Medizinische Hochschule Hannover

Final Report on the Project

"Investigation of the antibacterial properties of the product Parodont Creme®"

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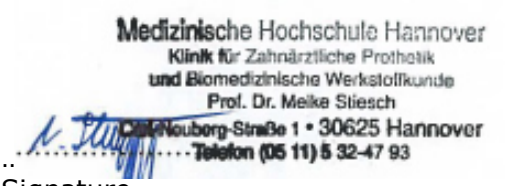

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1 General information

Project title

Investigation of the antibacterial properties of the product Parodont Creme®

Sponsor of the research project

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2 Work and Results Report

2.1 Introduction

The human oral flora comprises more than 700 different species of bacteria and is thus, along with the digestive tract, the habitat with the largest bacterial diversity in the human body. In the oral cavity, the bacteria form complex structural biofilm communities on hard and soft tissues; these communities are also referred to as plaques. The biofilms can trigger local and systemic infections in the human body which can have serious and partly life-threatening complications. Owing to special phenotypic properties of the biofilms and the special metabolic adaptation of bacteria living in biofilms, the therapeutic success of medicinal therapies is often inadequate. One significant characteristic of the biofilms is the polymer matrix that surrounds them – this matrix is comprised of proteins, DNA and/or polysaccharides and is formed by bacterial section. The polymer layer represents an effective (diffusion) barrier and prevents an effective immune response of the host as well as the ingress of antibacterial substances into the biofilm. This prevents lasting damage of the biofilm community. An important preventive measure is the maintenance of a healthy oral microflora, which counters the development not only of pathogenic biofilm communities, but also of inflammatory processes. Daily brushing of teeth, as well as the use of antibacterial oral hygiene products, support this process and are crucial in achieving long-lasting and sustained oral health.

The product Parodont Creme® is a cosmetic care product designed for local use inside the oral cavity. The ointment itself is used as a supportive therapeutic measure not only in case of inflammatory diseases, but also in daily oral hygiene. The main active component of the ointment is black cumin oil. Owing to a multitude of properties that promote healing, the oil has been used in traditional Western medicine for many decades. Its positive properties include, among others, antibacterial, antimycotic and anti-inflammatory effects. An antibacterial effect of black cumin oil on different oral bacterial species has already been described in the literature; however, the effect of the proprietary Parodont Creme® formulation has not been described yet. The aim of this study was to assess the antimicrobial effect of Parodont Creme® on the three oral bacterial species *Streptococcus gordonii, Streptococcus oralis* and *Streptococcus sanguinis*. These species are present in the healthy native microflora, but are also found in pathogenic periodontal biofilm communities.

2.2 Material and Methods

Investigational products

The investigational products were obtained from the manufacturer, Beovita GmbH & Co KG (Berlin), either in the original pack (Parodont Creme®) or as products filled straight from production (hydrophobic vehicle without an active substance).

Bacterial strains and cultivation conditions

The bacterial cultures used in this study were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). A list of the strains used is provided in Table 1. The precultures were grown statically at 37°C in Brain Heart Infusion Medium (BHI; Oxoid, Wesel, Germany) or on Columbia Blood Agar (CB; Oxoid, Wesel, Germany) with added 5% sheep blood under anaerobic conditions. All cultivations were prepared in an anaerobic workbench with external fumigation (DonWhitley, Concept 400; 10% H2, 10% C02, 80% N2). The typical duration of the cultivation, both in liquid media and in solid media, was 18-24 h.

Table 1 Overview of bacterial strains used

Species	**Strain**
Streptococcus sanguinis	DSM 20556
Streptococcus oralis	DSM 20627
Streptococcus gordonii	DSM 20568

Antibacterial testing - Agar diffusion test

Standardised bacterial suspensions were created for the antibacterial testing. To this end, individual bacterial colonies were harvested from the plates (preculture) and re-suspended in 10 ml of sterile 0.9% NaCl solution until a McFarland turbidity of 0.5 was reached (Remel™, Thermofisher, Waltham, USA). A sterile cotton swab was soaked with the bacterial suspension and the bacteria were smeared onto a CB agar plate. Agar was removed from the middle of the plate and near the edge of the plate using a 7 mm punch. The resulting holes were filled with approximately 120 µl of Parodont Creme® (middle of plate) or with 120 µl of a vehicle without an active substance (edge). The plates were cultivated for 5 days under anaerobic conditions; photographs were taken after 1 day and after 5 days. The experiments were carried out as three-fold determinations with 3 biological replicates. The diameter of the inhibitory zone was measured computer-assisted using the analytical software ImageJ (v1.37c). To this end,

a circular object was placed above the area of the inhibitory zone and the mean diameter of the inhibitory zone was calculated on the basis of the surface area. Statistical significances (significance level p≤ 0.05) were calculated by means of a t-test (2-tailed, independent samples) using the statistical analytical software SPSS (v24; IBM, Armonk, USA). The homogeneity of variances (Levene's test) and the normal distribution (Shapiro-Wilk test) were given in all cases and had been checked prior to conducting the t-test.

Antibacterial testing - Biofilms

In preparation for the test, sample specimens with a diameter of 12 mm were punched from a filter membrane (0.22 µm, GPW P04700; Merck Millipore, Billerica, USA), autoclaved and incubated for at least 24 h in PBS. In order to generate an initial biofilm on the surfaces of the membrane, the sample specimens were incubated for 18 h in a bacterial suspension under anaerobic conditions in 12 well plates (Cellstar; Greiner Bio-One, Kremsmünster, Austria). The suspension was manufactured from an overnight culture, which was washed (PBS), diluted (PBS) und set to a McFarland turbidity value of 0.5 (Remel™, Thermofisher, Waltham, USA). After that, the membranes were removed and any non-adhering bacteria were removed through rinsing with PBS. For the test, 50 µl of test substance was applied punctiform into each of the holes of a 12 well plate (Fig. 1A). Afterwards, the filter membranes were placed onto the test substance, whereby the side with the grown biofilm was positioned face up (Fig. 1B). An even distribution of the ointment on the lower side was ensured by gently applying pressure on the test specimens; the porous structure of the membrane provided for uninhibited diffusion of the active substances into the biofilm (on the top of the membrane). The membranes were incubated for 18 h each in 2 ml Todd-Hewitt Broth (Oxoid, Wesel, Germany) which had been supplemented with 0.5% sucrose and then rinsed with PBS. The membranes were transferred into a new 12 well plate and incubated with 2 ml of 0.001% resazurin solution for 45 min. The substance resazurin is an indicator dye which can be metabolically reduced to resorufin by the bacteria. Only vital, i.e. metabolically active cells, are able to reduce the dye to resorufin. The resulting fluorescence dye resorufin was quantitatively detected using a fluorescence reader (Tecan Infinite 200 pro, excitation: 530 nm, emission: 600 nm). Parodont Creme®, the vehicle, as well as an untreated membrane were assessed; three technical replicates each. As additional controls, Parodont Creme®, vehicle and the plastic surface of the Multiwell plates that were free from bacteria were tested with resazurin solution. To analyse statistical significances, the ANOVA test (univariate variance analysis, significance level p≤ 0,05) was carried out; the homogeneity of variances (Levene's test) and the normal distribution (Shapiro-Wilk test) had been checked beforehand.

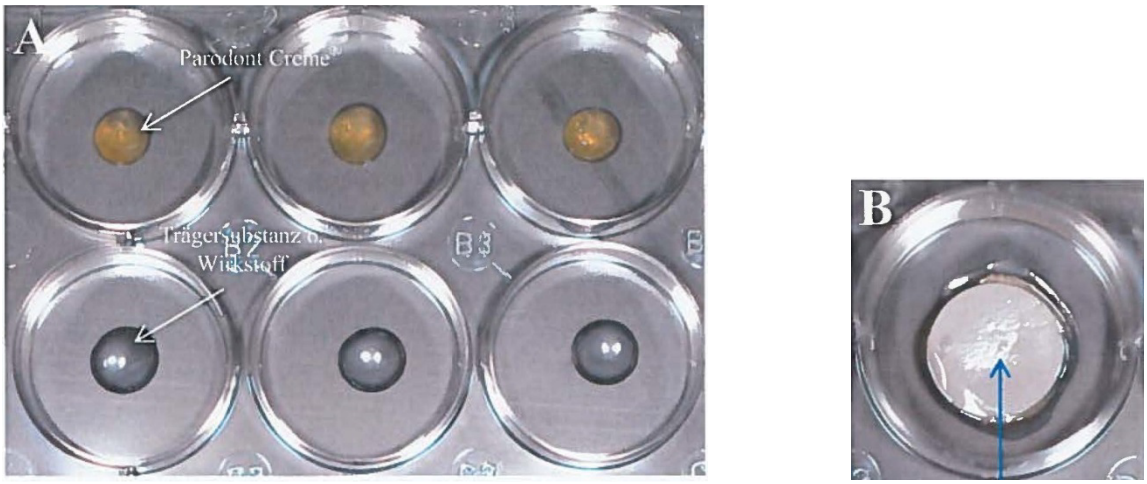

Fig. 1A/B

A shows an excerpt of a 12 well plate; 50 µl of Parodont Creme® (top row) and 50 µl of the vehicle without an active substance (bottom row) were applied to the floor of the wells; **B** shows a filter membrane with a bacterial growth film that was placed on the floor of the well plate (straight onto the ointment); gentle pressure with sterile tweezers ensured an even distribution of the product on the lower side of the membrane.

2.3 Results and Discussion

An antibacterial effect of Parodont Creme® on the bacterial species studied was confirmed under *in-vitro* conditions. However, the extent of the effect on the different bacterial species varied: *Streptococcus sanguinis* > *Streptococcus gordonii* > *Streptococcus oralis* (antibacterial effect in descending order; benchmark: diameter of inhibitory zone). An effect of the product on matured biofilms could not be demonstrated in the experiment. Here, no significant impairment of the bacterial metabolism was observed in any of the species, so that the product cannot be assumed to have anti-biofilm properties under the given experimental conditions. The results for the individual bacterial species are presented in detail below:

Streptococcus sanguinis

The observed antibacterial effect was greatest in *S. sanguinis*. The mean inhibitory zone diameters after 1 day and 5 days were identical (1 d= 6.86 cm ± 0.37 cm; 5 d =6.86 cm ± 0.30 cm; Fig. 2A). In the same way, the experiments with the vehicle without an active substance showed no inhibition of bacterial growth (Fig. 2B) and confirmed that the antibacterial effect was attributable to the black cumin oil. An effect on matured biofilms of the bacteria was not observed under the given conditions of the experiment; in other words, significant metabolic activity was measured in all samples irrespective of the type of treatment. The examinations showed no significant differences in the fluorescence intensities of the controls and of the samples treated with active substance ($p > 0.05$, Fig. 3A/B). A reduction of resazurin to resorufin by ingredients contained in the ointment formulation did not take place (Fig. 3B, (4), (8)).

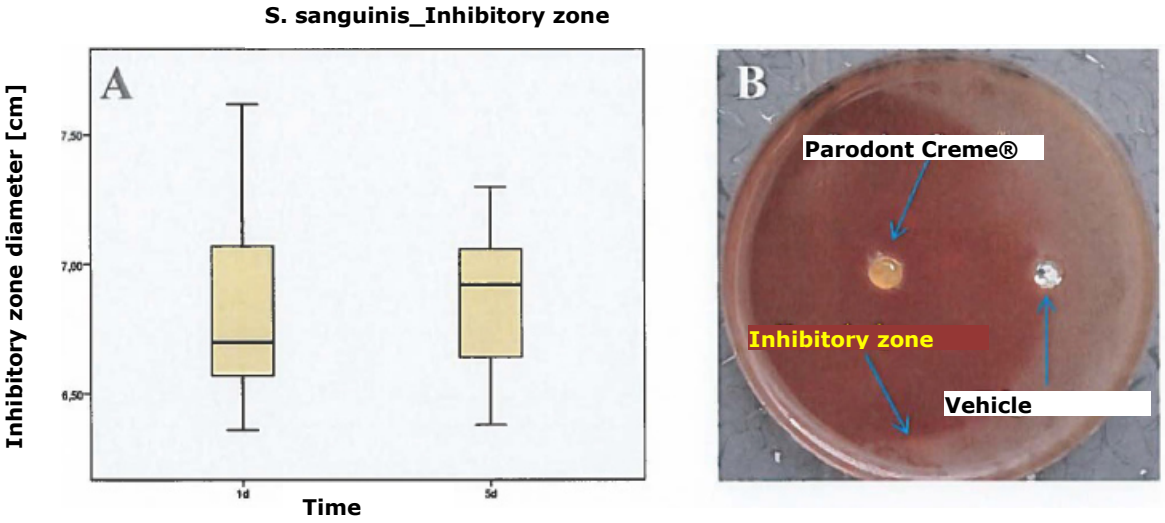

Fig. 2A/B

A shows a box plot diagram of the inhibitory zone diameter after 1 d (left) and 5 d (right); **B** shows an agar plate after 24 h of incubation with *S. sanguinis*, left hole with Parodont Creme® and marked inhibitory zone formation, right hole with vehicle without active substance and without a real inhibitory zone formation - the vehicle is partly located in the area of the inhibitory zone created by Parodont Creme®.

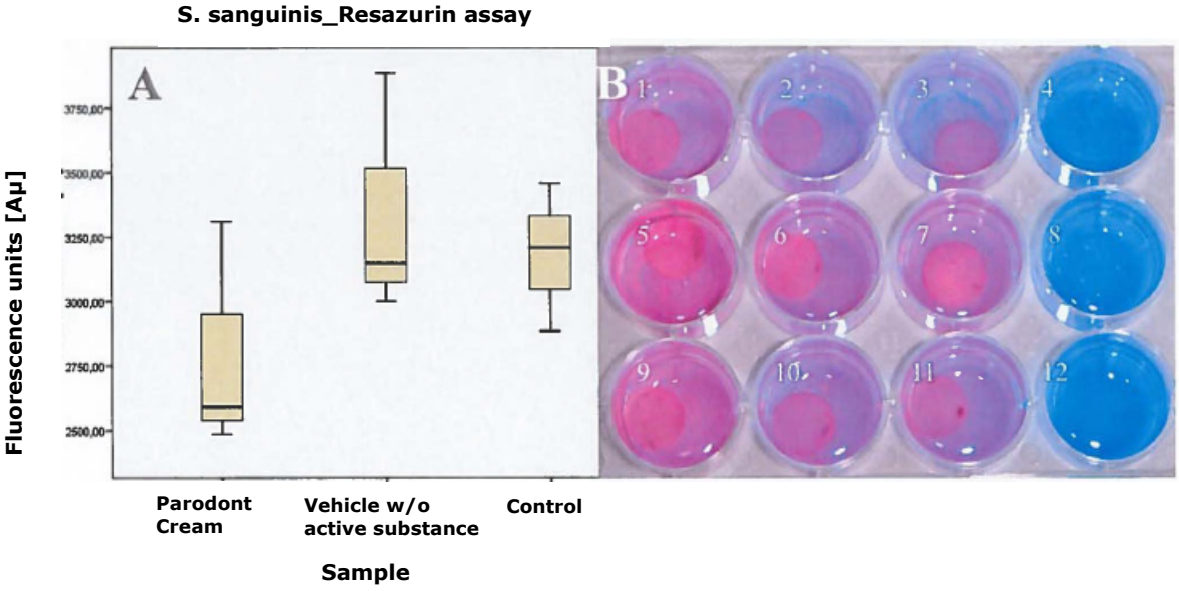

Fig. 3A/B

A shows a box plot diagram of the fluorescence intensities following incubation of colonised membranes *(S. sanguinis)* with Parodont Creme® (left), vehicle (middle) and without added substances (right) in resazurin solution; strong fluorescence equates to a high metabolic activity and is a sign of vital bacteria/biofilms; **B** shows a microtiter plate with colonised filter membranes following the treatment with resazurin solution; blue = no/little reduction of resazurin, purple = reduction of resazurin to resorufin; (1-3) colonised membrane incubated with Parodont Creme®, (4) Parodont Creme® without bacteria, (5-7) colonised membranes incubated with vehicle without active substance, (8) vehicle without bacteria, (9-11) colonised membrane without active substance (control), (12) resazurin solution without bacteria.

Streptococcus oralis

For the bacterium *S. oralis* the mean inhibitory zone diameter after 1 day was 2.86 cm (± 0.25 cm), after 5 days it was 2.78 cm (± 0.25 cm); an antibacterial effect on the bacterium was thus demonstrated. The differences in the size of the inhibitory zone on day 1 and day 5 were not statistically significant (p> 0.05; Fig. 4A/B). In the same way, no formation of an inhibitory zone was observed for the vehicle gel without an active substance. An effect on matured biofilms of the bacterium *S. oralis* could not be demonstrated in the resazurin assay; the differences between the groups (product/vehicle gel/control) were not statistically significant (p> 0.05, Fig. 5A/B).

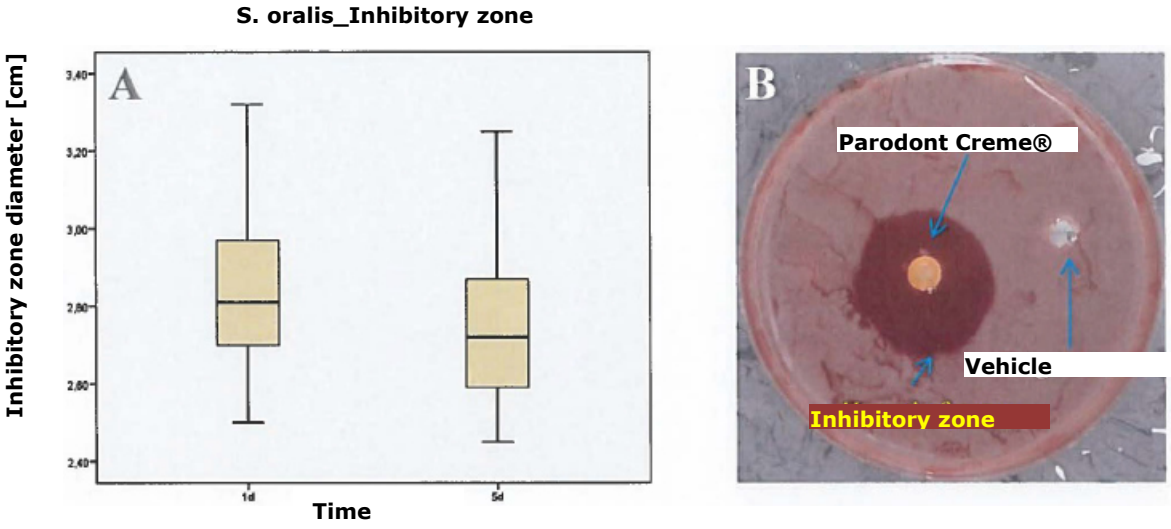

Fig. 4A/B

A shows a box plot diagram of the inhibitory zone diameter after 1 d (left) and 5 d (right); **B** shows an agar plate after 24 h of incubation with *S. oralis*, left hole with Parodont Creme® and marked inhibitory zone formation, right hole with vehicle without active substance and without inhibitory zone formation.

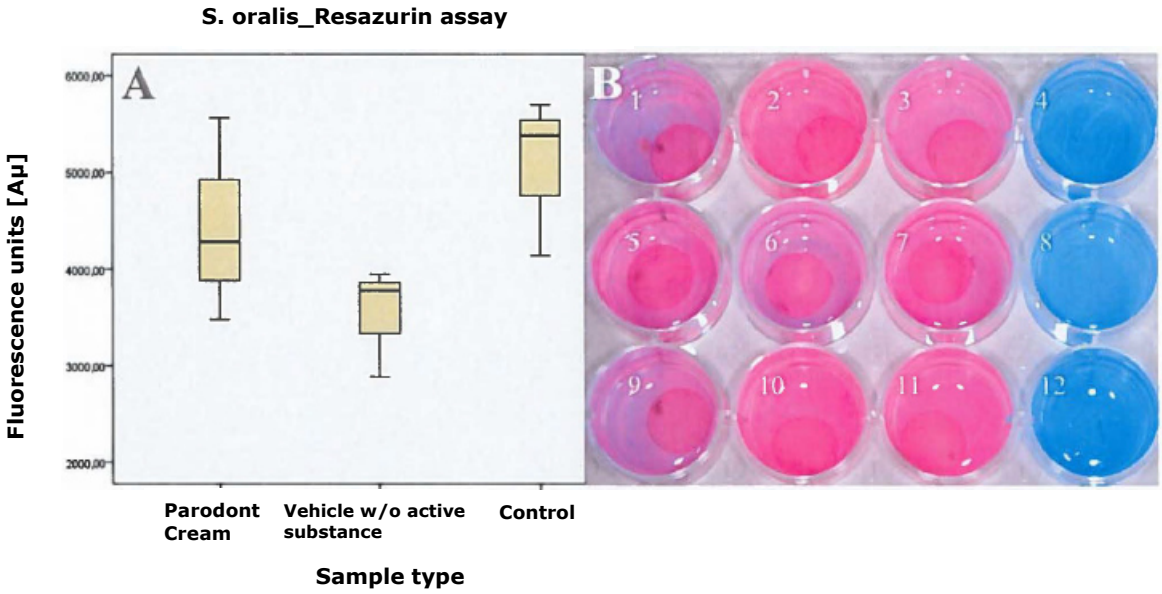

Fig. 5A/B

A shows a box plot diagram of the fluorescence intensity following incubation of colonised membranes *(S. oralis)* with Parodont Creme® (left), vehicle (middle) and without added substances (right) in resazurin solution; strong fluorescence equates to a high metabolic activity and is a sign of vital bacteria/biofilms;
B shows a microtiter plate with colonised filter membranes following the treatment with resazurin solution; blue = no/little reduction of resazurin, purple = reduction of resazurin to resorufin; (1-3) colonised membrane incubated with Parodont Creme®, (4) Parodont Creme® without bacteria, (5-7) colonised membranes incubated with vehicle without active substance, (8) vehicle without bacteria, (9-11) colonised membrane without active substance, (12) resazurin solution without bacteria.

Streptococcus gordonii

The mean inhibitory zone diameter in the tests with *S. gordonii* on day 1 after plating was 3.33 cm (± 0.18 cm). On day 5, a decrease to 3.08 cm (± 0.17 cm) was measured. This decrease in the inhibitory zone diameter was statistically significant (p= 0.011), and confirmed a decrease in the antibacterial effect over time (Fig. 6A/B). However, since the product can be applied daily, in practical use the loss of effect is probably not relevant. An effect on biofilms of the oral primary coloniser *S. gordonii* could not be demonstrated. The differences between the three groups (product/vehicle gel/control) were not statistically significant (Fig. 7A/B).

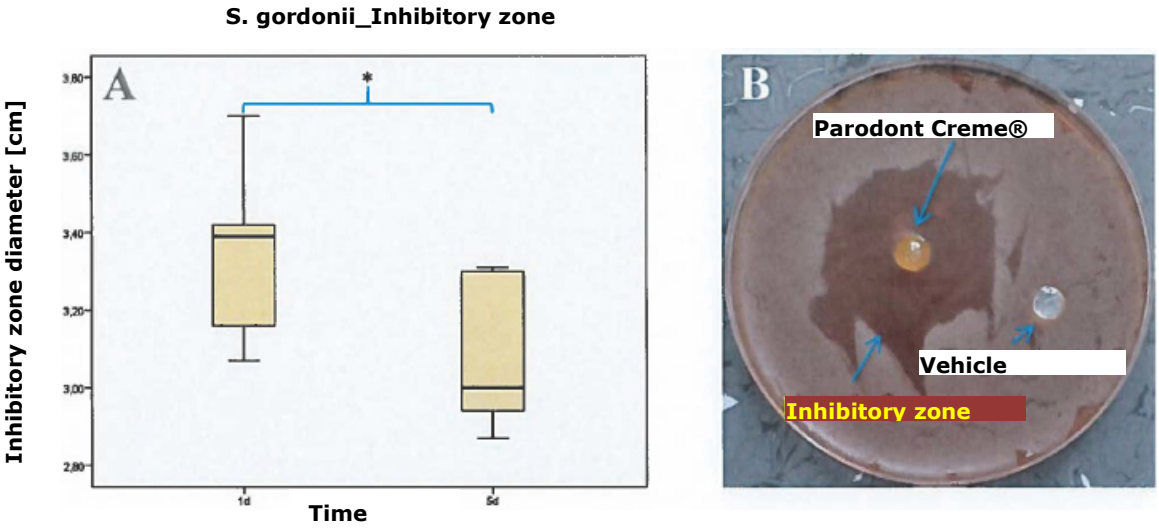

Fig. 6A/B

A shows a box plot diagram of the inhibitory zone diameter after 1 d (left) and 5 d (right), statistically significant outcomes marked with (*); **B** shows an agar plate after 24 h of incubation with *S. gordonii*, left hole with Parodont Creme® and marked inhibitory zone formation, right hole with vehicle without active substance and without inhibitory zone formation.

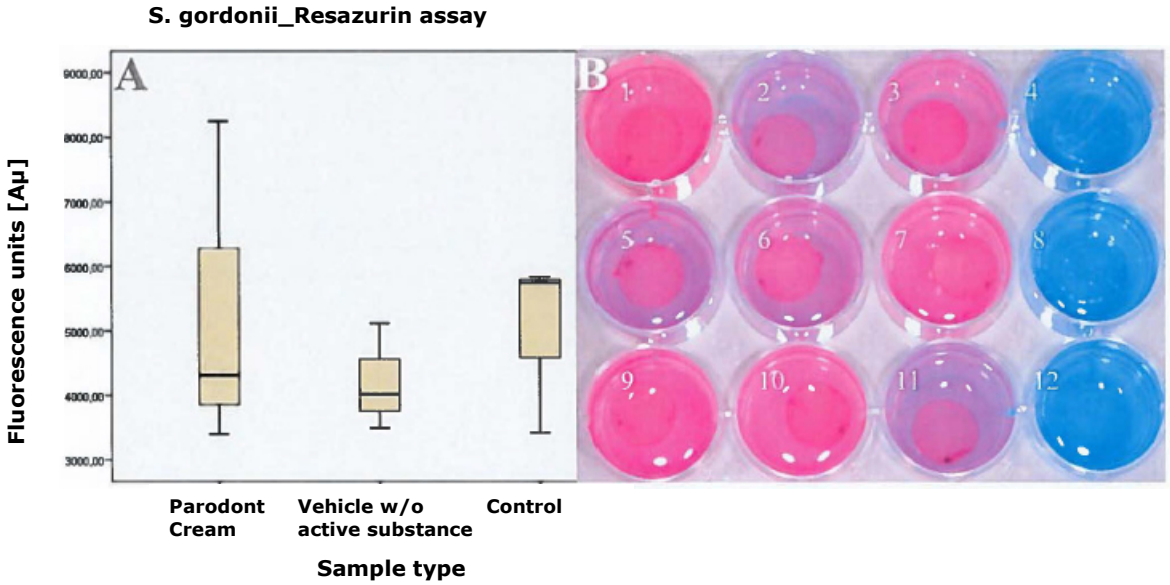

Fig. 7A/B

A shows a box plot diagram of the fluorescence intensity following incubation of biofilm-colonised membranes *(S. gordonii)* with Parodont Creme® (left), vehicle (middle) and without added substances (right) in resazurin solution; strong fluorescence equates to a high metabolic activity and is a sign of vital bacteria/biofilms; **B** shows a microtiter plate with colonised filter membranes following the treatment with resazurin solution; blue = no/little reduction of resazurin, purple = reduction of resazurin to resorufin; (1-3) colonised membrane incubated with Parodont Creme®, (4) Parodont Creme® without bacteria, (5-7) colonised membranes incubated with vehicle without active substance, (8) vehicle without bacteria, (9-11) colonised membrane without active substance, (12) resazurin solution without bacteria.

3 Summary

Under the given experimental conditions, an antibacterial effect of the product Parodont Creme® on the bacterial species *S. gordonii, S. sanguinis* and *S. oralis* was demonstrated in vitro. An effect against matured biofilms of the same species, on the other hand, was not confirmed in the experiments.