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Original Research

Development of a Postbiotic-Based Orodispersible Film to Prevent Dysbiosis in the Oral Cavity

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Abstract

Background: Oral diseases affect over three billion peopleand are among the most commonly observed infections worldwide. Recent studies have shown that controlling the ecology of the oralome is more effective in reducing the risk of caries than the complete removal of both harmful and beneficial microorganisms. This work aimed to develop a strategy for preventing dysbiosis in the oral cavity by applying a postbiotic-based orodispersible film. **Methods**: *Lactiplantibacillus plantarum* 226V and *Lacticaseibacillus paracasei* L26 were cultured in De Man–Rogosa–Sharpe (MRS) broth for 48 hours, followed by centrifugation and filtration. Then, the resultant postbiotics were then subjected to various dilutions (10% (v/v), 20% (v/v), 40% (v/v), 60% (v/v) and 100% (v/v)) and co-incubated with *Streptococcus mutans*. Antimicrobial efficacy, minimal inhibitory concentration, the time required to inhibit *S. mutans* growth, and antibiofilm properties of the postbiotics were assessed. Subsequently, an orodispersible film comprising polymers and plasticizers, namely Xanthan gum, maltodextrin, and glycerol, was developed as a vehicle for postbiotic delivery. Formulation optimization, physical property evaluation, and cytotoxicity against the TR146 human oral cell line (TR146 cell line) were conducted. **Results**: Postbiotics demonstrated antimicrobial and antibiofilm activity against *S. mutans* following 24-hour co-incubation. The minimal inhibitory concentration for combined postbiotics administration was 20% (v/v). Remarkably, 79.6 $\pm$ 8.15% inhibition of biofilm formation was achieved using 100% (v/v) of the postbiotic derived from *L. plantarum* 226V. Incorporating postbiotics did not compromise the dissolution time of orodispersible films, all exceeding 20 minutes. Furthermore, solubility improved following postbiotic addition, facilitating ease of handling. Importantly, postbiotic-impregnated orodispersible films were non-cytotoxic when exposed to the TR146 cell line. **Conclusions**: These findings underscore the potential of orodispersible films loaded with postbiotics as a promising potential intervention for oral dysbiosis.

Keywords: oral dysbiosis; postbiotics; *Streptococcus mutans*; antibiofilm capacity; orodispersible films

1. Introduction

Dental caries is the most prevalent infection worldwide among oral diseases, with more than 3.5 billion people experiencing it at least once in their lifetime [1]. Poor oral health impacts mouth well-being and has significant implications for overall health [2]. Studies have linked inadequate oral hygiene to various systemic conditions, including cardiovascular issues, chronic obstructive pulmonary disease, bone resorption, inflammatory bowel disease, and neurodegenerative diseases [3–5].

Oral health relies heavily on maintaining a balanced oral microbiota. When this equilibrium is disrupted, dysbiosis occurs, and certain microbiota bacteria undergo overgrowth, leading to biofilm formation [6–9]. The essential characteristic of biofilm formation is the bacterial ability to adhere to surfaces [10]. Moreover, it is crucial to consider that dysbiosis happens before the first symptoms of the oral disease appear, underscoring the importance of effective preventive measures in controlling this condition [11].

As the biofilm starts to form and the environment changes, there is loss in the diversity of the microbiota, which contributes to dysbiosis. Of note is the ability of these bacteria to adhere, a fundamental property for the bacteria in the biofilm to disrupt the homeostasis of the oral cavity. It is important to understand that the first colonizers of the biofilm are usually gram-positive bacteria, namely *Streptococcus mutans*, which ultimately serve as a bridge for other bacteria to bind, worsening the dysbiotic state [10].

The main bacteria found in dental biofilms include *Streptococcus*, *Actinomyces*, *Prevotella*, *Porphyromonas*, *Tannerella*, and *Fusobacterium* spp. [6]. These bacteria produce a matrix rich in glucans and exopolysaccharides (EPS), facilitating their adherence to one another and surrounding tissues [9]. Additionally, biofilms contain endotoxins, namely lipopolysaccharides (LPS), which elicit an inflammatory response in the host. As a protective structure, biofilms prevent chemical agents from reaching the microorganisms, thereby increasing antibiotic resistance [9,12].

The conventional approach for treating cavities involves mechanically removing the lesion and dental plaque. However, this method removes both beneficial and harmful microorganisms, disrupting microbial balance and creating opportunities for the adherence of dental pathogens to the oral surface by facilitating the obtention of nutrients due to the lack of competition [13]. In contrast, recent re-

search has explored postbiotics as a potential solution for preventing oral dysbiosis. Postbiotics are inactive microorganisms, their components, and metabolites that promote health when administered [14–16]. Common postbiotic metabolites include short-chain fatty acids (SCFAs) and organic acids, namely acetic, lactic, butanoic, and propionic acids, as well as antimicrobial molecules, such as bacteriocins [16–19], amino acids [20], EPS, cell wall peptides and lysates, varied enzymes [19,21], flavonoids, and phenolic compounds [22,23]. Recent studies have shown that postbiotics can influence commensal microorganisms in the oral cavity, helping to restore balance [14,24]. The administration of postbiotics in the oral cavity can be associated with an anti-biofilm capacity against *S. mutans*, a desirable characteristic for controlling dysbiosis. This effect can be correlated with the presence of teichoic acids produced by *Lactobacillus* spp. [17].

Furthermore, postbiotics present the ability to reduce the levels of oral pathobionts, with SCFAs potentially inhibiting bacterial growth by disrupting their membrane [12,18,24]. Postbiotics derived from lactic acid bacteria (LAB) often contain bacteriocins, which exhibit inhibitory activity against pathogens [16]. In this work, the postbiotics were obtained from *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei*, two lactic acid bacteria that produce bacteriocins and have been considered adequate to use in the oral cavity [1].

One significant advantage of postbiotic administration is the absence of live microorganisms, eliminating the risk of transmitting resistance genes or causing infection in vulnerable groups, such as the elderly, children, and pregnant women [14,16,18,24]. Moreover, postbiotics offer a longer shelf life than probiotics and require less storage and transportation facilities [14,16,24,25].

The effectiveness of postbiotics in promoting oral health depends on the method of administration, which determines their contact time within the oral cavity and how effectively they release their beneficial properties. Orodispersible films (ODF) are a promising delivery method due to their ability to quickly and easily deliver active ingredients. ODFs should be non-toxic, biocompatible, and have no expected adverse effects after use [26–32]. Furthermore, the manufacturing of ODF is simple, brief, and cost-effective [32].

Although ODFs show promise for administering postbiotics and meeting necessary release conditions, further research is needed to clarify their impact on oral dysbiosis. Therefore, our study aimed to address this gap by developing an ODF incorporating optimized postbiotics. To achieve this goal, we (*i*) assessed the *in vitro* antimicrobial and antibiofilm capabilities of specific postbiotics, including those derived from *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, and a combination of both, to determine their effectiveness in combating oral pathogens, (*ii*) investigated the biological properties of the postbiotics to gain insights into their mechanisms of action and potential health benefits; (*iii)* optimized the formulation and manufactured the orodispersible films to ensure their efficacy and stability; and (*iv*) analyzed the physicochemical characteristics of the films to understand their composition and properties.

With this work, we aimed to advance in innovative strategies for maintaining oral health and offer potential alternatives to traditional antibiotic therapy for managing oral dysbiosis.

2. Materials and Methods

2.1 Preparation of Postbiotics and Postbiotic-Based Orodispersible Films

Lactiplantibacillus plantarum 226V and *Lacticaseibacillus paracasei* L.26 were obtained as a DELVO-PRO freeze-dried, concentrated starter cultures from DSM (Moorebank, Australia). To obtain the postbiotic solutions, *Lactiplantibacillus plantarum* 226V and *Lacticaseibacillus paracasei* L.26 were grown in De Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) and isolated in MRS agar (Biokar Diagnostics, Beauvais, France) as previously described by Sornsenee *et al*. [17] with some modifications. Briefly, after obtaining isolated colonies of both species, each was inoculated in a 15 mL falcon in MRS broth (Biokar Diagnostics, Beauvais, France) and incubated at 37 °C for 48 h.

After that period, the falcon tubes were centrifuged at 8000 g (15 min at 4 °C) using a Hettich centrifuge (Hettich, Tuttlingen, Germany). The supernatant was then filtered with a 0.22 µm membrane to obtain the probiotics' cell-free supernatant (CFS), the postbiotic solution. To ensure that the supernatant did not contain any cells, the solution was plated in MRS agar (Biokar Diagnostics, Beauvais, France) using the drop method, and the growth was observed after incubation of 24 h at 37 °C.

To incorporate the CFSs postbiotics in the orodispersible films, the postbiotic solutions were added to the polymeric solution 2, adjusting the amount of water to maintain the final volume constant.

2.2 Antimicrobial Activity of Postbiotics against S. mutans

To evaluate the postbiotic's antimicrobial activity, the growth rate of *S. mutans* 45091 with a concentration of 10^9 colony-forming units (CFU)/mL was evaluated under distinct postbiotic conditions, according to Jung *et al*. [33]: those obtained from *L. plantarum,* those from *L. paracasei*, and a mixture of both, in the concentrations of 10% (v/v), 20% (v/v), 40% (v/v), 60% (v/v) and 100% (v/v). Different concentrations were selected based on the concentration-dependent activity of the postbiotics. Postbiotics were tested in the maximum range possible (0–100%) to understand this characteristic.

Initially, *S. mutans* was grown in BHI broth (Biokar Diagnostics, Beauvais, France) and isolated in BHI agar (Biokar Diagnostics, Beauvais, France) plates. After obtaining isolated colonies, *S. mutans* was regrown in BHI broth (Biokar Diagnostics, Beauvais, France) until a 10^9 CFU/mL concentration was reached. Then, a co-culture of the *S. mutans* in BHI broth (Biokar Diagnostics, Beauvais, France) and the CFS postbiotic solution were mixed in a 15 mL falcon tube in a 1:1 ratio and incubated for 24 h at 37 °C. Following that time, a 20 µL sample was taken from each condition and plated in BHI agar (Biokar Diagnostics, Beauvais, France) in triplicate. The results were expressed as positive or negative growth. Afterwards, with an inoculum of 10^9 CFU/mL, a 96-well plate with all the different postbiotic concentrations was inoculated to determine the postbiotic's antimicrobial activity against *S. mutans*. The essay was performed in triplicate. The plate was then incubated at 37 °C while measuring the Optical Density (OD) at $\lambda = 600$ nm for 24 h in a Multiskan GO plate reader (Thermo Scientific, Waltham, MA, USA).

To understand the standard growth rate of *S. mutans*, positive control of the microorganism and negative controls of each medium were used. The values were then averaged to obtain the mean results, which were used to analyze the effects of the different postbiotics on the *S. mutans* growth rate.

2.3 Monitoring the Reduction of S. mutans Co-Cultured with Postbiotics

2.3.1 Minimal Inhibitory Concentration

The methodology from Drumond *et al*. [34] was followed to determine the minimal inhibitory concentration (MIC). For that, samples of *S. mutans* were grown in MRS broth at 37 °C until reaching an OD at $\lambda = 600$ nmof 1.0, which corresponds to a concentration of 10^9 CFU/mL, and further decimal dilutions were performed to obtain the concentration of 10^6 CFU/mL. Afterwards, in a 96-well microplate, 100 µL of *S. mutans* together with 100 µL of the three postbiotic solutions in five different concentrations: 10% (v/v), 20% (v/v), 40% (v/v), 60% (v/v) and 100% (v/v) were mixed and incubated at 37 °C for 24 h. A positive control of *S. mutans* and MRS broth was used. After this period, the OD was measured at $\lambda = 600$ nm in a Multiskan GO plate reader (Thermo Scientific, Waltham, MA, USA). The minimal inhibitory volume was defined as the lowest volume of postbiotic that inhibited the growth of *S. mutans*.

2.3.2 Time-Kill Assay

The time-kill assay was conducted according to Rossoni *et al*. [35], with modifications regarding the incubation period. *S. mutans* was grown until a 10^9 CFU/mL concentration was reached. The bacteria were then incubated at 37 °C with the postbiotic solutions in their minimal inhibitory concentration. At different time points (0, 1, 2, and 4 h), a sample of 1 mL was taken from the mixture. After serial dilutions, these were plated (20 µL), in duplicate, in BHI agar and incubated at 37 °C for 24 h. The plates were then counted to determine the CFU/mL. A positive control (*S. mutans*) was also added to plates, incubated, and counted.

2.4 Antibiofilm Capacity of the Postbiotics against S. mutans

2.4.1 Antibiofilm Formation Inhibition

S. mutans was cultured in BHI broth until a 10^9 CFU/mL concentration was reached. For the biofilm formation assay, 100 µL of *S. mutans* suspension (above) was added to each well of a 96-well microplate. Various postbiotics were then introduced at 5 different concentrations: 10% (v/v), 20% (v/v), 40% (v/v), 60% (v/v) and 100% (v/v). The microplates were incubated at 37 °C for 72 h to allow the biofilm formation. After the incubation period, the content of each well was carefully removed, and the wells were washed with Ringer solution to ensure that only the adhered biofilm remained. Following the protocol by Costa *et al*. [36], the biofilms were stained with 0.1% crystal violet, and the plates were left to dry at room temperature for 24 h. Finally, the wells were resuspended in glacial acetic acid (30%), and the OD was measured at 630 nm.

2.4.2 Mature Biofilm Inhibition

The mature biofilm inhibition test was performed based on the methodology described by Sornsenee *et al*. [17] with some modifications. *S. mutans* was grown in BHI broth until a 10^9 CFU/mL concentration was reached. Then, 100 µL were placed in a 96-well microplate and incubated for 5 days at 37 °C to allow biofilm to reach its mature state. The CFS postbiotic solutions were then added to the formed biofilm in different concentrations and left to react for 72 h at 37 °C. The remaining steps were performed as described above.

2.5 Formulation of the Orodispersible Film

Orodispersible films were produced following the solving-casting method, according to Shah *et al*., 2022 [37], with some modifications. In a beaker, 2.0 g of Xantham gum (Sigma-Aldrich, Darmstadt, Germany) was added to 140 mL of deionized water and left in a magnetic stirrer for at least 4 h (Solution 1). In another beaker, 2.0 mL of Glycerol (VWR chemicals, Solon, OH, USA) was added to 58 mL of deionized water and stirred for 2 h. Following that time, the rest of the excipients were added: 0.2 g of Citric acid (Merck, Darmstadt, Germany) followed by 3.0 g of Maltodextrin (Sigma-Aldrich, Darmstadt, Germany) and stirred for 2 h (Solution 2) at 50 °C. Finally, Solution 2 was poured slowly over Solution 1 with constant stirring until complete homogenization. The final solution was left to rest until the complete disappearance of the bubbles formed during magnetic agitation, then spread over a plastic con-

tainer and left to dry for 48 h at room temperature. Afterwards, the samples were cut using a box cutter into 1 × 1 cm^2 squares for further analysis.

2.6 Analysis of the Physical Properties of the Orodispersible Film

2.6.1 Surface Morphology and Appearance

The surface appearance was observed after the ODFs were dried, before and after cutting, to understand if the surface was homogenous, without bubbles, and transparent. The observations were performed for all the samples, with and without the postbiotic solution, and the results were compared.

2.6.2 Disintegration Time and pH

The disintegration time was performed according to Shah *et al*., 2022 [37], with a few modifications. The orodispersible films were cut into squares measuring 1 × 1 cm^2 to measure the disintegration time. 100 mL of deionized water was added to a beaker. Then, the previously cut ODFs were added, and the stopwatch was started. After the film wholly dissolved, the stopwatch was stopped.

Each measurement was performed in triplicate, and the mean value was calculated. The results were compared to the orodispersible films without the impregnation of postbiotics, which served as a control.

The pH was then measured, according to Salawi [32]. After the complete disintegration/dissolution of the ODFs in water, the pH was measured using a Crison basic 20 pH probe (Crison, Barcelona, Spain). Each measurement was performed in triplicate, and the average value was calculated. The results obtained with the ODFs impregnated with CFS postbiotics were compared to those obtained with the ODFs without the impregnation of postbiotics to understand the pH variation.

2.6.3 Thickness

According to Batista *et al*. [38], the thickness of the ODFs was measured using a My20 micrometer (Adamel lhomargy, Saint-Baldoph, France). Squares of 1 × 1 cm^2 were cut from the orodispersible films, and three randomly selected points were measured on each sample to assess the homogeneity of the films. The mean thickness value was calculated and compared to control films without postbiotics.

2.6.4 Film Weight

According to Choi *et al*. [39], the mass was measured using an analytical scale (Sartorius, Göttingen, Germany) to understand the variation of the ODF's weight with the addition of the three conditions of the postbiotic solution. Each measurement was performed in triplicate, and the values were averaged to obtain the mean value. The ODFs without the addition of the postbiotics were used as a control.

2.6.5 Hydration, Moisture Loss, and Solubility

The percentages of hydration, moisture loss, and solubility were measured according to Al-Naamani *et al*. [40] with modifications. The orodispersible films, previously cut into 1 × 1 cm^2 squares, with and without postbiotics, were weighed under the same conditions (W_1). The ODFs were then submerged in 10 mL of deionized water for 1 hour and weighed again after removing the excess water with a paper towel (W_2). To obtain the solubility and moisture loss percentage values, the ODFs were stored in the incubator at 37 °C for 24 h, and one last measure was conducted after that period (W_3).

All measurements were performed in triplicate on an analytical scale (Sartorius, Göttingen, Germany), and the values were averaged to obtain the mean values.

The percentages of hydration, moisture loss, and solubility were calculated using the following equations:

$$\% \text{ hydration } = \left(\frac{W_2 - W_1}{W_1}\right) \times 100 \quad (1)$$

$$\% \text{ moisture loss } = \left(\frac{W_2 - W_3}{W_2}\right) \times 100 \quad (2)$$

$$\% \text{ solubility } = \left(\frac{W_1 - W_3}{W_1}\right) \times 100 \quad (3)$$

2.6.6 Contact Angle

The contact angle of the ODFs was determined through the sessile drop technique using a tensiometer (Attension Theta, Biolin Scientific, Sweden). For that, 5 µL of deionized water was dispensed on the film samples, and the angle formed between the baseline and the lines tangent to the water droplet. The values were recorded for 1 min, and the average value was calculated to perform the analysis. The ODFs without CFS postbiotic solution were used as a control.

2.7 Cell Culture and Cytotoxicity Evaluation of the Postbiotic-Based Orodispersible Film on Human Oral Cells

TR 146 human buccal carcinoma cell line was used as an *in vitro* model of the human epithelial mucosa. The cell line was purchased from Sigma-Aldrich (# 10032305) and validated by STR profiling and tested negative for mycoplasma (Mycostrip 50, InvivoGen, San Diego, CA, USA). After defrosting, it was maintained in HAMS F12 medium (Bio West) with 10% of Fetal Bovine Serum (FBS, Biowest), and 1% of Penicillin-Streptomycin-Fungizone

solution (Penstrep, Lonza). The TR146 cell line was then maintained in T75 flasks at 37 °C in a 5% CO_2 humidified atmosphere during the experimental time.

TR146 cells were plated onto a 24-well microtiter plate at 10^5 cells/mL of density to perform the cytotoxicity assay and allowed to attach overnight. The medium was replaced, and two pieces of each film with a 0.5 cm diameter were added to the wells in triplicate per condition. Five conditions were tested: (1) control cells (cells were cultured only with medium, without any film); (2) control of the film (cells were cultured in the presence of film without postbiotics); (3) CFS *L. plantarum* (cells were culture in the presence of film with CFS postbiotics obtained from *L. plantarum*); (4) CSF *L. paracasei* (cells were culture in the presence of film with CFS postbiotics obtained from *L. paracasei*), and (5) CSF both (cells were culture in the presence of film with a mix of CFS postbiotics obtained from both probiotics). After 24 hours of incubation, the metabolic activity of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Sigma-Aldrich). For this purpose, the culture media was removed and replaced with 450 µL of HAMS F12 and 50 µL of MTT solution per well. These were incubated for 4 h at 37 °C in a CO_2 incubator. The culture media was discarded, and 500 µL of DMSO per well was added to dissolve the formazan crystals. Subsequently, the plates were agitated for 10 minutes at room temperature. The absorbance was measured at λ = 570 nm using a microplate reader (Synergy 4, Biotek). Three independent assays in triplicate for each condition were performed, and the cell viability was calculated in percentage.

2.8 Statistical Analysis

All analyses were done with GraphPad Prism version 10.1.1 software (GraphPad Software, Inc., San Diego, CA, USA). The antimicrobial and antibiofilm activity were analyzed using a two-way ANOVA followed by Dunnett's multiple comparison test to assess significant differences. For the analysis of the ODF's physical properties and cytotoxicity, the one-way analysis of variance (ANOVA) method was used for multiple comparisons, followed by Tukey's post hoc test after testing for the normal distribution of all data.

All experiments were performed in triplicate. The results are expressed as means, and the corresponding standard deviations were calculated. Values were considered statistically different at a p-value ≤ 0.05.

3. Results and Discussion

3.1 Monitoring the Reduction of Viable Numbers of S. mutans in Co-Culture with Postbiotics

Minimal Inhibitory Concentration and Time-Kill Assay

The minimal inhibitory concentration (MIC) assay was conducted with all postbiotic solutions to determine the concentration (v/v) required to inhibit the growth of *S. mutans*. This is a crucial assessment since the dysbiotic environment improves the growth of *S. mutans*, and its metabolic activity creates an anaerobic environment that allows other pathogens, such as *Porphyromonas gingivalis* and *Treponema denticola*, to grow. The resulting acid production culminates in an enhanced demineralization process of the dental enamel, ultimately forming a cavity [1].

The minimal inhibitory concentration was defined as the lowest postbiotic concentration, resulting in no visible growth of *S. mutans* (Fig. 1A). For CFS postbiotics from *L. plantarum*, the (MIC) was 40%. At the same time, for those from *L. paracasei*, it was 60%. Interestingly, CFS postbiotics from both probiotics exhibited the lowest concentration needed for complete bacteria inhibition, with only 20% required to inhibit *S. mutans*' growth. This suggests an advantage in obtaining CFS postbiotics from two probiotic species due to their synergetic behavior.

Additionally, a time-kill assay was performed to evaluate the growth inhibition or death of *S. mutans* in co-culture with different postbiotics (Fig. 1B). Notably, postbiotics demonstrating higher antimicrobial activity showed a quicker time to kill *S. mutans*. Both CFS obtained from *L. plantarum* and those from both probiotics took 2 h before no growth after plating and counting could be observed. In comparison, CFS postbiotics from *L. paracasei* required 4 h until no growth was detected.

A positive control was used in both tests to ensure the growth of *S. mutans*. However, it is important to notice that the CFU/mL of S. mutans was adjusted to 10^6 in the MIC assay.

These findings are promising for incorporating postbiotics into ODFs, as they exhibit rapid antimicrobial activity without requiring significant time to exert their effects, thus making them suitable for oral cavity administration.

The results obtained in this study are in accordance with those in the literature [12,17,34,35].

3.2 Antimicrobial Capacity of the Postbiotics against S. mutans

Regarding the antimicrobial measurements performed with the postbiotics against *S. mutans*, it was critical to understand how the co-culture of the different postbiotics in distinct concentrations affected the microorganism's growth rate. Firstly, a co-culture of various concentrations of *S. mutans* with the three postbiotics (at 50% concentration) was plated, and growth inhibition was observed (data not shown). Based on these results, the growth rate of *S. mutans* at a concentration of 10^9 CFU/mL was evaluated for each postbiotic during 24 h.

Growth Rate Measurement of S. mutans

In co-culture with CFS postbiotic from *L. plantarum,* the growth rate of *S. mutans* varied notably with increasing postibiotic concentration (see Fig. 2A). Interestingly, even

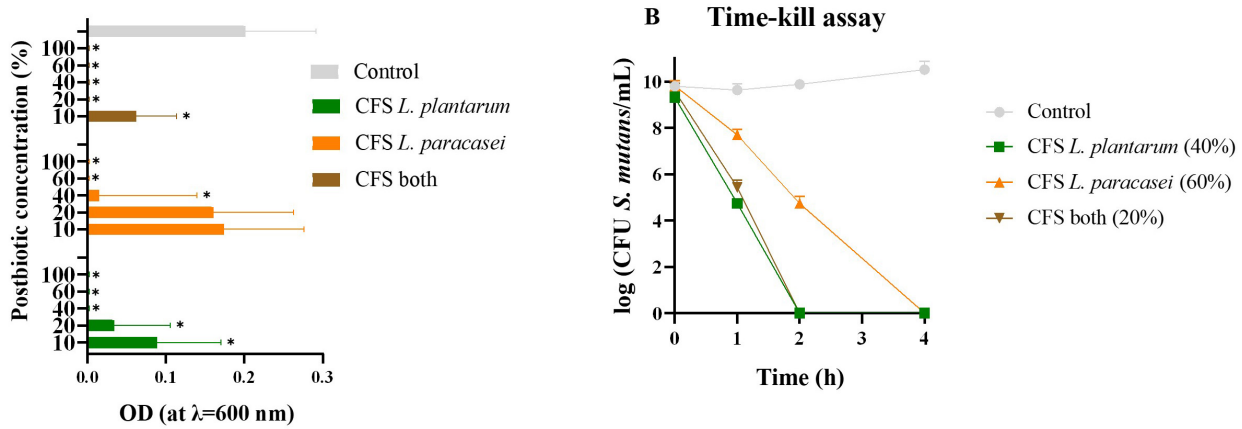

Fig. 1. Minimal inhibitory concentration and Time-kill assay. (A) Optical density (OD) variation of *S. mutans* measured at λ = 600 nm with different cell-free supernatant (CFS) postbiotic concentrations. (B) Values of the logarithm of the Colony-Forming Units (CFU) of *S. mutans* in co-culture with different CFS postbiotics over 4 hours. The data is presented as mean $\pm$ SD. * indicates significant differences between the samples ($p < 0.05$) and the control. The conditions are classified as Control (*S. mutans* without postbiotics); CSF *L. plantarum* (*S. mutans* in co-culture with postbiotics obtained from *L. plantarum*); CSF *L. paracasei* (*S. mutans* in co-culture with postbiotics obtained from *L. paracasei*) and CSF both (*S. mutans* in co-culture with postbiotics obtained from both *L. plantarum* and *L. paracasei*).

at 10% concentration (sub-MIC) of CFS postbiotic, a discernible difference was observed. When a concentration of 40% was reached, the growth was minimal, and no pronounced difference was noted between the higher concentrations (40, 60, and 100%). These findings align with those of the MIC (Fig. 1A). Similarly, in co-cultures with CFS postbiotics from *L. paracasei*, a decline in *S. mutans* growth rate was observed with increasing postbiotic concentration (Fig. 2B). However, only at a high concentration (100%) did the postbiotic notably reduce bacterial growth. This indicates that the antimicrobial activity of the *L. paracasei* postbiotic was less effective compared to that of *L. plantarum*, corroborating the results of the MIC assay (Fig. 1A).

Analysis of co-cultures with postbiotics obtained from both probiotics revealed a significant impact on *S. mutans* growth rate (Fig. 2C). Notably, the postbiotic mixture from both probiotics exhibited higher antimicrobial activity than individual postbiotics. Once again, these findings are consistent with those of the MIC assay, indicating that a lower concentration of the postbiotic mixture is needed to effectively inhibit *S. mutans* growth (Fig. 1A). From Fig. 2, it is clear that at 10% (sub-MIC) concentration, inhibition of S. mutans can be observed in all tested CFS's.

3.3 Antibiofilm Capacity of the Postbiotics against S. mutans

Studying the potential antibiofilm activity is crucial in controlling oral cavity homeostasis, considering *S. mutans*' ability to adhere to oral mucosa and dental surfaces, leading to biofilm formation. Two key aspects must be addressed: inhibiting biofilm formation and disrupting mature biofilms. The former is vital for preventive approaches, while the latter is essential for co-adjuvant therapy. Given that disease symptoms manifest later than dysbiosis onset, postbiotics could be applied in an asymptomatic oral cavity already experiencing dysbiosis. Moreover, microorganisms become more resistant to therapies once the biofilm forms. Hence, understanding the mature biofilm disintegration/inhibition capacity of different postbiotic solutions tested in this study is imperative.

The results obtained in our study are in accordance with other findings [12,17,33].

3.3.1 Biofilm Formation Inhibition

The antibiofilm activity correlated directly with postbiotic concentration (Fig. 3A) in inhibiting biofilm formation. The CFS postbiotic from *L. plantarum* exhibited the highest inhibition percentage at 100% concentration (79.6 $\pm$ 8.15%). Following closely, the CFS postbiotic from both probiotics showed 79.4 $\pm$ 15.48% inhibition at 100% concentration. Conversely, the CFS postbiotic from *L. paracasei* demonstrated the lowest activity, reaching its highest inhibition percentage (72.2 $\pm$ 7.48%) at 60% concentration instead of the maximum.

Interestingly, the postbiotic from *L. plantarum* displayed a notable inhibition percentage (75.5 $\pm$ 7.24%) at its minimal inhibitory concentration (40%). However, the CFS postbiotic from both probiotics showed a lower inhibition rate (34.3 $\pm$ 12.43%) at its minimal inhibitory concentration (20%). Despite these substantial inhibition rates, none of the postbiotics reached statistical significance re-

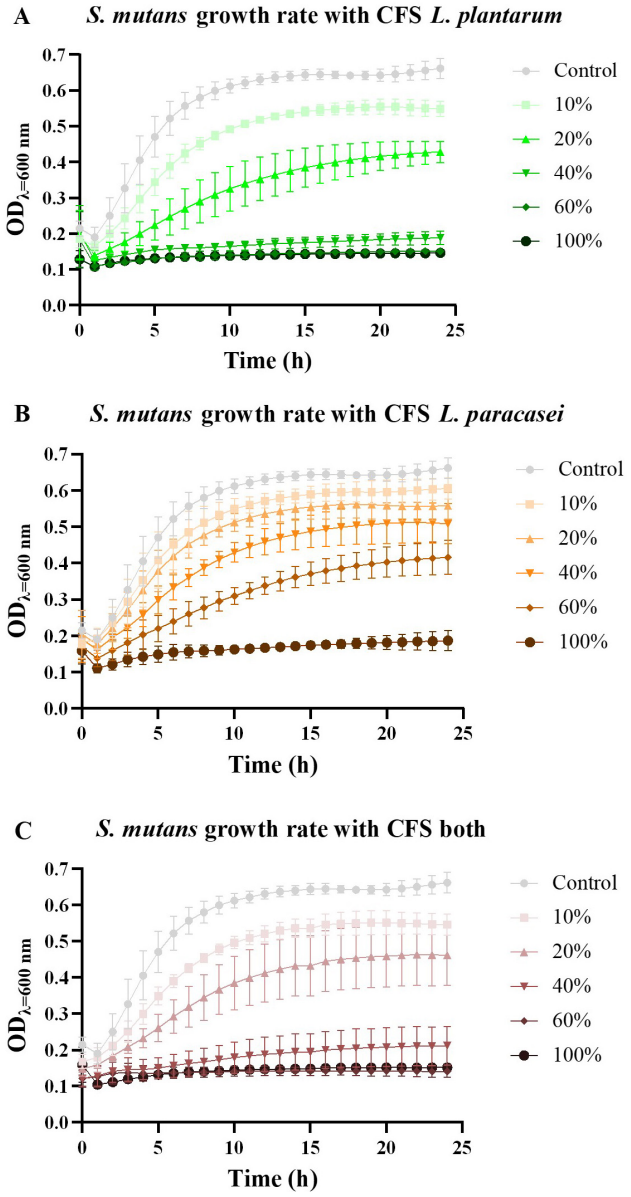

Fig. 2. Growth rate measurement. (A) The Optical Density (OD) variation of *S. mutans* measured at λ = 600 nm over 24 hours represents the growth rate of *S. mutans* in co-culture with CFS postbiotics obtained from *L. plantarum*. (B) The OD variation of *S. mutans* measured at λ = 600 nm over 24 hours represents the growth rate of *S. mutans* in co-culture with CFS postbiotics obtained from *L. paracasei*. (C) The OD variation of *S. mutans* measured at λ = 600 nm over 24 hours represents the growth rate of *S. mutans* in co-culture with CFS postbiotics obtained from both probiotics. The data is presented as mean ± SD. The control represents the growth of *S. mutans* without postbiotics.

garding biofilm inhibition percentage, indicating that while they showed considerable inhibition rates, further investigation may be needed to confirm their relevance.

3.3.2 Mature Biofilm Inhibition

The mature biofilm inhibitory properties of the tested postbiotic were expected to be lower compared to biofilm formation inhibition due to the heightened resistance of bacteria in mature biofilms to treatment. As shown in Fig. 3B, none of the tested postbiotics reached 50% inhibition, indicating the significant challenge in removing mature biofilms.

Curiously, mature biofilm inhibition activity appeared unrelated to postbiotic concentration. The highest inhibition percentage (44.7 ± 9.90%) was achieved with the CFS postbiotic from *L. plantarum* at only 20% concentration, which is lower than the MIC. In contrast, CFS postbiotics from *L. paracasei* and the postbiotic solution from both probiotic bacteria displayed anti-biofilm activity against mature biofilms only at 100% concentration, with values of 6.1 ± 4.87% and 28.8 ± 7.17%, respectively. Despite these findings, no statistical significance was observed in the mature biofilm inhibition assay. Biofilm formation shows progressive development; it begins with reversible adhesion, followed by irreversible adherence and consequent maturation. As the biofilm reaches its mature state, an equilibrium is formed between the microorganisms involved in this process [2], rendering it harder to break down. This explains the lower inhibition achieved with mature biofilms compared to forming biofilms.

However, the potential activity demonstrated by CFS postbiotics from *L. plantarum* warrants further analysis. The lack of correlation between antibiofilm activity and concentration requires deeper investigation, as these results were unexpected.

3.4 Physical Characteristics of the Orodispersible Film

3.4.1 Surface Morphology and Appearance

The orodispersible film formulation was optimized for elasticity, appearance, and maneuverability. According to Mura *et al*. [41], an ODF that presents elasticity and flexibility ensures a pleasurable sensation in the oral cavity. The optimized formulation was based on Cugini *et al*. [42], who stated that film-forming polymers should constitute up to 50% of the total concentration, followed by up to 20% of plasticizers, 10% of sweetening agents, and 10% of saliva stimulants. In this sense, the final optimized concentration of the film-forming solution was 25% (m/v) film-forming polymers (Xantham gum and Maltodextrin), 15% (m/v) plasticizer agent (Maltodextrin and Glycerol), 1% (m/v) saliva stimulant (Citric acid), and 1% (v/v) sweetening agent (Glycerol). After drying, the postbiotic-free ODFs presented adequate handling; they were thin and easy to cut. Regarding color, the ODFs were transparent. However, the same was not observed regarding the ODFs impregnated with the postbiotic solutions. During the formulation of the ODF, a significant number of bubbles were formed in the solutions; however, when left to rest before pouring, the complete disappearance of the bubbles was

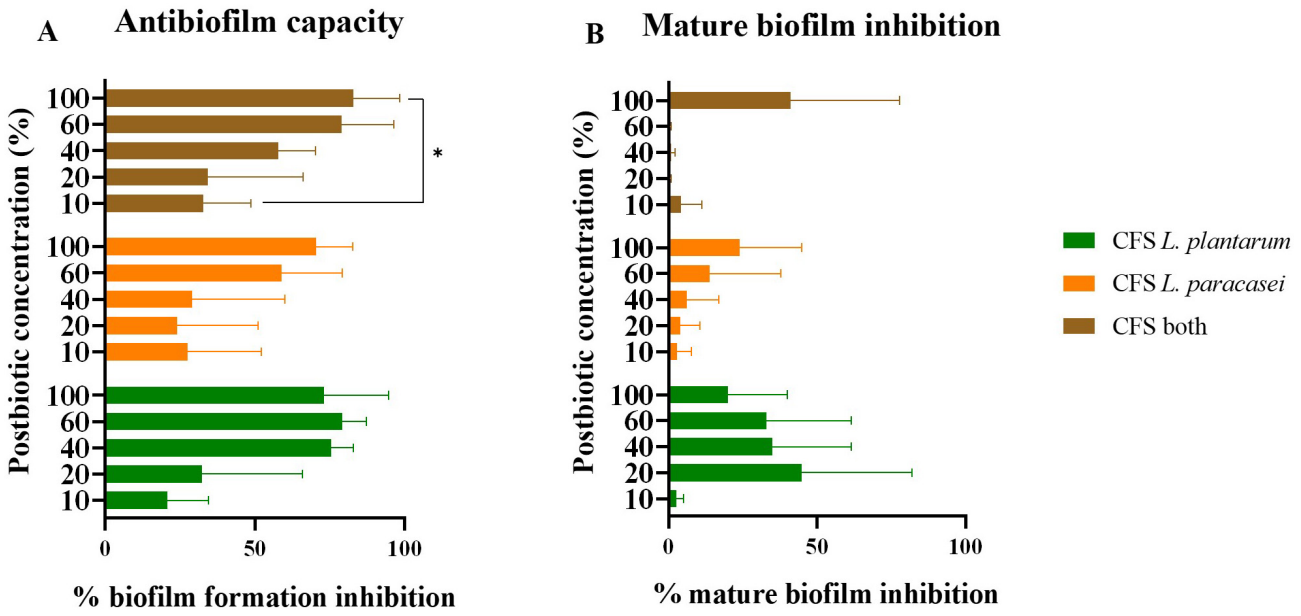

Fig. 3. Antibiofilm capacity. (A) Inhibition percentage of *S. mutans* biofilm formation with different CFS postbiotics in distinct concentrations. (B) Inhibition percentage of *S. mutans* mature biofilm formation with different CFS postbiotics in distinct concentrations. * indicates significant differences between the samples ($p < 0.05$). The data is presented as mean ± SD. The conditions are classified as CSF *L. plantarum* (*S. mutans* in co-culture with postbiotics obtained from *L. plantarum*); CSF *L. paracasei* (*S. mutans* in co-culture with postbiotics obtained from *L. paracasei*) and CSF both (*S. mutans* in co-culture with postbiotics obtained from both *L. plantarum* and *L. paracasei*).

noted. However, the bubbles persisted in the solution impregnated with the postbiotic, even after the rest period; they were darker and not homogenous. Nonetheless, the ODFs impregnated with CFS postbiotics were more accessible to handle since they were thicker and less gelatinous.

The choice of natural polymers was based on the fact that they are biodegradable and biocompatible, do not present toxicity for the oral cavity, and and they are generally recognized as safe (GRAS) by the FDA [43]. Maltodextrin was chosen based on the appearance it provides the films; however, its mechanical properties are often limited [37]. To overcome this problem, xantham gum was used concomitantly as a film-forming polymer.

3.4.2 Disintegration Time and pH

A significant value to determine regarding ODFs is the disintegration time, i.e., the time it takes to dissolve entirely after contact with the oral cavity. There are still no guidelines regarding the dissolution time. However, the time should be long enough to ensure that the postbiotics can be delivered to the oral cavity and exert their activity. Still, according to Lordello *et al.* [28], the disintegration time is directly related to the polymer concentration in the ODF.

The ODF without the impregnation of CFS postbiotics served as a control and presented a dissolution time of 24 ± 2 min. It was observed that there was no significant difference between the disintegration times of the samples when compared to the control; the CFS postbiotic obtained from *L. plantarum* and *L. paracasei* both dissolved in 24 ± 3 min, and the CFS postbiotic obtained from both probiotics dissolved in 25 ± 2 min. According to these results, there were also no statistically significant differences between the postbiotics tested (Fig. 4A).

However, it is essential to remember that *in vitro* behavior differs drastically from *in vivo* performance. If the ODFs are applied to the oral cavity, the time it takes to dissolve them is expected to decrease since they will be affected by deglutition, speech, and the normal movement of the tongue.

The pH was measured after the complete dissolution of the ODFs; data is shown below in Fig. 4B. The ODF without the impregnation of the postbiotics served as a control and showed a pH value of 4.33 ± 0.50. The pH value of the ODF impregnated with CFS postbiotics obtained from *L. plantarum* was significantly lower (3.86 ± 0.07) compared to the control. The remaining ODFs were not statistically different from the control. However, an increase in the pH of the ODFs impregnated with CFS postbiotics from *L. paracasei* was noted (4.58 ± 0.11), which was significantly higher than the ODF with postbiotics from *L. plantarum*.

This demonstrates that, probably during growth, *L. plantarum* produced acidic metabolites, such as lactic acid, which offers more antimicrobial activity against pathogens. It is also likely that *L. plantarum* produces more acidic metabolites during growth, which can be noted when com-

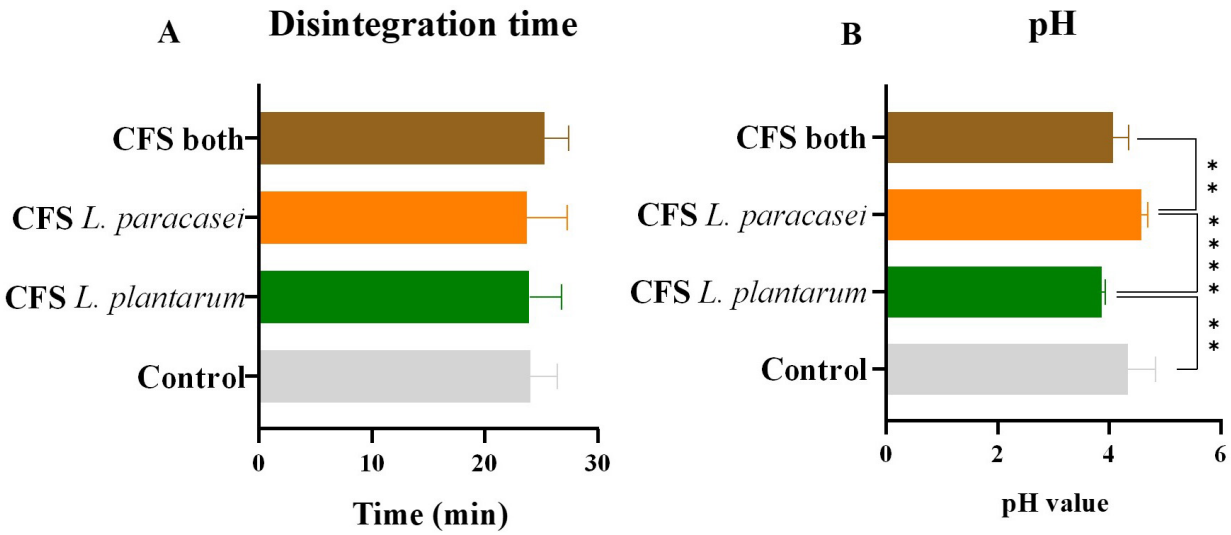

Fig. 4. Disintegration time and pH values. (A) Time, in minutes, until complete dissolution of ODFs and (B) pH values of the different oral dispersible films (ODFs) impregnated with postbiotic solutions. The data is presented as mean ± SD. ** indicates significant differences between samples ($p < 0.01$) and **** ($p < 0.0001$). The conditions are classified as Control (ODF without the impregnation of CFS postbiotics); CSF *L. plantarum* (ODF containing CSF postbiotic obtained from *L. plantarum*); CSF *L. paracasei* (ODF containing CSF postbiotic obtained from *L. paracasei*) and CSF both (ODF containing CSF postbiotic from both *L. plantarum* and *L. paracasei*).

paring the antimicrobial activity with the postbiotic obtained from *L. paracasei*. The ODF impregnated with CFS postbiotics obtained from both probiotics had a pH value of 4.06 ± 0.28, which was not statistically different from those obtained from *L. plantarum* or the control but significantly lower than those obtained from *L. paracasei*.

The films' pH was desired to be closer to neutral (around 7) [44]. However, lower pH values were expected since the chosen probiotics are LAB that produce acid during growth [28]. Cytotoxicity assessments showed that none of the tested ODFs presented increased cytotoxicity when in contact with oral cavity cells. This demonstrates that the low pH does not present a safety limitation. However, its application could be limited since it might exacerbate the already acidic environment in an unhealthy oral cavity. Additionally, some acids produced during *Lactobacillus* spp. growth are responsible for disrupting the bacterial membrane, which could translate into an absent antimicrobial activity after the neutralization of the ODFs.

The low pH of the control was due to the addition of citric acid, which acts as a saliva stimulant. These low pH values might contribute to the films' antimicrobial activity and the maintenance of a desired salivary flow, which can contribute to removing harmful bacteria in the oral cavity. However, multiple factors could influence this activity, and further analysis is needed to determine if the antimicrobial effect persists after neutralizing the film-forming solutions.

3.4.3 Thickness and Weight

Another physical property of the ODFs assessed was the variation of their thickness before and after the addition of the different CFS postbiotic solutions (Fig. 5A).

The ODF without impregnating the postbiotic solutions was considered a control, presenting a thickness of 0.175 ± 0.080 µm. When compared to the ODFs after impregnation with postbiotics, a statistically significant increase in the ODFs thickness was noted, namely 0.327 ± 0.041 µm for ODF with CFS postbiotic obtained from *L. plantarum* and 0.346 ± 0.024 µm for ODF with CFS postbiotic obtained from *L. paracasei*. Regarding the ODF impregnated with CFS postbiotics obtained from both probiotics, it was not as thick (0.266 ± 0.029 µm) but equally different from the control. The thickness increase was directly related to the easiness of maneuvering and cutting the ODFs into 1 × 1 cm^2.

Measurements were taken using an analytical scale to understand the variations in film weight before and after incorporating different postbiotic solutions. The ODF without any CFS postbiotic solution served as a control. All ODFs showed significant weight increases with incorporating CFS postbiotics (Fig. 5B).

The control weight was 0.0210 ± 0.0007 g; the ODF impregnated with CFS postbiotic from *L. plantarum* increased to 0.0427 ± 0.0019 g. The ODF impregnated with CFS postbiotics from *L. paracasei* weighed 0.0492 ± 0.0029 g, significantly more than the control but not statistically different from the *L. plantarum* sample. The ODF with CFS postbiotics from both probiotics increased its weight to 0.0346 ± 0.0072 g, but this was not significantly different from ODF impregnated with CFS postbiotics obtained from *L. plantarum*.

As expected, the weight variations were similar to thickness changes observed with different postbiotic solutions. However, some authors suggest that the increased

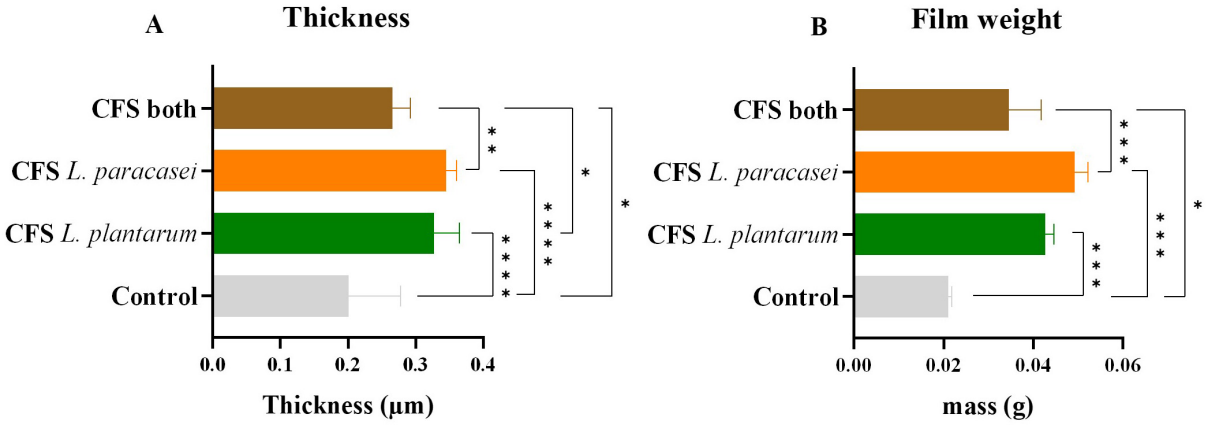

Fig. 5. Thickness and film weight. (A) Thickness values, in µm, of the different samples of postbiotic-impregnated ODFs. (B) Film weight values, in grams, of the different samples of postbiotic-impregnated ODFs. The data is presented as mean ± SD. * indicates significant differences between samples ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$). The conditions are classified as Control (ODF without the impregnation of CFS postbiotics); CSF *L. plantarum* (ODF containing CSF postbiotic obtained from *L. plantarum*); CSF *L. paracasei* (ODF containing CSF postbiotic obtained from *L. paracasei*) and CSF both (ODF containing CSF postbiotic from both *L. plantarum* and *L. paracasei*).

weight and thickness after CFS postbiotics impregnation could exceed the recommended levels for optimal behavior in the oral cavity [38,45].

3.4.4 Hydration, Moisture Loss, and Solubility

Another critical factor to consider is the hydration percentage or swelling capacity. This value is directly related to the hygroscopic properties of the ODF. It is affected by the film-forming polymers and can influence the physical characteristics of the final product [37,45]. Films with high glycerol concentrations display lower mechanical stress since it increases their hygroscopic tendency [37].

The results showed a significant drop in hydration percentage when CFS postbiotics were added to the film-forming solution (Fig. 6A). The control ODF had a hydration percentage of 1710 ± 128%, highlighting its hygroscopic nature. The ODF impregnated with CFS postbiotics from both probiotics had the highest hydration percentage at 800 ± 119%, compared to 552 ± 44% and 423 ± 5% for the ODFs with CFS postbiotics from *L. plantarum* and *L. paracasei*, respectively.

Alongside hydration, moisture loss and solubility percentages were also measured. Higher solubility was found in samples with lower hydration, which is desirable for better postbiotic dissolution in the oral cavity. Increasing solubility is desired since it allows for better dissolution of the postbiotics in the oral cavity. The control ODF had a solubility of 39 ± 2%. In contrast, the ODFs with CFS postbiotics from *L. plantarum*, *L. paracasei*, and both probiotics had solubility percentages of 58 ± 3%, 57 ± 1%, and 56 ± 1%, respectively, with no statistically significant differences between them (Fig. 6B). Increased solubility is crucial for ODFs to dissolve easily without water.

Moisture loss variability between samples was less substantial but still statistically significant. The control ODF had a moisture loss percentage of 96 ± 1%. The ODFs with CFS postbiotics from *L. plantarum*, *L. paracasei*, and both probiotics had moisture loss percentages of 93 ± 1%, 92 ± 1%, and 95 ± 1%, respectively (Fig. 6C). These values indicate the stability of the ODFs over time, as they reflect the weight constancy after incubation. Despite high moisture loss values indicating some instability, a desired decrease was observed with adding CFS postbiotics. Our results are according to those found in the literature [28].

3.4.5 Contact Angle

The water contact angle, which indicates the hydrophilicity of the ODFs, was the final physical property analyzed (Fig. 7). This angle, formed between the ODF base and the tangent to the water droplet's exterior plane, can impact dissolution time and hydration percentages. Lower contact angles signify higher hydrophilicity. As anticipated, the contact angle decreased after adding CFS postbiotics, correlating with increased solubility. The control ODF, without CFS postbiotics, had a contact angle of 54.6 ± 2.2°. However, this difference was insignificant compared to the addition of CFS postbiotics obtained from *L. paracasei* (47.3 ± 5.1°). The lowest contact angle was measured for the ODF impregnated with CFS postbiotics from *L. plantarum* (39.7 ± 2.3°), followed by the ODF with CFS postbiotics obtained from both probiotics (44.7 ± 0.5°) ($p < 0.01$ and $p < 0.05$, respectively).

3.5 Cytotoxicity of the Postbiotic-Based Orodispersible Film on Human Mouth Cells

ODFs present a promising avenue for maintaining oral cavity homeostasis, with postbiotics impregnated in the

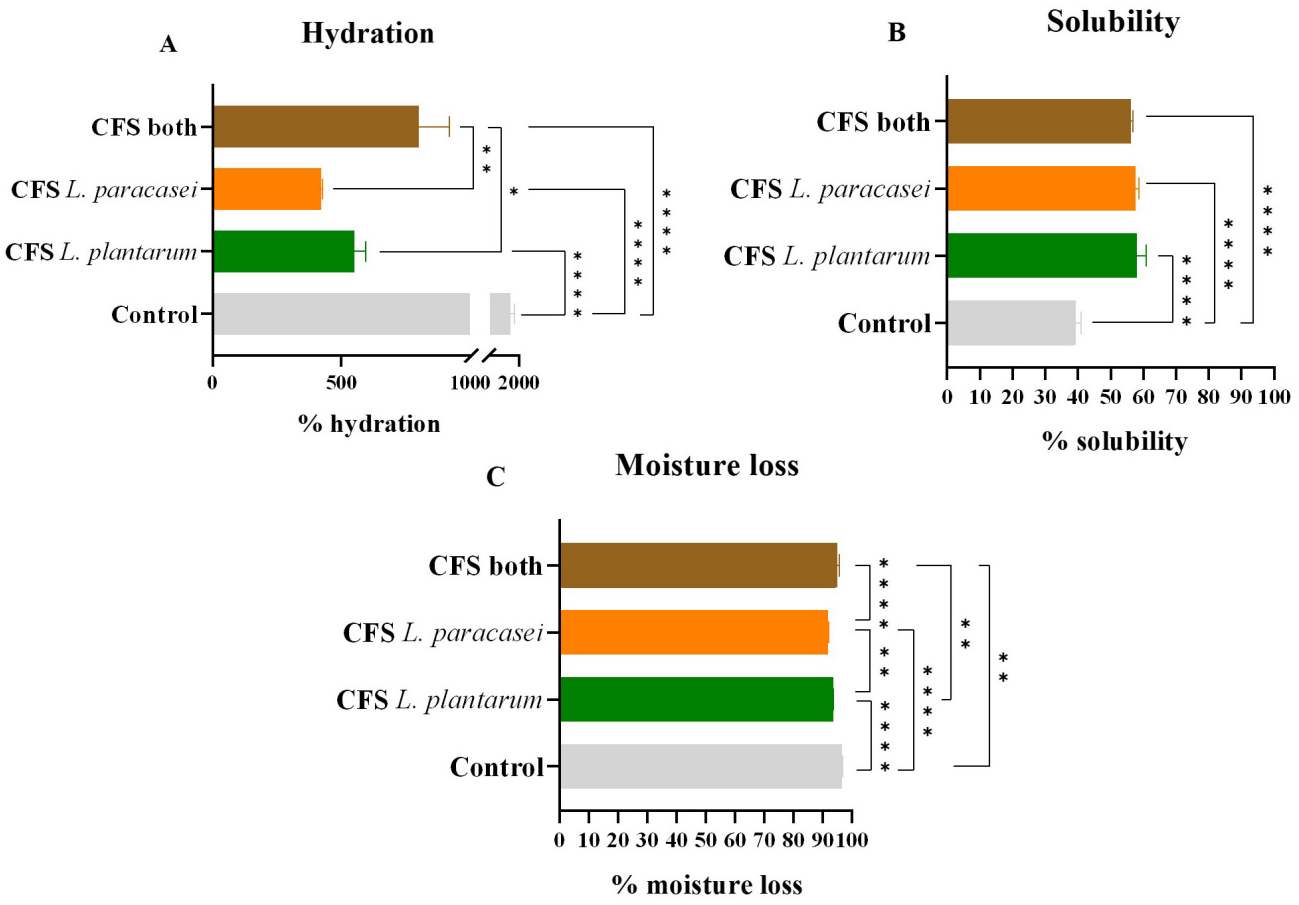

Fig. 6. Hydration, moisture loss, and solubility percentages. (A) Hydration percentage values of the different samples of postbiotic-impregnated ODFs. (B) Solubility percentage values of the different samples of postbiotic-impregnated ODFs. (C) Moisture loss percentage values of the different samples of postbiotic-impregnated ODFs. The data is presented as mean ± SD. * indicates significant differences between samples ($p < 0.05$), ** ($p < 0.01$) and **** ($p < 0.0001$). The conditions are classified as Control (ODF without the impregnation of CFS postbiotics); CSF *L. plantarum* (ODF containing CSF postbiotic obtained from *L. plantarum*); CSF *L. paracasei* (ODF containing CSF postbiotic obtained from *L. paracasei*) and CSF both (ODF containing CSF postbiotic from both *L. plantarum* and *L. paracasei*).

film-forming solution exhibiting antimicrobial and potential antibiofilm activity upon oral administration. To ensure safety (cell viability maintenance) when in contact with the different ODFs tested, a cytotoxicity assay was conducted, with two defined controls: a cell control without adding ODFs with or without postbiotics and a control of ODFs without impregnating CFS postbiotics. The loss of cell viability between controls was not statistically significant, decreasing from 100 ± 0% to 77.9 ± 13%.

Across the three postbiotics tested, cell viability decreased in all samples, with the highest loss attributed to the CFS postbiotic from *L. plantarum* (56.2 ± 11.1%), as it can be observed in Fig. 8. The CFS postbiotic from *L. paracasei* showed a cell viability percentage of 62.2 ± 6.4%. Interestingly, CFS postbiotics from both probiotics exhibited the least cytotoxicity (72.6 ± 9.8%). Notably, none of the cell viability values dropped below 50%, indicating the safety and non-toxic nature of the ODFs.

These findings underscore the potential of ODFs impregnated with postbiotics as a preventive treatment option for controlling dysbiosis in the oral cavity, mainly when *S. mutans* is the major pathogen involved.

These results are in accordance with those obtained in the literature [17,45].

4. Conclusions

Oral diseases stem from an imbalance in the oral cavity microbiome known as oral dysbiosis. To address this, preventive measures should prioritize restoring the natural balance of the microbiota rather than eliminating it.

In our study, we developed an ODF incorporating postbiotics derived from *L. paracasei* and *L. plantarum*. Evaluating the antimicrobial properties of these postbiotics revealed significant activity against *S. mutans*, varying effectiveness based on their concentration. Postbiotics from both sources demonstrated superior antimicrobial effects

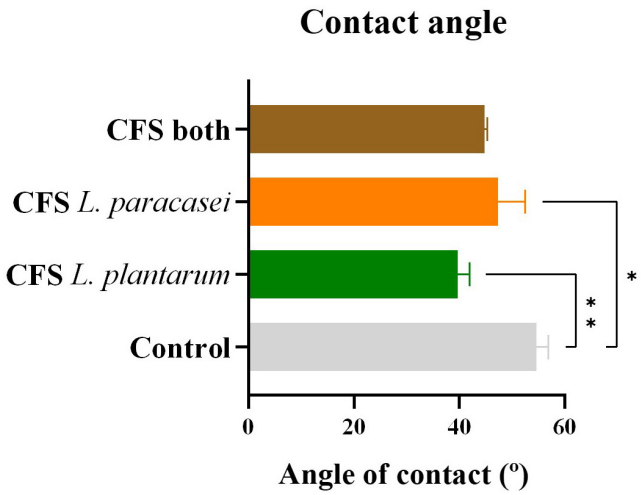

Fig. 7. Contact angle. Angle of water contact of the different samples of postbiotic-impregnated ODFs. The data is presented as mean ± SD. * indicates significant differences between samples ($p < 0.05$) and ** ($p < 0.01$). The conditions are classified as Control (ODF without the impregnation of CFS postbiotics); CSF *L. plantarum* (ODF containing CSF postbiotic obtained from *L. plantarum*); CSF *L. paracasei* (ODF containing CSF postbiotic obtained from *L. paracasei*) and CSF both (ODF containing CSF postbiotic from both *L. plantarum* and *L. paracasei*).

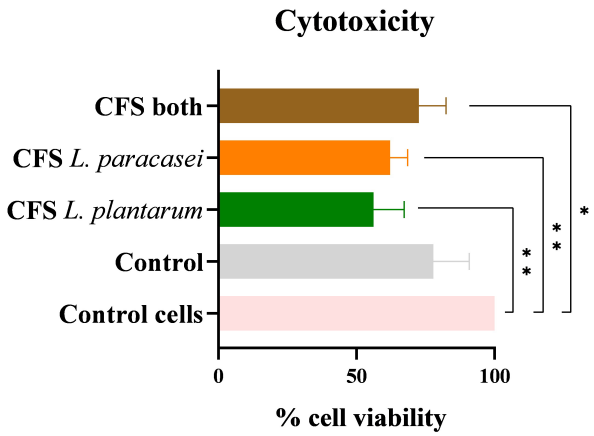

Fig. 8. Cytotoxicity. Percentage of cell viability of TR146 cell line in contact with ODFs impregnated with the different CFS postbiotics, representing cytotoxicity. The data is presented as mean ± SD. * indicates significant differences between samples ($p < 0.05$) and ** ($p < 0.01$). The conditions are classified as Control (cells cultured in the presence of orodispersible films without the impregnation of postbiotics); Control cells (TR146 cells cultured with HAMS F12 medium, without any ODF); CSF *L. plantarum* (cells cultured in the presence of ODF containing CSF postbiotic obtained from *L. plantarum*); CSF *L. paracasei* (cells cultured in the presence of ODF containing CSF postbiotic obtained from *L. paracasei*) and CSF both (cells cultured in the presence ODF containing CSF postbiotic from both *L. plantarum* and *L. paracasei*).

when compared to those from *L. paracasei* alone, suggesting potential synergies. Additionally, these postbiotics displayed antibiofilm properties, particularly when applied during biofilm formation. Furthermore, these ODFs showed no cytotoxicity to human oral cells.

However, it is essential to recognize that there are limitations that should be overcome before considering this approach. The lack of antimicrobial activity against mature biofilms is concerning since this condition is prevalent in a dysbiotic environment. Additionally, the low pH attained can be considered a limitation since it can aggravate the dysbiosis in an already unhealthy oral cavity, worsening the disease or the dysregulated condition. Lastly, more detailed studies of ODF's physical characteristics should be performed, considering that the lack of understanding of the mechanisms by which it delivers the active compounds in the oral cavity is a lenient limitation. Additionally, it should be understood how the postbiotics behave in the long term to determine their viability accurately.

All things considered, further investigations are warranted to understand their physical characteristics, storage requirements, and shelf life post-impregnation with postbiotics. Optimization of ODF formulation and delivery methods are also essential.

Notably, *in vitro* findings may not fully translate to *in vivo* scenarios due to environmental factors unique to the oral cavity, underscoring the need for clinical trials to assess ODF efficacy. For this, preclinical work should be performed with a three-dimensional model of the oral cavity that takes into account the salivary flow and the mastication effects in the ODF. It is also vital to carry out clinical tests in the future to determine the actual extent of the benefits of using postbiotic-impregnated ODFs.

Lastly, standardized guidelines for postbiotics acquisition and formulation are necessary to ensure safe and effective application in improving oral health.

Availability of Data and Materials

The data utilized and/or examined in the present study can be obtained from the corresponding author upon reasonable request.

Author Contributions

MBR and FKT designed the research study. MBR and CSO performed the cell culture assays. MBR analyzed the data and wrote the manuscript. CSO and FKT provided supervision and directions to the manuscript. CSO and FKT contributed with writing-review, editing and validation. All authors contributed to editorial changes in the manuscript and approved the final version of manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

According to the Portuguese Catholic University's Ethical Commission for Health (CES-UCP), this study does not involve human or animal subjects, therefore, ethical committee approval is not necessary.

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Conflict of Interest

The authors declare no conflict of interest.

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