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Mango peel as a potential enzyme inducer in *Trichoderma harzianum*: a strategy for cariogenic biofilm degradation and reuse of industrial waste

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Water-insoluble exopolysaccharides (I-EPS) are a virulence factor for dental biofilms. It has already been demonstrated that mango pulp induces the secretion of glucan-hydrolytic enzymes in the fungus *Trichoderma harzianum*, and that they have an effect on I-EPS from young biofilms. **Aim:** Evaluate the effect of mango peel as an enzyme inducer in *T. harzianum*, and the effect of enzymes secreted on mature biofilms. **Methods:** Fractions of the peel (PL) and ethanol-precipitated pulp (PP) of Tommy Atkins mangoes were sterilized and added to a culture medium containing *T. harzianum* for induction of hydrolytic enzymes. After 192 h, the culture medium was centrifuged and the supernatant (enzyme extract) was used as treatment on *S. mutans* biofilms (n=9): a) NaCl 0.9 %; b) 0.12 % chlorhexidine digluconate; and c) extract of enzymes induced by PL or PP. Acidogenicity, bacterial viability, quantification of insoluble polysaccharides, and three-dimensional analysis of the biofilm by scanning electron microscopy (SEM) was performed. Data were analyzed by ANOVA followed by the Tukey test (α=5 %). **Results:** The hydrolytic enzymes did not alter the metabolism or bacterial viability of the biofilm ($p<0.05$). Although the images obtained by SEM suggest some degree of matrix degradation, the quantification of I-EPS for the PL and PP groups did not differ from the control group ($p>0.05$), suggesting a slight effect on the disorganization of the mature *S. mutans* biofilm. **Conclusion:** The results suggest that mango peel fraction can induce secretion of mutanase by *T. harzianum*, however in an insufficient amount to generate significant degradation on cariogenic biofilm.

Keywords: Biofilms. Biotechnology. Glucans. Mangífera. Waste management.

Introduction

Strategies for dental caries control such as reduction of frequency of sucrose consumption and fluoride use to balance the de- and re-mineralization process are well established[1]. Antimicrobial agents such as chlorhexidine, have proven effective in oral bacteria management. However, strategies distinct from the biofilm-bactericidal effect have been proposed[2], including enzymatic biofilm destabilization[3]. Although the role of *Trichoderma harzianum* is recognized to produce industrial enzymes with a broad range of applications supporting the bio-based economy, the potential of these enzymes to produce antibiofilm effect is not well established for mature cariogenic biofilms.

Biofilm virulence is enhanced by the presence of water-insoluble exopolysaccharides (I-EPS) and their relation with dental caries is recognized[4-6]. I-EPS is a glucan with a predominance of α-1→3 bonds[7] and performs a structural role in biofilm development and accumulation since they are not degradable for oral microorganisms[8] due to the absence of bacterial α-(1→3)-glucanases.

Fungi such as *Trichoderma harzianum* have been used as an important source for the production of hydrolytic enzymes such as α-(1→3)-glucanases (mutanases), which may represent new ways to control the biofilm matrix. Mutanases are inducible enzymes, which means that α-(1→3) bonds may increase their specific induction. Previous studies have already described the presence of glucans with α-(1→3) main chain and α-(1→4) branches isolated from mango pulp[9,10]. Although there are indications that mango pulp induces the secretion of mutanases in *T. harzianum* and that they have an effect on young biofilms[10], there are no studies that have evaluated the effect of mango peel as an enzyme inducer, nor the effect of enzymes induced by mango by-products on mature biofilms.

Thus, considering that the cariogenicity of the biofilm increases with the number of insoluble glucans[4] and that the degradation of such polymers would be a determining factor for the control of dental caries, the objective of this work was to evaluate the potential of mango peel as an enzyme inducer in *Trichoderma harzianum*, studying the effect of enzymes produced on cariogenic biofilm. In this way, instead of being a polluting source[11,12], mango industrial waste could become a source of mutanase enzyme inducers, being easily available for the production of enzymes that would degrade the oral biofilm, directly impacting the control of caries.

Materials and Methods

Obtaining raw mango fractions

Five undamaged Tommy Atkins mangoes were acquired in a mature stage suitable for consumption, and in complete physiological development, in the retail market in the city of Ribeirão Preto (São Paulo, Brazil). The fruits were washed in running water, followed by the separation of peels (80.40 g) and pulps (2412 g). They were transferred to different beakers containing 92 % ethanol, where they remained for 15 min. After complete ethanol evaporation, the peel fraction (PL), cleaned of pulp

residues, was transferred to another beaker containing 100 mL of absolute ethanol. After 20 min the ethanol had evaporated to dryness; the fraction was milled and reserved (33.1 g). A fraction of the pulp (650 g) was homogenized in a blender with 1L of Milli-Q water, followed by filtration through tissue. The filtrate was precipitated with three volumes of ethanol. After refrigeration for 24 h, the material was centrifuged, lyophilized, reserved, and nominated PP (33.4 g).

Enzyme induction

The study involved fermentations of different fractions of mango with the fungus *T. harzianum*. The fractions PP and PL were weighed, and 10 mg aliquots were placed in a 1.5 mL Eppendorf tube, then sent for irradiation at the Center for Nuclear Energy in Agriculture – CENA (University of São Paulo, Piracicaba, Brazil) with a dose of 100 Gy, and a dose rate of 0.169 kGy/h, for 35 min and 29 s (Gammacell 220 ®, MDS Nordion, Ottawa, Canada).

For induction, *T. harzianum* isolated from silage was grown in inclined tubes with potato dextrose agar at 30 °C. After seven days, a fungal spore suspension in 10 mL of sterile ultrapure water was obtained by scraping the agar surface. The spores were counted and inoculated in flasks containing 10 mL of sterile medium with 0.7 % K_2HPO_4 (w/v); 0.2 % KH_2PO_4 (m/v); 0.01 % $CaCl_2$ (m/v); 0.01 % $MgSO_4.7(H_2O)$ (m/v); 0.1 % yeast extract (w/v); 0.01 % peptone (m/v); and 0.01 % sodium citrate (m/v), at pH 6. The final spore concentration was $5x10^6$ conidia/mL. The material was incubated in a horizontal shaker (Incubator Chamber with Orbital Shaking MA420, Marconi Laboratory Equipment, Piracicaba, Brazil) at 30 °C with 120 rpm continuous agitation. After 18 h, the flasks were centrifuged at 12000 x *g*, 4 °C for 15 min. The supernatant was discarded and the precipitate containing the mycelium from each flask was resuspended in 10 mL of culture medium containing 0.01 % of yeast extract (w/v), 5 % Khanna's salts solution (v/v)[13], and 0.1 % sterile I-EPS or PP or PL (w/v). The flasks were placed in the horizontal shaker at 30 °C at 120 rpm for 192 h. After the incubation period, the material was centrifuged (12000 x *g*, 4 °C for 15 min). Each experimental group had 6 experimental units (flasks) and the supernatants from the six flasks were pooled together. The mix was filtered through a sterile syringe filter unit (PVDF, 0.20 μm, 25 mm, GVS Filter Technology, Morecambe, UK) and the clarified solution containing the enzymes was divided into aliquots intended for characterization of the extract (mutanase assay) and antibiofilm effect evaluation. All the aliquots were frozen at -21 °C until use. For the aliquots directed to antibiofilm analysis, they were thawed and heated through a water bath at 40 °C.

Mutanase assay

In order to diminish the ramifications and increase the proportion of (1→3)- α linkages, I-EPS were pretreated with dextranase of *Penicillium* sp. (25 U/mg, Sigma-Aldrich, St. Louis, USA) as described elsewhere with slight modification[10]. Briefly, 50 U of dextranase per mg of I-EPS were solubilized in a solution 0.2 % I-EPS in acetate buffer. The hydrolysis occurred at 37 °C, pH 6. After 72 h, the resulting suspension was centrifuged (1,000 x *g*, 10 min, room temperature). The supernatant was discarded and the

precipitate was resuspended in 500 µL of acetate buffer (pH 5.5, 0.2 mol/L). Then, 250 µL each of pretreated I-EPS solution was added to 250 µL of active and inactive fungal enzymatic extract (100 °C, 30 min, Drybath Stdrd blckm, Thermo Scientific, Massachusetts, USA) and were incubated at 40 °C for 3 h. The reaction was stopped by adding 100 µL of DNS solution and the supernatant was quantified for reducing sugars according to Miller[14] (1959). The absorbance of the samples was then measured using a spectrophotometer at 440 nm against their blanks. The protein content for the fungal extract was quantified applying the Bradford method[15], using bovine serum albumin as a standard.

Biofilm assay

The *S. mutans* biofilm in a "feast or famine" model simulates the fluctuation conditions of exposure to fermentable carbohydrates in the oral cavity, and therefore, is considered highly pathogenic.

Biofilms were formed on glass slides using the strain *S. mutans* UA159 (ATCC 700610), as described by Ccahuana-Vásquez and Cury[16] (2010). After reactivation, 10 colonies were transferred to an ultra-purified culture medium (UTYB) containing 1 % glucose (w/v) and incubated at 37 °C and 5 % CO_2 to form the starter. After 18 h, they were transferred to a sterile flask containing UTYB medium and 0.1 mmol/L glucose. Subsequently, 5 mL of this mixture was pipetted into wells of the 12-well plate followed by the insertion of glass slides (2.1 x 1.9 cm) and incubated at 37 °C and 5 % CO_2 for 24 h.

After initial growth of the biofilm, the glass slides were exposed for 1 min, eight times per day (08:00, 09:30, 11:00, 12:00, 13:30, 15:00, 16:00, and 17:30 h) to 10 % sucrose. After each cariogenic challenge, they were washed three times with 0.9 % NaCl and replaced in the ultra-purified culture medium wells. On the third day of growth, in addition to sucrose challenges, the biofilms from each group (n=9) were treated for one min, twice per day (8:00 and 17:00 h) for another two days, with one of the following treatments: a) NaCl 0.9 % (negative control); b) 0.12 % chlorhexidine digluconate (positive control); c) extract of enzymes induced by PL; and d) extract of enzymes induced by PP.

On the morning of the 6th day, the biofilms were harvested. Each glass slide was scraped with a sterile spatula and 10 mg of biofilm was introduced into a microcentrifuge tube containing 1.0 mL of saline solution. After homogenization, the biofilm harvest was performed by sonication at 7 W for 30 s[17]. The resulting suspension was used for biofilm analysis.

Biofilm analysis

Biofilm acidogenicity

During the biofilm assay, the culture media were changed daily and their pH was determined prior to disposal in order to assess the acidogenicity of the biofilms[18]. The pH measurement was performed using a pH microelectrode (Thermo Fischer Scientific, Waltham, USA) coupled to a pHmeter (ORION Benchtop 710A pH/ION Meter, Thermo Fischer Scientific, Waltham, USA) calibrated with pH standards of 4.0 and 7.0, performed directly inside the wells.

Bacterial viability

An aliquot of 100 µL of the suspension of biofilms was diluted in 0.9 % NaCl in series up to 10^{-7}. Then, two drops of 20 µL of each dilution were plated on BHI agar (Oxoid Ltd., Hants, UK) to determine the number of viable microorganisms. The plates were incubated at 37 °C with 5 % CO_2 for 24 h. Colony forming units (CFU) were counted with the aid of a stereoscopic magnifying glass and the results were expressed as CFU/mg of biofilm.

I-EPS degradation assay

From 1 mL suspension of biofilm, I-EPS were extracted according to Aires et al.[17] (2008). Resulting dry samples of I-EPS were resuspended in 500 µL of 1 mol/L NaOH. The amount of total carbohydrates was quantified by the phenol-sulfuric acid method[19], using glucose as a standard. The results were normalized by the wet weight of biofilm.

SEM analysis

In order to obtain representative images of the biofilms after treatment, biofilms were grown on 2.5 x 1 cm glass slides in the wells of a 24-well plate, according to the "feast and famine" protocol. The slides were stabilized as described by Krzyściak et al.[20] (2017). After air-dry overnight, the slides were transferred to copper disks and processed by carbon evaporation (2A, 2.10^{-1} mbar vacuum) then dusted with gold (120 s, 40 mA) in Bal-Tec SCD 050 equipment (Bal-Tec, CA, USA). The samples were analyzed using a scanning electron microscope (Zeiss EVO 50, Carl-Zeiss-Stiftung, Jena, Germany). The protocol was conducted in duplicate.

Statistical Analysis

The SAS system (SAS Institute Inc., Cary: NC, 2014) performed all the statistical tests considering a significance level of 5 %. Data were analyzed using analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons of means. The adherence of the residuals to Gaussian distribution was evaluated using the Shapiro-Wilk test, the skewness and kurtosis coefficients, and graphs (histogram and QQ-Plot).

A summary of the experimental design is shown in Figure 1.

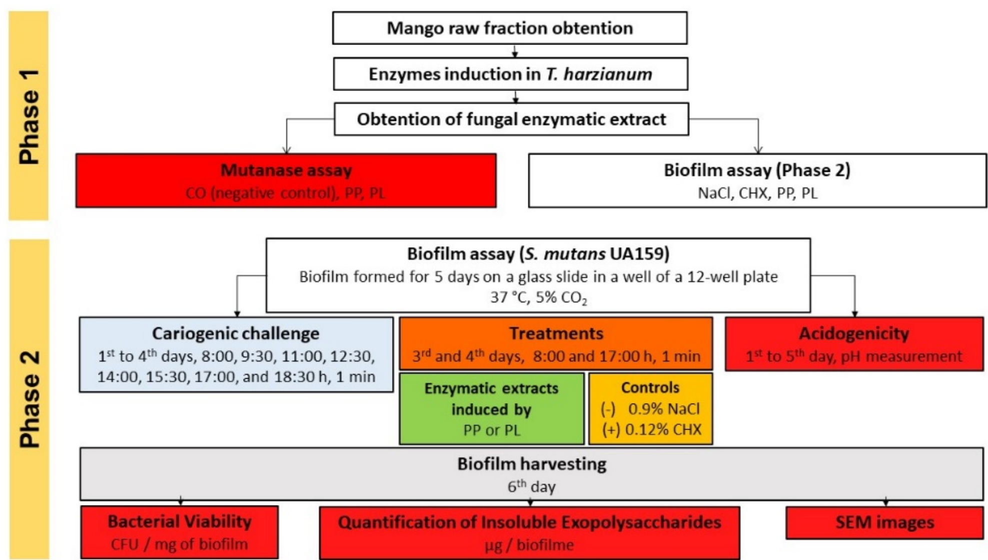

Figure 1. Experimental design flow chart from mango fraction acquisition until biofilm harvesting. The analysis performed are shown in red. CO (Negative Control) = fungus in culture medium not exposed to substrates, NaCl = 0.9 % sodium chloride solution, CHX = 0.12 % chlorhexidine digluconate, PP: fungal extract induced by mango ethanol-precipitated pulp; PL: fungal extract induced by mango peel.

Results

Mutanase assay

The specific activity assay sought to identify the enzymatic potential of the extracts for the degradation of α-(1→3) glucans (shown in Table 1). The enzyme activity indirectly indicates through the amount of product released the number of active enzymes under certain conditions (pH, substrate concentration, temperature). The specific activity of an enzyme indicates in addition to the activity, the degree of purity of this enzyme in a mixture. Thus, the specific activity refers to the amount of substrate converted to product by the active enzyme per unit of time and total protein mass. Through the obtained results, it is possible to observe that there are mutanases in the enzymatic extracts induced by peel and ethanol-precipitated pulp, suggesting that these mango fractions have inducing potential in *T. harzianum*. However, the amounts of enzymes produced among the total amount of proteins are smaller than those observed in the control group.

Table 1. α-1,3-glucanase activity in mU per mg of protein present in the fungal extract after 192 h of exposure to different mango substrates. A unit of mutanase activity (U) represents the amount of enzyme needed to produce reducing sugars equivalent to 1 μmol of glucose per minute. CO (Negative Control) = fungus in culture medium not exposed to substrates. PP: fungal extract induced by mango precipitated pulp; PL: fungal extract induced by mango peel. Distinct capital letters indicate statistically significant differences ($p < 0.05$) among groups (mean ± SD; n = 9).

Group	Specific Activity (mU/mg of protein)
CO	130.39 ± 64.8^{A}
PP	102.7 ± 13.66^{A}
PL	92.31 ± 29.37^{A}

Biofilm assay

Acidogenicity is the ability of a bacterium to produce acid by lactic fermentation, which is an indicator of biofilm metabolism. Data demonstrate (shown in Table 2) that all treatments varied pH between the first (pH around 6) and 6th day (pH around 4), except in the CHX group (positive control) which had low variation (0.31 ± 0.17) indicating that the extracts, unlike chlorhexidine, preserve bacterial metabolism. In addition, no action was observed in the number of CFU, suggesting that the fungal extracts do not influence bacterial viability as shown in Figure 2.

Table 2. pH variation between experimental days 1 and 6 in the biofilm culture media according to different treatments. Distinct capital letters indicate statistically significant differences ($p < 0.05$) among groups (mean ± SD; n = 9). Negative control - NaCl: 0.9 % NaCl, Positive Control - CHX: 0.12 % chlorhexidine digluconate, PP: fungal extract induced by mango ethanol-precipitated pulp; PL: fungal extract induced by mango peel.

Treatment group	ΔpH
NaCl	2.52 ± 0.07^{A}
CHX	0.31 ± 0.17^{B}
PP	2.47 ± 0.15^{A}
PL	2.48 ± 0.11^{A}

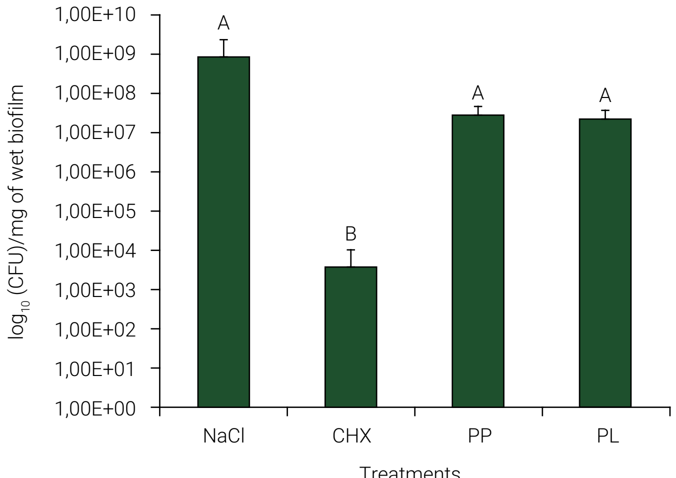

Figure 2. CFU counts per mg of biofilm (log_{10}(CFU)/mg of biofilm wet weight) according to the treatments. Distinct capital letters indicate statistically significant differences ($p < 0.05$) among groups (mean ± SD; n = 9). Negative control - NaCl: 0.9 % NaCl, Positive Control - CHX: 0.12 % chlorhexidine digluconate, PP: fungal extract induced by mango ethanol-precipitated pulp; PL: fungal extract induced by mango peel.

The quantification of insoluble extracellular polysaccharides in the matrix of biofilms after treatment suggests that there is insufficient enzymatic action on the biofilm matrix to notably reduce the amount of I-EPS (shown in Figure 3). Also, the groups Precipitated Pulp (PP) and Peel (PL) presented similar performances, which corroborate mutanase assay data. The dataset obtained indicates that even though mango

peel and pulp fractions are potential enzyme inducers in *T. harzianum*, the amount produced could not significantly decrease the number of insoluble glucans.

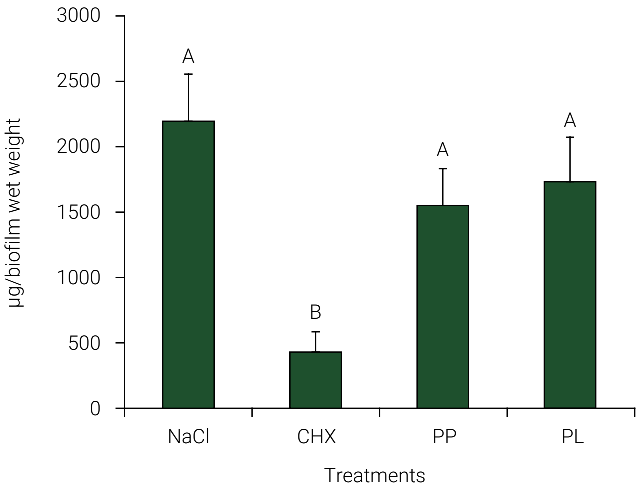

Figure 3. Water-insoluble extracellular polysaccharides (µg/ wet biofilm) according to the treatments. Distinct capital letters indicate statistically significant differences ($p < 0.05$) among groups (mean ± SD; n = 9). Negative control - NaCl: 0.9 % NaCl, Positive Control - CHX: 0.12 % chlorhexidine digluconate, PP: fungal extract induced by mango ethanol-precipitated pulp; PL: fungal extract induced by mango peel.

At the 200x magnification (shown in Figure 4), it was possible to observe conglomerates (microcolonies or “clusters”) with bacterial cells joined and surrounded by smooth-looking material, the matrix rich in insoluble glucans (indicated by the black arrow). When observing the images of the CHX group when compared to the NaCl group (negative control), it is possible to notice that the microcolonies are sparse and less voluminous. At 10Kx magnification, the scarcity of cells resulting from the bactericidal action of chlorhexidine is remarkable. The little matrix present is, for the most part, remnants of production in the first 48 h of the experiment in which there was no exposure to treatments. The presence of cracks in the CHX group images implies a biofilm less voluminous, and consequently more susceptible to dehydration during sample preparation. At a higher magnification, more exposed cells with some spots of a visible concentrated matrix are observed.

For the PP group, images at lower magnification do not show cracks as seen in the CHX group images, indicating a slightly thicker biofilm. However, in an enlarged view, an organization with areas of matrix concentration covering the bacteria and intensely uncovered areas is visible, suggesting enzymatic degradation. Images of the PL group, in turn, present robust and voluminous microcolonies close to the aspect viewed in the NaCl group. At a higher magnification, the diversity of matrix deposition areas next to less covered areas is visible, which may indicate low enzymatic action, resulting in a low degree of degradation.

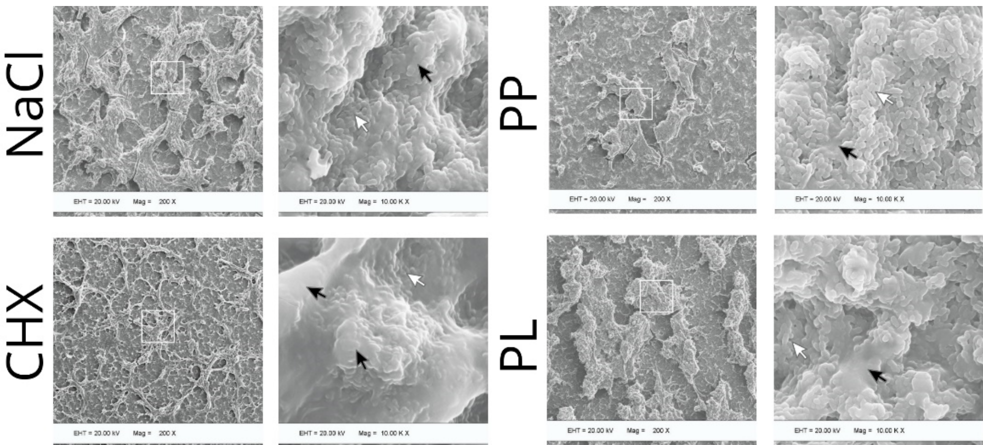

Figure 4. Representative SEM images of bacterial cells (white arrow) and glucans (black arrow) within biofilms formed by *S. mutans* UA159 on glass slide surface in "feast-famine" regimen. The label on the left indicates the sample group according to the fungal extract or control solution used as treatment. Each square refers to the region presented in a close-up view in the image on its right. White tags indicate electron high tension (EHT) voltage and magnification (Mag) for image acquisition. Negative control - NaCl: 0.9 % NaCl, Positive Control - CHX: 0.12 % chlorhexidine digluconate, PP: fungal extract induced by mango ethanol-precipitated pulp; PL: fungal extract induced by mango peel.

Discussion/Conclusion

I-EPS is a major component of cariogenic biofilm matrix and a critical virulence factor for dental caries. Thus, degradation of I-EPS is a key strategy to dismantle cariogenic biofilms. Here, we investigated the potential of mango by-products as inducers of mutanase secretion on *T. harzianum* and the *effect* of short-term exposure to mutanase on mature *S. mutans* biofilm through mutanase activity, quantification of matrix water-insoluble polysaccharides, bacterial viability, acidogenicity, and morphology by Scanning Electron Microscopy (SEM), observing that the mango fractions used were a suitable inducer for *T. harzianum,* even though the mutanase produced were insufficient to provide a relevant effect on the cariogenic biofilm matrix.

With the advent of biotechnological interventions, heterologous overexpression in suitable hosts, immobilization on novel matrices, and tailoring of fungal enzymes are being pursued. *Trichoderma* secretome is composed of an arsenal of enzymes such as proteases, chitinases, pectinases, xylanases, β-glucanases, and mutanases, among other carbohydrate-active enzymes – CAZy[21]. The more complex the substrate, the more enzymes it takes to degrade it. Peel (PL) and ethanol-precipitated pulp (PP) of Tommy Atkins mango used in this study are crude fractions and could induce a range of fungal enzymes. Although preliminary, this study showed a promising strategy that should be explored in Dentistry to control dental caries by degrading the biofilm matrix.

Ripe Tommy Atkins mango may contain 0.9 to 1.38 g of protein per 100 g of raw pulp in addition to 12.8 g of carbohydrate and 0.22 g of lipid[22-24]. Meanwhile, in the peel the amount of protein can vary +- 4 % with the dry weight, a mix of carbohydrates comprises more than 90 % of the weight, in addition to other components such as

cellulose, hemicellulose, lignin, carotenoids, α-tocopherol, and polyphenols[25,26]. The variability of components requires the fungus to secrete different enzymes but in relation to mutanase, the results show that mutanase specific activity is not different compared to the control group, and inferior to that found by Wiater et al.[10] (2013) that described a specific activity of 992.64 mU/mg (33.75 mU/mL and protein concentration of 34 μg/mL). However, the substrate used for induction was a purified polysaccharide and the proportion used was 2.5 times higher than that used in the present study (2.5 mg/mL versus 1 mg/mL). Thus, the results suggest that the refinement of mango fractions (purity) and the increase in the proportion between substrate and medium (availability) may be an interesting future strategy for this bioprocess in order to obtain an extract with a higher amount of active mutanases.

The results demonstrate that treatment with mutanases did not modify the number of viable bacteria and their metabolism, which agrees with the literature[27,28]. Recently, time-lapse confocal imaging data[28] showed that treatment with mutanase and dextranase leads to biofilm matrix collapse and bacterial dispersion. *In vitro* studies have signaled that dispersed bacteria could colonize other surfaces and restart the biofilm cycle[29,30]. However, for oral biofilms, other factors, including dynamics due to constant salivary flow and periodic swallowing of saliva[31,32] should be considered. Thus, the detachment of oral biofilm provoked by enzymes could be a feasible approach to control biofilm mass as well as improve antimicrobial performance by removing matrix protection and exposing bacteria[28,33,34]. This strategy should be considered in further studies using other mango fractions.

Although our results imply that the treatments with the experimental groups were not enough to degrade insoluble polysaccharides, SEM images suggest that there is some enzymatic action on the biofilm matrix even after a short exposure. Probably, the quantity of enzymes produced could not significantly decrease the number of insoluble glucans in the experimental groups. Wiater et al.[10] (2013) analyzed the effect on the biofilm using a mixture of pulp polysaccharide-induced mutanase associated with commercial dextranase in a mixed biofilm within 24 h of formation. After 3 h of exposure to the enzyme mixture, only 18.2 % of the biofilm remained adhered to the glass slide. Although our work used a highly cariogenic validated monospecies model, and an even shorter exposure time (one minute) than the previous study, a tendency towards a decrease in the number of structural polysaccharides is still noticeable. This observation constitutes relevant data for these enzymes' application as an adjuvant in daily oral care products.

Attachment of microorganisms such as *S. mutans* can occur to natural surfaces such as teeth or artificial surfaces in experimental models (glass slides). The model we used is rich in glucans, which also play an important role in adhesion[35]. A study[36] showed that there is no significant change in bacterial colonization on abiotic surfaces (hydroxyapatite, stainless steel, glass) if sucrose is present. The presence of sucrose on any of the surfaces increased the amount of *S. mutans* biofilm in relation to the control. Also, the feast and famine model responds both to demineralization and to the use of agents that act on bacterial biofilms[16]. This model used with glass slides was also able to respond to positive (CHX) and negative (NaCl) controls,

as can be seen in Figures 2 and 3. Further investigations should focus on isolating the polysaccharides of interest from the peel and pulp fractions and combining them to increase the amount of the target substrate and intensify the fermentation process. Furthermore, other industrial wastes resulting from mango processing should have their biotechnological potential explored, representing a promising source for prospecting new substances with biological relevance. The development of new ways of obtaining mutanases may pave the way for the development of formulations for oral hygiene with matrix-targeting enzymes and antimicrobials capable of overcoming the problem of bacterial dissemination caused by the matrix degradation and holistic treatment of pathogenic biofilms. In summary, the findings of our study suggest that mango peel fractions were able to induce expression of mutanases in *T. harzianum* but in insufficient amounts to have a relevant effect on insoluble glucans in the cariogenic biofilm.

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Statements & Declarations

Competing Interests

The authors have no conflicts of interest to declare.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Statement of Ethics

Ethics approval was not required for this study.

Author Contribution

Jéssica Silva Peixoto Bem: Conceived and designed the experiment, performed the experiment, analyzed and discussed the data, wrote the draft manuscript; **Ana Cristina Morseli Polizello:** performed the experiment, analyzed and discussed the data; **Hamilton Cabral:** Conceived and designed the experiment, analyzed and discussed the data; **Nathalia Gonsales da Rosa-Garzon:** performed the experiment,

analyzed and discussed the data; **Carem Gledes Vargas Rechia:** performed the experiment, analyzed and discussed the data; **Carolina Patrícia Aires:** Conceived and designed the experiment, analyzed and discussed the data, wrote the draft manuscript. All authors reviewed and approved the final version of the manuscript.

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