0.98              | 200              |
|                               | Control 2×              |                 |                        |                  |                   |                  |
|                               | Sb 2×                   |                 |                        |                  |                   |                  |
| Pseudoramibacter alactolyticus| Control 1×              | 0.003           | 223.16                 |                | 11.71             |                  |
|                               | Sb 1×                   |                 |                        |                  |                   |                  |
|                               | Control 2×              |                 |                        |                  |                   |                  |
|                               | Sb 2×                   |                 |                        |                  |                   |                  |
| Prevotella intermedia         | Control 1×              |                 |                        |                  |                   |                  |
|                               | Sb 1×                   |                 |                        |                  |                   |                  |
|                               | Control 2×              |                 |                        |                  |                   |                  |
|                               | Sb 2×                   |                 |                        |                  |                   |                  |
| Streptococcus oralis          | Control 1×              |                 |                        |                  |                   |                  |
|                               | Sb 1×                   |                 |                        |                  |                   |                  |

The symbol (-) indicates zero growth of the microorganism under such conditions. 1) The values indicate the change in growth rate (μ), expressed as a percentage of the controls (values >100% indicate stimulation of growth; values <100% indicate inhibition of growth; values =100% indicate no effect). 2) The values indicate the change in generation time (GT), expressed as a percentage of the controls (values >100% indicate inhibition of growth; values <100% indicate stimulation of growth; values =100% indicate no effect). 3) The values indicate the change in latency period (LP), expressed as a percentage of the controls (values >100% indicate increased latency; values <100% indicate decreased latency, values =100% indicate no effect).

Fig. 1 Growth curves of the species in group 1. Optical density is plotted against time (in hours). The curves obtained by adding BHI 2× and non-concentrated BHI medium are shown in blue and green, respectively. The curves obtained by adding concentrated (2×), non-concentrated (1×), and diluted supernatant (0.2×) are shown in purple, orange, and violet, respectively. The species represented are: a) Streptococcus mutans; b) Streptococcus salivarius; c) Streptococcus sobrinus; d) Pseudoramibacter alactolyticus; e) Prevotella intermedia; and f) Streptococcus oralis.

BHI, brain heart infusion.
supernatant of *S. dentisani* at 1× and 2× concentrations, reaching an OD lower than that of the respective controls (0.4 versus 0.66 and 0.72 in the case of the controls at 1× and 2× concentration, respectively). *Lactobacillus casei* (*L. casei*) showed very similar behavior (0.48 versus 0.7 and 0.55 in the case of the controls at 1× and 2× concentration, respectively). In contrast, *Olsenella uli* (*O. uli*) exhibited different growth dynamics. In effect, it started to grow in a way similar to the controls in the presence of 1× supernatant, although upon reaching an OD of 0.45, it entered the stationary phase, slowing its growth and reaching slightly lower cell densities (0.62 versus 0.72 in the case of the controls). This same effect, but with greater magnitude, was observed with the concentrated supernatant (2×): the culture entered the stationary phase much faster, with an even lower final OD (0.55 versus 0.85 in the case of the controls).

### Table 3 Maximum growth rate, generation time, and latency period of the species in group 2

| Microorganism                | Maximum growth rate (µ) | Effect on µ (%) a | Generation time (min) | Effect on GT (%) b | Latency period (h) | Effect on LP (%) c |
|-----------------------------|-------------------------|-------------------|-----------------------|-------------------|-------------------|-------------------|
| *Actinomyces naeslundii*     | Control 2×              | 0.01              | 56.40                 | 356.3             | 4.93              | 137.5             |
|                             | Sb 2×                   | 0.003             | 200.93                | 51.33             | 4.31              |                   |
|                             | Control 1×              | 0.01              | 47.54                 | 92.6              | 4.93              | 114.4             |
|                             | Sb 1×                   | 0.01              | 65.72                 | 1.23              |                   |                   |
|                             | Sb 2×                   | 0.003             | 204.74                | 311.5             | 3.08              | 250.4             |
|                             | Control 1×              | 0.006             | 115.22                | 64.4              | 1.23              | 100               |
|                             | Sb 1×                   | 0.009             | 122.96                | 1.23              |                   |                   |
|                             | Sb 2×                   | 0.006             | 118.78                | 96.5              | 2.46              | 100               |
|                             | Control 1×              | 0.007             | 104.96                | 113.8             | 1.85              | 75.2              |
|                             | Sb 1×                   | 0.01              | 64.17                 | 0.62              |                   |                   |
|                             | Sb 2×                   | 0.005             | 131.23                | 204.5             | 1.85              | 298.4             |
| *Candida albicans*          | Control 1×              | 0.02              | 80.96                 | 159.3             | 6.16              | 250.4             |
|                             | Control 2×              | 0.06              | 12.20                 | 2.46              |                   |                   |
|                             | Sb 2×                   | 0.002             | 293.92                | 2409.2            | 3.7               | 150.4             |
|                             | Control 1×              | 0.007             | 94.58                 | 1.85              |                   |                   |
|                             | Sb 1×                   | 0.003             | 203.35                | 2.46              |                   |                   |
|                             | Sb 2×                   | 0.008             | 80                    | 1.85              |                   |                   |
|                             | Control 1×              | 0.02              | 34.28                 |                   |                   |                   |
|                             | Sb 1×                   | 0.01              | 49.56                 | 2.46              |                   |                   |
|                             | Sb 2×                   | 0.008             | 88.82                 | 0.62              |                   |                   |
| *Enterococcus faecalis*     | Control 1×              | 0.005             | 141.43                | 1.23              |                   |                   |
|                             | Sb 1×                   | 0.01              | 250                   | 159.2             |                   |                   |
|                             | Sb 2×                   | 0.004             | 250                   | 38.6              |                   | 133               |
| *Fusobacterium nucleatum*   | Control 1×              | 0.01              | 105.44                | 3.7               |                   |                   |
|                             | Sb 1×                   | 0.01              | 142.9                 | 54.13             | 4.31              | 116.5             |
|                             | Sb 2×                   | 0.01              | 142.9                 | 53.5              |                   |                   |
|                             | Control 1×              | 0.007             | 71.93                 | 3.7               |                   |                   |
| *Porphyromonas endodontalis*| Control 2×              | 0.008             | 92.37                 |                   |                   |                   |
|                             | Sb 2×                   | 0.003             | 249.88                | 0.62              |                   |                   |
|                             | Control 1×              | 0.007             | 79.87                 | 0.62              |                   |                   |
| *Porphyromonas gingivalis*  | Control 2×              | 0.009             | 100.05                | 1.23              |                   |                   |
|                             | Sb 2×                   | 0.007             | 77.8                  |                   |                   |                   |
| *Streptococcus anginosus*   | Control 2×              | 0.009             | 75.95                 | 1.85              |                   |                   |
|                             | Sb 2×                   | 0.005             | 146.13                | 2.46              |                   |                   |
|                             | Control 1×              | 0.01              | 54.6                  | 1.23              |                   |                   |
| *Streptococcus gordonii*    | Sb 1×                   | 0.005             | 50                    | 1.85              |                   |                   |
|                             | Sb 2×                   | 0.008             | 86.14                 | 4.31              |                   |                   |
|                             | Control 2×              | 0.001             | 125                   | 7.39              |                   |                   |
| *Tanerella forsythia*       | Sb 1×                   | 0.008             | 55.5                  | 3.7               |                   |                   |
|                             | Sb 2×                   | 0.01              | 29.06                 | 4.93              |                   | 133.2             |

The symbol (−) indicates zero growth of the microorganism under such conditions. a) The values indicate the change in growth rate (µ), expressed as a percentage of the controls (values >100% indicate stimulation of growth; values <100% indicate inhibition of growth; values =100% indicate no effect). b) The values indicate the change in generation time (GT), expressed as a percentage of the controls (values >100% indicate inhibition of growth; values <100% indicate stimulation of growth; values =100% indicate no effect). c) The values indicate the change in latency period (LP), expressed as a percentage of the controls (values >100% indicate increased latency; values <100% indicate decreased latency, values =100% indicate no effect).
Fig. 2  Growth curves of the species in group 2. Optical density is plotted against time (in hours). The curves obtained by adding brain heart infusion (BHI) 2× and non-concentrated BHI medium are shown in blue and green, respectively. The curves obtained by adding concentrated (2×) and non-concentrated supernatant (1×) are shown in purple and orange, respectively. The species represented are: a) Actinomyces naeslundii; b) Candida albicans; c) Corynebacterium matruchotii; d) Eikenella corrodens; e) Enterococcus faecalis; f) Fusobacterium nucleatum; g) Porphyromonas endodontalis; h) Porphyromonas gingivalis; i) Streptococcus anginosus; j) Streptococcus gordonii; and k) Tannerella forsythia.

Table 4  Maximum growth rate, generation time, and latency period of the species in group 3

| Microorganism  | Maximum growth rate (μ) | Effect on μ (%) | Generation time (min) | Effect on GT (%) | Latency period (h) | Effect on LP (%) |
|----------------|-------------------------|-----------------|-----------------------|-----------------|-------------------|-----------------|
| Dialister invisus | Control 2× 0.005 | Sb 2× 0.006 | Control 1× 0.006 | Sb 1× 0.007 | Control 2× 0.003 | Sb 2× 0.003 | Control 1× 0.003 | Sb 1× 0.002 | Control 2× 0.005 | Sb 2× 0.004 | Control 1× 0.004 | Sb 1× 0.006 |
|                 |                         | 120             | 106.72                | 82.8            | 0.62              | 4.93            | 248.22                | 94              | 3.7              | 75.1              | 175.29                | 119.9            | 7.39              | 120 |
| Lactobacillus casei | Control 2× 0.006 | Sb 2× 0.003 | Control 1× 0.003 | Sb 1× 0.002 | Control 2× 0.005 | Sb 2× 0.004 | Control 1× 0.004 | Sb 1× 0.006 |
|                 |                         | 116.7           | 102.52                | 85.1            | 1.23              | 63              | 303.62                | 115.8           | 3.08             | 83.2              | 118.85                | 63              | 5.54              | 100 |
| Olsenella uli   | Control 2× 0.006 | Sb 2× 0.004 | Control 1× 0.004 | Sb 1× 0.006 |
|                 |                         | 150             | 118.85                | 63              | 5.54              | 188.71           | 119.9            | 7.39              | 120 |

The symbol (-) indicates zero growth of the microorganism under such conditions. 1) The values indicate the change in growth rate (μ), expressed as a percentage of the controls (values >100% indicate stimulation of growth; values <100% indicate inhibition of growth; values =100% indicate no effect). 2) The values indicate the change in generation time (GT), expressed as a percentage of the controls (values >100% indicate inhibition of growth; values <100% indicate stimulation of growth; values =100% indicate no effect). 3) The values indicate the change in latency period (LP), expressed as a percentage of the controls (values >100% indicate increased latency; values <100% indicate decreased latency, values =100% indicate no effect).

Fig. 3  Growth curves of the species in group 3. The colors of the curves are the same as described in Fig. 2. The species represented are: a) Dialister invisus; b) Lactobacillus casei; c) Olsenella uli.
Species unaffected by the presence of the supernatant of S. dentisani
Lastly, of the studied microorganisms, two species, Actinomyces odontolyticus (A. odontolyticus) and P. micra, were detected that were resistant to the antimicrobial effect of the supernatant of S. dentisani (Table 5). The growth curves revealed the same growth dynamics both in the presence and absence of the supernatant (Fig. 4). A. odontolyticus showed very similar growth under all four tested conditions, with a slightly increased generation time in the presence of the supernatant as compared with the controls. The practically nonexistent latency period was not modified, since the bacterium was seen to grow immediately under all four conditions. P. micra also showed very similar growth rates under all the tested conditions, although the latency period increased considerably in the presence of 2× supernatant, and its generation time was slightly shorter (Table 5).

Morphological changes induced by the supernatant of S. dentisani in certain tested species (SEM)
Exposure of the microorganisms to the supernatant of S. dentisani induced structural changes at the membrane level consistent with the mechanism of action of the bacteriocins. In the case of F. nucleatum, dramatic cell lysis including the release of cell contents (Fig. 5a, b) was observed. P. intermedia showed cell membrane alterations with the appearance of small vesicles and destructuring of the cell wall (Fig. 5c, d). E. corroden showed cell agglutination in the presence of concentrated supernatant (2×) (Fig. 5e, f). The cell membranes of E. faecalis showed destructuring in the presence of concentrated supernatant (2×), yielding a sticky appearance, with total lysis of some individual cells (Fig. 5g, h). P. micra showed no structural changes in the presence of concentrated supernatant (2×) (Fig. 5i, j). S. mutans showed multiple pores as compared with the controls (Fig. 5k, l).

**Table 5 Maximum growth rate, generation time, and latency period of the species in group 4**

| Microorganism         | Maximum growth rate (μ) | Effect on μ (%) | Generation time (min) | Effect on GT (%) | Latency period (h) | Effect on LP (%) |
|-----------------------|-------------------------|----------------|----------------------|-----------------|--------------------|-----------------|
| Control 2×            | 0.01                    |                | 58.76                | 0.01            | 115.7              | 115.7           |
| Sb 2×                 | 0.01                    | 100            | 67.96                | 70              | 150.3              | 150.3           |
| Control 1×            | 0.01                    |                | 62.38                | 1×              | 0.007              | 1×              |
| Sb 1×                 | 0.007                   | 70             | 93.76                | 0.007           | 142.46             | 0.007           |
| Control 2×            | 0.005                   |                | 124.93               | 120             | 87.7               | 124.93          |
| Sb 2×                 | 0.006                   | 120            | 94.47                | 0.007           | 92.56              | 92.56           |
| Control 1×            | 0.007                   |                | 142.46               | 1×              | 87.7               | 142.46          |
| Sb 1×                 | 0.007                   | 100            | 92.56                | 98              | 0.62               | 92.56           |

The symbol (-) indicates zero growth of the microorganism under such conditions.

1)The values indicate the change in growth rate (μ), expressed as a percentage of the controls (values >100% indicate stimulation of growth; values <100% indicate inhibition of growth).
2)The values indicate the change in generation time (GT), expressed as a percentage of the controls (values >100% indicate inhibition of growth; values <100% indicate stimulation of growth).
3)The values indicate the change in latency period (LP), expressed as a percentage of the controls (values >100% indicate increased latency; values <100% indicate decreased latency).

**Fig. 4** Growth curves of the species in group 4. The colors of the curves are the same as described in Fig. 2. The species represented are: a) Actinomyces odontolyticus and b) Parvimonas micra.

**Discussion**
Infections caused by biofilms constitute a public health problem, since they account for 80% of all human infections (20). Microorganisms embedded in biofilms modify their properties, and as a result, 10-1,000 times higher concentrations of antimicrobial agents are required to secure treatment efficacy as compared with the planktonic form of the microorganisms (1). The most commonly used substances with antimicrobial action are sodium hypochlorite at room temperature or warmed to 60°C, chlorhexidine, chitosan (16,17), and calcium hydroxide as an intracanal medication or combined with chlorhexidine as an irrigating agent (21). Combinations of different substances have also been used for irrigation purposes, such as a combination of doxycycline, citric
New procedures have recently been introduced as complementary to chemo-mechanical therapy, such as photodynamic therapy (PDT) (23,24), which involves the application of a photosensitizing agent that becomes activated upon exposure to light of a specific wavelength (630-700 nm), inducing the production of reactive oxygen species that in turn cause oxidative damage to the target cells. PDT has been shown to be effective in the cases of necrosis with bacteria resistant to antibiotics, and results in a substantial reduction in the bacterial count when applied following chemical-mechanical preparation of the canals (25). Gram-positive bacteria are more susceptible to PDT due to their physiological characteristics, since gram-negative bacteria have an external membrane that isolates them from the environment. Similarly, planktonic microorganisms are far more vulnerable to PDT than microorganisms present in biofilms (26).

In recent years, there has been an increase in the number of studies on antimicrobial peptides (AMPs) for the treatment of infections and the prevention of microbial resistance (27,28). It has been demonstrated that certain AMPs possess activity against the microorganisms implicated in pulp and periapical infectious disease. As a result, these peptides are becoming a promising option to complement chemical-mechanical instrumentation in the treatment of root canals (28). An example of the use of these peptides is nisin, which is naturally produced by the bacterium *Lactococcus lactis* (*L. lactis*), and is used as a preservative in the food industry (29). The biological properties of nisin suggest that it may be adequate and effective in the prevention of dental caries and for the treatment of root canals. Moreover, it is relatively safe, since it is quickly degraded and inactivated by the digestive enzymes when accidentally swallowed (30). This molecule has been mixed with irrigants such as MTAD (a combination of tetracycline, citric acid, and detergent Tween 80), forming MTADN, and with chlorhexidine-in both cases improving the bactericidal properties of the irrigants, regardless of whether the bacteria are present in planktonic form or in biofilms (31).
The present study shows that the supernatant of *S. dentisani* exerts an inhibitory effect upon most of the selected microorganisms due to their prevalence in root canal infection. The concentrated supernatant (2×) caused total growth inhibition in some cases (e.g., *S. mutans* and *S. sobrinus*), while in other cases, it was seen to slow and/or limit microbial growth. Although the growth rate of some of the species was not affected by exposure to the supernatant, the final microbial load was decreased. This was the case with *O. uli*, an emergent pathogen in endodontic infection (32) that proves difficult to eliminate. In only two species (*A. odontolyticus* and *P. micra*) did the supernatant of *S. dentisani* appear to be ineffective or require higher doses to exert an effect.

In this regard, the inhibition of certain pathogens of great relevance was observed in endodontic infectious processes, such as *E. faecalis*, which exhibited a 97% decrease in its growth rate, or the yeast *C. albicans*, which appears to be sensitive to the supernatant. The inhibition of microorganisms that are crucial in biofilm formation, such as *C. matruchotii*, which appears to constitute the main structural element of dental plaque (19,20), or *F. nucleatum*, which is the bacterium with the largest number of interactions and coaggregations among the different oral species (33), defines the supernatant of *S. dentisani* as a promising anti-biofilm agent.

It is important to note that the supernatant obtained by the same procedure described here was previously tested for bacteriocin presence (12). Briefly, treatment with peptidase eliminated the inhibitory effect, confirming that the inhibition was due to molecules of a peptidic nature (Fig. 6).

The SEM images showed that the effects on the cell membrane differed from one species to another, although in all cases, the changes were consistent with the mechanism of action of bacteriocins as hydrophobic and polar molecules that destabilize cell membranes or walls. This mechanism of action is believed to be responsible for making the development of bacterial resistance more difficult than with conventional antibiotics (32). The SEM images indicate different degrees of inhibition, with species that suffer total cell lysis (e.g., *Fusobacterium*), as well as species where conspicuous pores are seen (e.g., *S. mutans*), and others that suffer membrane alterations indicative of damage or stress but without fully lethal effects at the studied doses (e.g., *E. corrodens*).

The present study opens new perspectives for the use of *S. dentisani* in the prevention and treatment of different oral disease conditions, since in addition to its known anti-caries effect (12), it inhibits or slows the growth of microbial species implicated in endodontic infections. Further studies are needed to confirm whether the promising inhibitory effects demonstrated *in vitro* in pure cultures are also observed in samples taken directly from endodontic infections, which are polymicrobial and involve the presence of biofilms. Such studies may lead to clinical trials involving application of the product as part of endodontic treatment.

As a limitation of the present study, the inhibition tests were performed against a single strain (in most cases the type strain) of each selected species. However, the variability among strains belonging to the same species can be large; therefore, there may be non-tested strains of the same species that are resistant to treatment, as has been demonstrated in oral bacteria exhibiting intra-specific variations in sensitivity to human antimicrobial peptides (30). Another limitation of this experimental protocol refers to the use of pure cultures. Although this approach allows controlled and standardized testing of the inhibition of each species, the organisms that infect root canals are rarely found alone but instead form complex biofilms containing multiple microbial species that are usually protected from the action of antimicrobial agents by the extracellular matrix (33). Therefore, it would be advisable to evaluate the efficacy of the supernatant of *S. dentisani* against complex biofilms *in vitro* or clinical samples of endodontic infections, where the microstructural characteristics of root canals may have a strong influence on the capacity of the treatment to reach the microorganisms.

In conclusion, the present results indicate that the supernatant of *S. dentisani* exerts antimicrobial effects upon the most common microorganisms in planktonic form implicated in root canal infections. The SEM findings, in turn, show that the inhibitory effect of the supernatant of *S. dentisani* differs among species and is consistent with the mechanism of action of AMPs, which should be characterized in future studies with respect to large-scale production considering the limitations for their extraction, isolation, and purification (34).

**Conflict of interest**
The authors have no conflict of interest to declare.

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