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Antibacterial and cytotoxic effects of chlorhexidine combined with sodium DNA on oral microorganisms: an *in vitro* study using *Dictyostelium discoideum*

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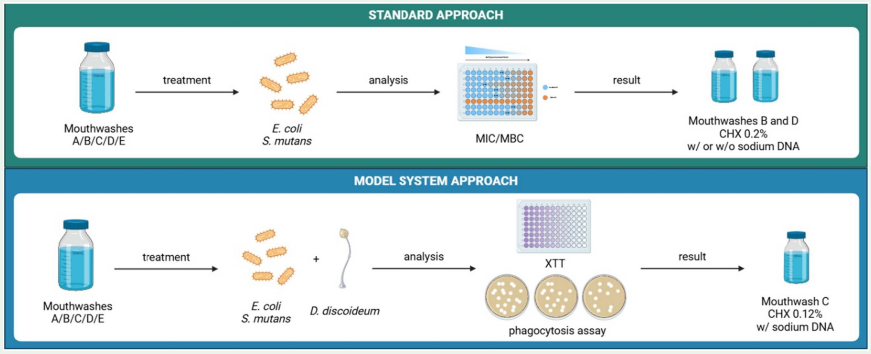
ABSTRACT

Background: Chlorhexidine (CHX) is widely used in oral care for its broad-spectrum antimicrobial activity but can cause significant side effects. Sodium DNA has emerged as a potential adjunct capable of modulating cellular responses.
Aim: This study assessed whether sodium DNA enhances the antibacterial and anti-biofilm activity of 0.20% and 0.12% CHX mouthwashes against *Streptococcus mutans* and *Escherichia coli*, and evaluated their effects on the viability and phagocytic activity of *Dictyostelium discoideum*, a model for mammalian phagocytes.
Results: All CHX-containing mouthwashes were bactericidal against *S.mutans*, regardless of sodium DNA, whereas CHX-only formulations were more effective against *E.coli* in time–kill assays. All formulations inhibited biofilm formation at 50–0.01%. In *S. mutans*, early biofilms were strongly inhibited (50–0.39%), whereas mature biofilms were less affected. In *E. coli*, sodium DNA enhanced inhibition of both biofilm formation (50–1.56%) and mature biofilms (50–3.12%). The 0.12% CHX–sodium DNA formulation most effectively modulated *D.discoideum* viability and phagocytic activity, and metabolomics showed that sodium DNA reduced CHX-induced metabolic stress.
Conclusions: This study integrates antimicrobial, antibiofilm, cellular, and metabolomic analyses to assess CHX with sodium DNA. Sodium DNA reduces CHX-induced cytotoxicity and metabolic stress while maintaining antimicrobial activity, offering insights for optimizing oral hygiene formulations through combined microbial and host-cell evaluation.



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Background

Periodontitis is a chronic multifactorial disease of the periodontal apparatus triggered by a dysbiotic microbiota followed by a subsequent host inflammatory response, leading to periodontal tissue destruction and tooth loss if not properly and preventively treated [1,2]. The presence of periodontal pathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actynomycetemcomitans* and related bacteria including *Streptococcus mutans* and *Klebsiella pneumoniae* have been shown to determine, together with several periodontal bacteria, a concomitant low-grade negative stimulus that can spread out periodontal infections to the bloodstream. This event could negatively affect both oral and systemic health in certain predisposed individuals [3–5]. During the last few decades, periodontitis has been correlated, through a bilateral relationship, with a plethora of systemic diseases, including cardiovascular diseases, diabetes, preterm birth, haematological and bone marrow diseases [6–8]. Salivary samples from patients with periodontal disease revealed facultative anaerobic Gram-positive strains (*Streptococcus spp.*, *Staphylococcus aureus*, *Staphylococcus epidermidis*) and Gram-negative species (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*). Their presence, facilitated by gingival mucosal damage, is linked to reduced host inflammation and rapid disease progression [9].

Likewise, in recent years it has been clarified that the early treatment of periodontal disease primarily relies on the effective removal of supra- and subgingival biofilms. In this regard, the recently published guidelines of the European Federation of Periodontology (EFP) clearly suggest that the first step of periodontal treatment, which is traditionally performed through reinforced oral hygiene practices aimed at biofilm reduction, remains crucial throughout all steps of treatment for the management of stage I–III periodontitis patients [10]. Several new and potentially efficacious adjunctive treatments have been investigated for periodontal treatment, such as probiotics, anti-inflammatory agents, and local antiseptics, with uncertain results [8–10].

More specifically the efficacy of CHX against oral bacteria, mycetes and viruses is widely known, as well as its ability to adsorb to tooth structures and oral epithelium firmly. In this regard, the S3 EFP guidelines, which focused on primary measures of periodontal treatment, concluded that the adjunctive use of chemical antiplaque agents such as chlorhexidine (CHX), in addition to traditional oral hygiene practices, provides clear and immediate benefits in reducing gingival indices in periodontitis patients [10]. These guidelines suggest that adjunctive antiseptics may be considered, specifically through mouth rinses formulation for a limited period of time in periodontitis therapy, as adjuncts to mechanical debridement in specific cases [10].

However, CHX use must be weighed alongside its less desirable effects, including extrinsic staining of teeth, antimicrobial resistance to antiseptic agents and the rare, but fatal, allergic reactions to CHX. CHX presents relevant drawbacks, including the potential to disrupt the commensal microbiota, induce cytotoxicity on oral fibroblasts and keratinocytes, and cause genotoxic effects, including primary DNA damage in leucocytes and oral mucosal cells [11–14]. The reported evidence of CHX-related cytotoxic impact on mammalian cells, mainly due to its detrimental effects on microbial ecology leading to overgrowth of *Firmicutes* and a decrease of nitrate-reducing bacteria [15], and associated resistance genes that could potentially lead to cross-resistances to antibiotics [14]. These limitations have prompted interest in identifying adjuvants that can preserve or enhance the antimicrobial efficacy of CHX while mitigating its cytotoxicity.

In this regard, sodium DNA, a molecule composed of native and purified gonadic deoxyribonucleic acid, has emerged as a promising adjuvant due to its dual role: as a structural precursor for nucleic acid biosynthesis and as a modulator of cell proliferation, growth factor release, and immune response [16]. In vitro and clinical studies suggest that sodium DNA can protect oral epithelial cells against oxidative and genotoxic stress, enhancing the effects of CHX by stimulating the production of cytokines and growth factors, which modulate nucleotide salvage A2, purinergic receptor, finally promoting a better reduction of gingival inflammation in patients with periodontitis [17,18]. These properties make sodium DNA an appealing adjuvant in oral antiseptics, potentially shifting the balance from cytotoxicity toward tissue protection.

Beyond these clinical effects, it is important to better understand the underlying biological mechanisms. CHX, while effective as a broad-spectrum antiseptic, interacts not only with bacterial membranes but also with eukaryotic cells, promoting reactive oxygen species (ROS) formation, disrupting phospholipid bilayers, and in some cases altering DNA integrity [12]. Sodium DNA has been suggested to act as a regenerative substrate and may influence cellular processes, although specific mechanisms and effects

require further investigation [19]. This duality highlights the rationale for studying CHX and sodium DNA in combination, balancing antimicrobial potency with host cell protection.

In addition to microbiological assays, innovative eukaryotic models are valuable for assessing the cytotoxic and functional effects of oral antiseptics. In this regard, *Dictyostelium discoideum*, a soil-dwelling social amoeba, represents an ideal model. *D. discoideum* life cycle comprises a unicellular stage, during which amoebae feed on bacteria or axenic media and a starvation-induced multicellular phase that culminates in the formation of a fruiting body with a stalk and a spore-filled sorus. This unique alternation between growth and development phases allows researchers to independently study both processes within the same organism Moreover, *D. discoideum* combines rapid growth, ease of culture, versatile genetic manipulation with live-cell imaging, and modern 'omics' approaches, enabling detailed analysis of cellular mechanisms. These features make it particularly suitable to dissect conserved, cell-autonomous processes such as chemotaxis, phagocytosis, macropinocytosis and phago-lysosomal trafficking [20]. Interestingly, it can phagocytose bacteria similarly to mammalian macrophages, and conserves key molecular pathways involved in vesicle trafficking and innate immunity [21,22]. Unlike isolated cell models, *D. discoideum* enables simultaneous evaluation of both cell viability and phagocytic function after exposure to bioactive agents, making it particularly suitable for screening the effects of CHX-based formulations [23]. Thanks to its genetic tractability and physiological similarity to professional phagocytes, *D. discoideum* has been widely adopted for drug screening and the study of host–pathogen interactions [24,25]. Based on these premises, the present study aimed to evaluate the antimicrobial effects of CHX formulations (0.12 and 0.20%), with or without sodium DNA against *S. mutans* DSM 20523 and *E. coli* ATCC 25922 as well as their effects on the viability and phagocytic function of *D. discoideum* Although *E. coli* is not primarily an oral or periodontal bacterium; it is efficiently ingested by *D. discoideum* and is therefore commonly employed to study bacterial phagocytosis and gene expression in this model [23]. Recent work demonstrated that *D. discoideum* recognises and kills *E. coli* through specific phagocytic and bacetriocidal pathways involving conserved effectors such as NADPH oxidase and lysozymes, highlighting its relevance as a standardised and genetically tractable bacterial model [26]. Moreover, these strains were selected as standardised and representative models for Gram-positive and Gram-negative bacteria, respectively. *S. mutans* DSM 20523 is a well-established oral biofilm former and contributes to dysbiosis in the oral cavity, while *E. coli* ATCC 25922, a reference strain for antimicrobial susceptibility testing, provides a reproducible model to assess antiseptic efficacy against Gram-negative bacteria that may transiently colonise damaged gingival tissues [27,28]. This combination allowed the evaluation of both the antimicrobial spectrum and potential differential effects related to differences in bacterial envelope architecture. Based on this evidence, the null hypothesis tested in this study was that CHX formulations, with or without sodium DNA, do not induce any significant changes in *D. discoideum* viability, phagocytic activity, or metabolic profile. The use of *D. discoideum* allowed the investigation of the broader biological impacts of these formulations beyond bacterial killing, including their influence on eukaryotic phagocyte-like cells.

Materials and methods

Bacterial strains and growth conditions

Streptococcus mutans DSM 20523 and *E. coli* ATCC 25922 strains were used to investigate the bactericidal activity of different mouthwashes. *S. mutans* DSM 20523 was cultivated on Mueller–Hinton (MH) (Oxoid, Basingstoke, UK) with 5% horse blood (Thermo Scientific, Basingstoke, UK) agar plates at 35 °C with an atmosphere containing 5% CO_2 for 24 h. *E. coli* ATCC 25922 was incubated overnight at 35 °C on Mac Conkey (Oxoid, Basingstoke, UK) agar plate under aerobic conditions.

D. discoideum cell culture

D. discoideum wild-type cells of Ax4 strain were cultured in suspension in axenic medium (Fey et al., 2007) at 23 °C under shaking at 150 rpm in a climatic cabinet equipped with gyratory shakers (Kühner,

Bielefeld, Switzerland). To perform *D. discoideum* assays, *E. coli* ATCC 25922, *S. mutans* DSM 20523 and *K. pneumoniae* ATCC 2008133 bacteria were grown on Nutrient agar (*N* agar) or SM agar plates (recipe available at dictybase.org) at 37 °C for 20 h.

Mouthwash formulations

In this study, five mouthwashes were tested, named A (placebo, similar in colour and structure to other formulations, Betafarma, Saronno, Italy), B and C, containing as active agents various percentages of CHX plus sodium DNA (0.2 and 0.12% respectively, BetafarmaCurasept SPA, Saronno, Italy) and two formulations containing only CHX as active agent, as D (0.20% CHX, Dentosan, Recordati, Milan, Italy) and E (0.12% CHX, Dentosan, Recordati, Milan, Italy).

In vitro susceptibility testing for S. mutans and E. coli

Minimum Inhibitory Concentration (MIC) values of all mouthwashes were determined by the standard broth microdilution method using an *inoculum* of about 5×10^5 CFU/mL, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [29]. Brain Heart Infusion Broth (BHI, Oxoid, Basingstoke, UK) and cation-adjusted Mueller–Hinton broth (CAMHB) (BD Difco, UK) were used as a medium for MICs determination of *S. mutans* and *E. coli*, respectively. The tested samples were used at serial concentrations ranging from 50 (v/v%) to 0.0124% of each mouthwash. The microplates were incubated at 35 °C for 24 h in 5% CO_2 and aerobic conditions for *S. mutans* and *E. coli*, respectively. Minimum Bactericidal Concentration (MBC) values of all mouthwashes were determined by broth microdilution methodology. Ten microliters of broth from each well with no visible growth after 24 h were plated onto BHI agar to determine the 99.9% killing endpoint. The plate counts of *S. mutans* and *E. coli* were incubated at 35 °C with 5% CO_2 and in aerobic conditions for 24 h, respectively. All experiments were performed in triplicate. The MBC was defined as the lowest concentration of mouthwash, yielding colony count <0.1% of the initial inoculum. Time–kill studies of each mouthwash were performed using 10 mL tube containing BHI broth and CAMHB for *S. mutans* and *E. coli*, respectively, either with each mouthwash at MBC concentration [30]. They were performed in triplicate using a starting *inoculum* of $1–5 \times 10^6$ CFU/mL. The kinetic growth of strains was tested at different time intervals: 0, 4, 6, 8 and 24 h and viable microbial cell counts (CFU/mL) on MH agar (Oxoid, Basingstoke, UK) plates were determined. Bactericidal activity was defined as a ≥3 decrease in CFU from the initial size of the inoculum by 24 h. Results were expressed as the mean value of the $\log_{10}$ CFU/mL ± standard deviation.

Assessment of the antibiofilm efficacy of mouthwashes against reference bacterial strains

Biofilm of *S. mutans* and *E. coli* were obtained as previously described with some modifications [31,32]. The precultures of *S. mutans* and *E. coli* were diluted 1:100 to obtain an inoculum of 107 CFU/mL in BHI + 2% of sucrose (BHIS) for *S. mutans* and 108 CFU/mL in Luria Bertani (LB) broth (BD Difco, UK) for *E. coli*. Antibiofilm activity was tested in three different conditions: (i) inhibition of biofilm formation, (ii) inhibition of biofilm formation after 4 h of incubation, and (iii) inhibition of mature biofilm. Test (ii) was performed only for *S. mutans* as previously reported with some modifications, because E. coli is a weak biofilm producer [27].

In all assays the antibiofilm activity of mouthwashes B, C, D and E was tested at scalar dilutions of the antimicrobial agents starting from a 1:1 ratio in a 96 flat bottom microtiter plate (Nunc, Thermo fisher), with a final volume of 100 µL per well as previously described [33]. For test (i) 100 µL of *S. mutans* in BHIS or *E. coli* in LB were added. For growth control, 200 µL of each strain were dispensed. For negative control, non-inoculated broths were allotted. After incubation at 37 °C for 24 h, the medium was carefully removed, and the plate was washed three times with sterile saline and air-dried. Two hundred microliters of 0.1% crystal violet were added to each well and incubated at room temperature for 15 min. After three washes with distilled water, the plate was air-dried and 200 µL of glacial acetic acid 33% were added to each

well. The plate was read with a BioTek Synergy H1 plate reader (Agilent Technologies, USA) at 500 nm for *S. mutans*, 600 nm for E. coli. The resulting OD values were analysed as previously reported, comparing the OD values of control wells (not inoculated medium) with the OD values of inoculated wells [32]. All conditions were performed in triplicate.

Similarly, for test (ii) 200 µL of *S. mutans* in BHIS were dispensed, incubated at 37 °C for 4 h to allow a partial biofilm formation. Then, the medium and planktonic cells were carefully removed and 100 µL of scalar dilutions of mouthwashes B, C, D and E were added. After incubation for 24 h, the plate was washed, stained and read at 500 nm as aforementioned. All conditions were performed in triplicate. For test (iii) 200 µL of *S. mutans* in BHIS or E. coli in LB were dispensed, incubated at 37 °C for 24 h to obtain mature biofilm. Then, the medium and planktonic cells were carefully removed and 100 µL of scalar dilutions of mouthwashes B, C, D and E were added. After an ulterior incubation of 24 h, the plate was washed, stained and read at 500 nm or 600 nm as aforementioned. All conditions were performed in triplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA) to compare the effects of different mouthwash concentrations on biofilm inhibition. Results were considered statistically significant at $p < 0.05$. Data are presented as mean ± standard deviation (SD) of three independent experiments. The results were graphed using GraphPad Prism version 9.0.2 (GraphPad Software, LLC, USA).

D. discoideum in co-culture with bacteria and treatments with mouthwashes

Phagocytosis was tested by mixing 100 *D. discoideum* cells and 300 µL (approximately 10^8 CFU/mL) of *E. coli*, *S. mutans* and *K. pneumoniae,* which serves as a negative control due to its reduced susceptibility to Dictyostelium phagocytosis. The mixture was placed on agar *N* plates with different mouthwashes, used at a final concentration of 2% for *E. coli* and *K. pneumoniae*, 1% for *S. mutans*. The count of *D. discoideum* colonies was performed after 3 days of incubation at 23 °C. A 3-day incubation time was chosen since is the standard time point at which *D. discoideum* colonies are visible. Three replicates were performed for each bacterial strain, and standard deviations were calculated based on the means of each sample. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis.

D. discoideum phagocytosis assay after treatment with mouthwashes

D. discoideum represents a primitive macrophage capable of ingesting, killing, and digesting at least one bacterium per minute. To test whether the use of *D. discoideum* as a professional phagocyte could increase the sensitivity of screening the bactericidal activity of mouthwashes against oral microbiota bacteria such as *S. mutans*, a total of 6×10^6*D. discoideum*. The cells in the axenic medium were treated with the different mouthwashes at a concentration of 2% for 1 min. After that, small drops of 20 µL of cells were deposited on agar *N* plates, where a monolayer of *E. coli* or *S. mutans* was previously grown. Once the drops were completely dry, plates were incubated at 23 °C for a maximum of 3 days. Subsequently, the radius of zones eaten by the amoeba was measured and their areas (cm^2) were calculated. Pictures were taken using a digital microscope imager (Celestron). Three replicates were performed for each bacterial strain in total. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis.

D. discoideum vitality assay after treatment with mouthwashes

D. discoideum cells were treated with previously described mouthwashes at a concentration of 2% in axenic medium for 1 min. Then, a total of 3×10^5 cells (in 100 µL of medium) were seeded in each well of a 96-well plate. Cell vitality was tested using the cell proliferation Kit XTT (Roche), according to manufacturer's protocol, by measuring the absorbance at 450 nm in a microplate reader. Tested conditions were seeded in triplicate and One-way ANOVA with Tukey's post-hoc test was used for statistical analysis.

Samples preparation for HPLC–HRMS analysis

Five million cells were pelleted and washed twice with PBS. The resulting dry pellet was resuspended in 250 µL of Milli-Q water and lysed by alternating immersion in liquid nitrogen and heating at 45 °C. An equal volume (250 µL) of acetonitrile containing 1% formic acid was then added, and the sample was vortexed for 2 min. The mixture was stored at −20 °C for 30 min to promote protein precipitation, followed by centrifugation at 13,000 rpm for 10 min. Finally, 200 µL of the clear supernatant was collected and transferred into a vial for HPLC-MS analysis.

HPLC–HRMS metabolomic analysis

The molecular composition of the samples was analysed by HPLC-HRMS using an HPLC-timsTOF Pro 2 system (Bruker Daltonics, Bremen, Germany) equipped with a Luna Polar C18 column (2.1 × 150 mm, 3 µm; Phenomenex, Bologna, Italy) and operated in binary gradient mode. Solvent A consisted of water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The gradient programme was as follows: flow rate 0.3 mL/min; 0 min, 5% B; 15 min, 100% B; 16 min, 100% B; followed by 4 min re-equilibration at 5% B. The HRMS was coupled to the HPLC via a VIP-HESI source, operating in both positive and negative ion modes, with the following parameters: end plate offset 500 V, capillary voltage 4.5 kV, nebuliser gas 2 bar, dry gas flow 8 L/min, dry temperature 230 °C, and sheath gas temperature 400 °C [34]. Data were acquired in full-scan mode (20–1,300 m/z) at a resolution of 30,000 (FTMS). Data-dependent acquisition (DDA) MS/MS experiments were performed in the same m/z range for both polarities, using Auto MS/MS with a dynamic spectra rate (16–20 Hz). The collision energy was set to 20 eV for ions between 50–1,000 m/z and 30 eV for those at 1,300 m/z. All the spectra were recorded in profile mode. Data processing and feature extraction were carried out with MetaboScape 2023b (Bruker Daltonics, Bremen, Germany).

Data pre-processing and analysis

The raw HPLC–MS data were processed using MetaboScape 2023b (Bruker Daltonics) to generate a feature table. Each feature is a combination of chromatographic peak characterised by its molecular ion mass, associated fragment ions, ion mobility value, and, when available, molecular annotation derived from these parameters. The table also includes the chromatographic peak areas for each sample. For downstream statistical analysis, only features associated with a molecular annotation were considered and imported into the online MetaboAnalyst 6.0 platform. Each experimental group consisted of three biological replicates. Data were normalised in MetaboAnalyst using $\log_{10}$ transformation and autoscaling prior to multivariate and univariate analyses. Principal component analysis (PCA) was performed to evaluate the distribution and clustering of the experimental groups in the principal component space. The statistical significance of individual metabolites was assessed by ANOVA (Tukey's post-hoc test, with a FDR = 0.05), and the results were visualised as a heatmap. Finally, pathway enrichment analysis was conducted to identify metabolic pathways significantly affected by the experimental conditions.

Results

Antimicrobial activity of mouthwashes

In this study, five mouthwashes were tested: formulation A (placebo), formulations B and C containing chlorhexidine (0.20 and 0.12%, respectively) in combination with sodium DNA, and two commercial formulations, D (0.20% CHX) and E (0.12% CHX), both without sodium DNA. In the first part of the study, we investigated the effects of the tested compounds using a classical microbiological approach. The *in vitro* activity of the mouthwashes against *Streptococcus mutans* DSM 20523 and *E. coli* ATCC 25922 is reported in Tables 1 and 2, respectively. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined and expressed as % v/v.

Table 1. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of mouthwashes against *S. mutans* DSM20523. A (placebo); B (0.2% chlorhexidine with sodium DNA); C (0.12% chlorhexidine with sodium DNA) D (0.20% chlorhexidine) and E (0.12% chlorhexidine); N.D., not determined.

Mouthwash	MIC (mg/l) (%v/v)	MBC (mg/l) (%v/v)
A	>50%	N.D.
B	0.049%	3.13%
C	0.049%	3.13%
D	0.024%	1.56%
E	0.049%	0.78%

Legend: A (placebo); B (0.2% chlorhexidine with sodium DNA); C (0.12% chlorhexidine with sodium DNA) D (0.20% chlorhexidine) and E (0.12% chlorhexidine); N.D., not determined.

Table 2. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of mouthwashes against *E. coli* ATCC 25922. A (placebo); B (0.2% chlorhexidine with sodium DNA); C (0.12% chlorhexidine with sodium DNA) D (0.20% chlorhexidine) and E (0.12% chlorhexidine); N.D., not determined.

Mouthwash	MIC (mg/l) (%v/v)	MBC (mg/l) (%v/v)
A	>50%	N.D.
B	0.024%	0.78%
C	0.10%	1.56%
D	0.05%	1.56%
E	0.10%	1.56%

Legend: A (placebo); B (0.2% chlorhexidine with sodium DNA); C (0.12% chlorhexidine with sodium DNA) D (0.20% chlorhexidine) and E (0.12% chlorhexidine); N.D., not determined.

All mouthwashes tested showed antibacterial activity against *S. mutans* and *E. coli,* with MIC values ranging from 0.024 to 0.049% and 0.05 to 0.10%, respectively. In particular, *S. mutans* was inhibited by 0.049% of mouthwashes B, C and E, and 0.024% of mouthwash D. Differently, *E. coli* was inhibited by 0.024% of mouthwash B, 0.05% of D and 0.10% of C and E. Mouthwash A showed no activity for both bacterial strains with MIC >50%, confirming that it is a placebo reagent. The MBC values against *S. mutans* were higher than MIC values for all mouthwashes, with MBC of 3.13% for mouthwash B and C, and 1.56 and 0.78% for D and E mouthwashes, respectively. Similarly, 0.78% of B and 1.56% of C, D and E were required for *E. coli* killing. No MBC determination was performed for A mouthwash.

Time–kill curves

After determining the MIC and MBC values of the tested mouthwashes, we further explored their antimicrobial activity through time–kill kinetics assays. Our results on the activity of the different formulations against *S. mutans* and *E. coli* are shown in Figure 1A and B, respectively. Bactericidal activity was defined as a decrease of the initial inoculum by at least 3 $\log_{10}$ CFU/mL and the MBC values of B, C, D and E mouthwashes were evaluated.

The bactericidal activity of mouthwashes B and C against *S. mutans* was observed at T6 (6 h), and at T8 (8 h) for both D and E (Figure 1A). At T24, the killing kinetics of mouthwashes B, C and D showed a strongly bactericidal activity, with 99.9% reduction in CFU/mL of initial inoculum. The mouthwash B tested against *E. coli* showed bactericidal activity with a 3 $\log_{10}$ decrease in CFU/mL at T24 (Figure 1B), while the mouthwash C showed

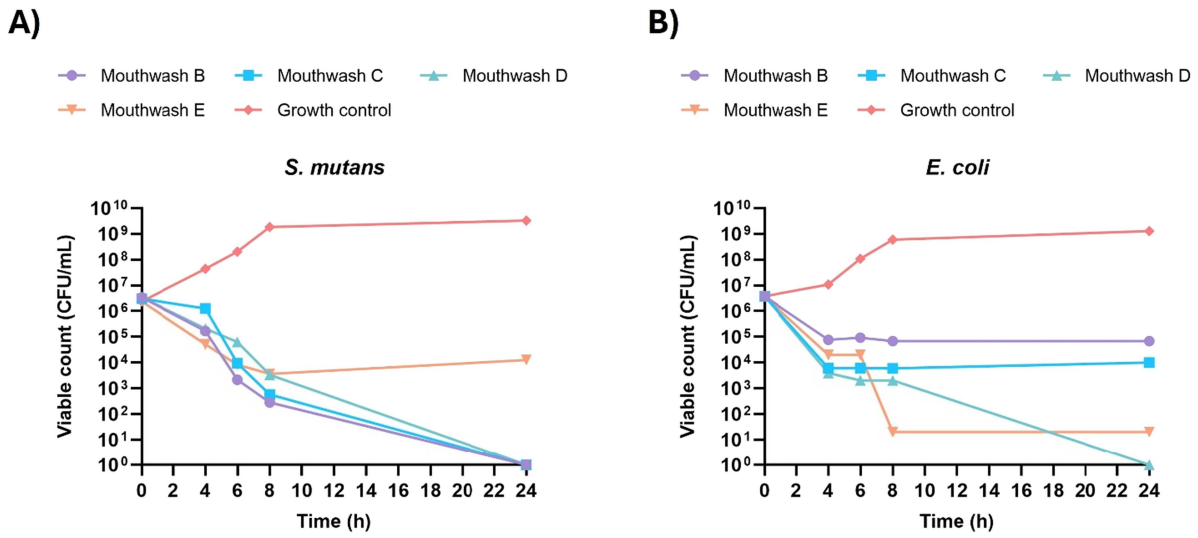

Figure 1. Time–kill curves of mouthwashes B, C, D, E and growth control against *S. mutans* DSM 20523 (A) and *E. coli* ATCC25922 (B). Time–kill assays were performed in triplicate using an initial inoculum of 1–5 × 10^6 CFU/mL in Brain Heart Infusion broth (*S. mutans*) or cation-adjusted Mueller–Hinton broth (*E. coli*). Each mouthwash was tested at its respective MBC concentration. Viable cell counts (CFU/mL) were determined at 0, 4, 6, 8, and 24 h by plating on Mueller–Hinton agar. Bactericidal activity was defined as a ≥3 log10 reduction in CFU/mL compared with the initial inoculum. Results are expressed as mean log10 CFU/mL ± SD.

a bacteriostatic activity with a reduction of 2 $\log_{10}$ of initial inoculum at T24. Viable counts were decreased by 3 $\log_{10}$ with mouthwash D after 4 h of contact, reaching maximum activity after 24 h, when no growth was detected. Remarkable bactericidal activity (5 $\log_{10}$ killing) occurred after 24 h with the mouthwash E tested.

Taken together, these results indicate that while all formulations were effective against *S. mutans*, CHX-only mouthwashes D and E showed rapid and pronounced bactericidal activity against *E. coli*.

Antibiofilm activity

The quantitative measurement of *S. mutans* and *E. coli* biofilm formation was assessed using crystal violet staining. The inhibition of biofilm of *S. mutans* was tested by three assays: (i) inhibition of biofilm formation, (ii) inhibition of biofilm formation after 4 h of incubation, and (iii) inhibition of mature biofilm. The results of test (i) are shown in Figure 2. A total inhibition of biofilm formation was detected for all mouthwashes tested across concentrations from 50 to 0.01% ($p < 0.0001$), confirming a strong broad-spectrum antibiofilm potential.

Figure 3 shows the evaluation of *S. mutans* biofilm inhibition after 4 h (ii). In particular, for mouthwash B (Figure 3A) and mouthwash D (Figure 3C), both containing the higher CHX concentration (0.20%), the most pronounced inhibitory activity was observed from 50 to 0.39%, with partial inhibition still evident at 0.19 and 0.05%. For mouthwash C (Figure 3B) and mouthwash E (Figure 3D), the strongest inhibitory activity was exerted from concentration 50 to 1.56%, whereas from 0.78 to 0.05% had lower inhibition.

Lastly, inhibition of mature biofilm of *S. mutans* is shown in Figure 4. A low inhibition was reported only for mouthwash D (Figure 4C) and E (Figure 4D) at 50 and 25%.

For *E. coli*, OD values of biofilm suggested that this strain is a weak biofilm producer, thus we performed only test (i) and test (iii) to assess the inhibitory activity of all mouthwashes. The former test resulted in the highest inhibition between 50 and 1.56% for mouthwash B, C and D (Figure 5A, B, and C), where biofilm formation was almost completely suppressed. From 6.25% onward OD values gradually increased, suggesting a partial loss of inhibitory effect at lower dilutions. Differently, for mouthwash E (Figure 5D) the strongest inhibition was between 50 and 25%, and moderate activity was detected from 12.5 to 1.56%. In general at lower concentrations, the inhibitory effect progressively

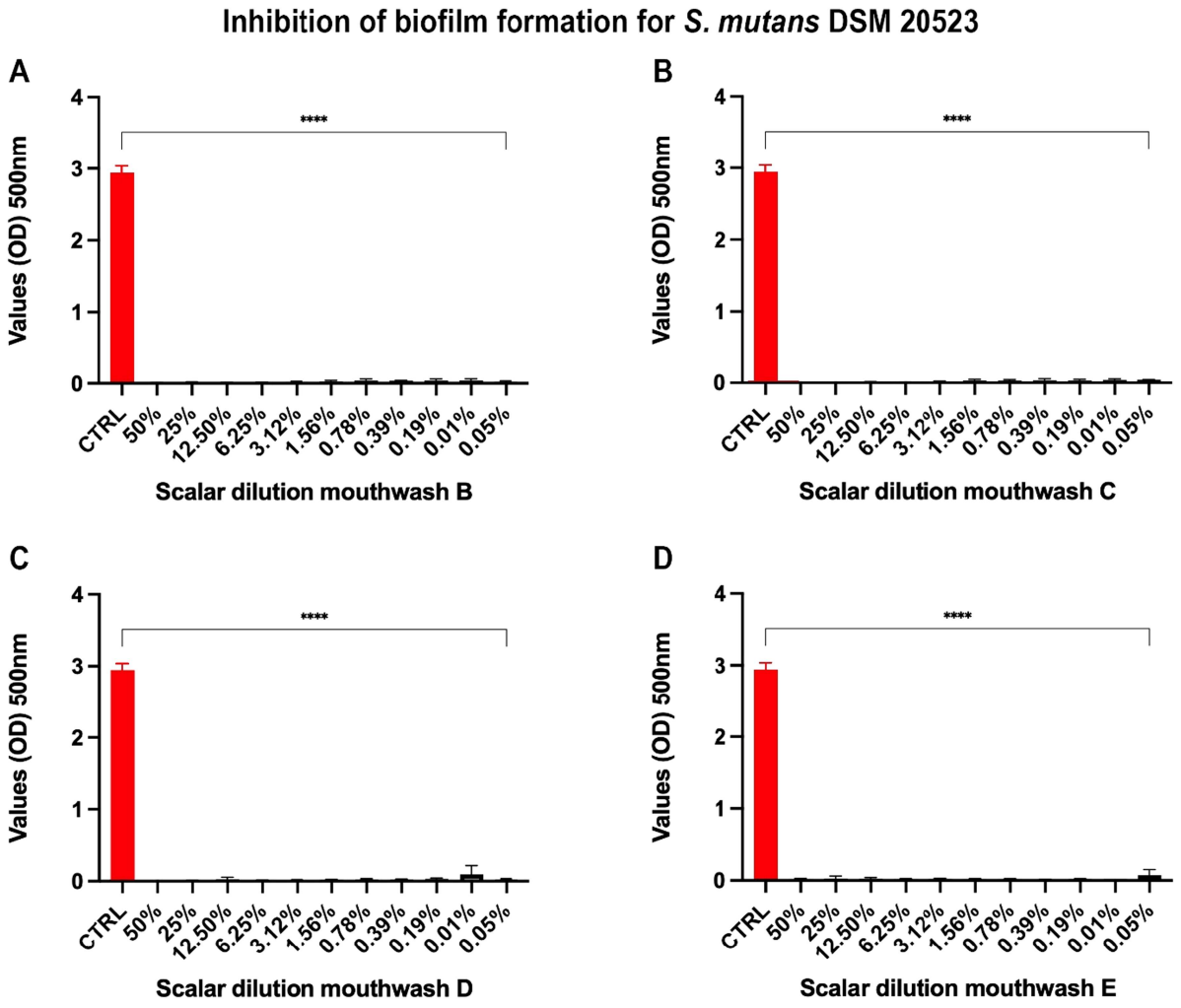

Figure 2. Inhibition of biofilm formation for *S. mutans* DSM 20523. OD values of *S. mutans* biofilm treated with mouthwash B, C, D and E. The mean optical density (OD500) of the control group was 2.94 ± 0.10, whereas treated samples exhibited OD values ranging from: A) 0.0038 ± 0.006 (50%) to 0.0408 ± 0.025 (0.01%) for mouthwash B; B) from −0.002 ± 0.002 (50%) to 0.042 ± 0.002 (0.05%) for mouthwash C; C) from 0.011 ± 0.001 (50%) to 0.095 ± 0.122 (0.01%) for mouthwash D; D) from 0.019 ± 0.008 (50%) to 0.071 ± 0.077 (0.05%) for mouthwash E. Statistical significance was analysed by ANOVA one-way and reported as p-value < 0.05 (*), p-value < 0.01 (**), p-value < 0.001 (***), p-value < 0.0001 (****), non-significative p-value > 0.05 (ns).

declined, especially in formulations lacking DNA, suggesting that sodium DNA might contribute to maintaining CHX bioactivity under more diluted conditions.

Inhibition of mature biofilm of *E. coli* assay showed that only CHX + sodium DNA formulations (B and C) (Figure 6A and B) exert strong antibiofilm activity between 50 and 3.12%, while a weak inhibition was detected by mouthwash D from 50 to 6.25% (Figure 6C). Finally, a moderate biofilm inhibition ranging from 50 to 0.39% was exhibited by mouthwash E (Figure 6D).

Bacterial activity testing by D. discoideum model

The antibacterial activity of mouthwashes was subsequently tested by a co-culture of *D. discoideum* cells and bacteria (*E. coli*, *S. mutans* and *K. pneumoniae)* on agar plates in the presence of different types of mouthwashes. *K. pneumoniae* was used as a negative control because of its ability to escape from *D. discoideum* phagocytosis while *E. coli* was included in the study since it is efficiently ingested by *D. discoideum* and is therefore commonly employed as positive control. After 3 days of incubation, we

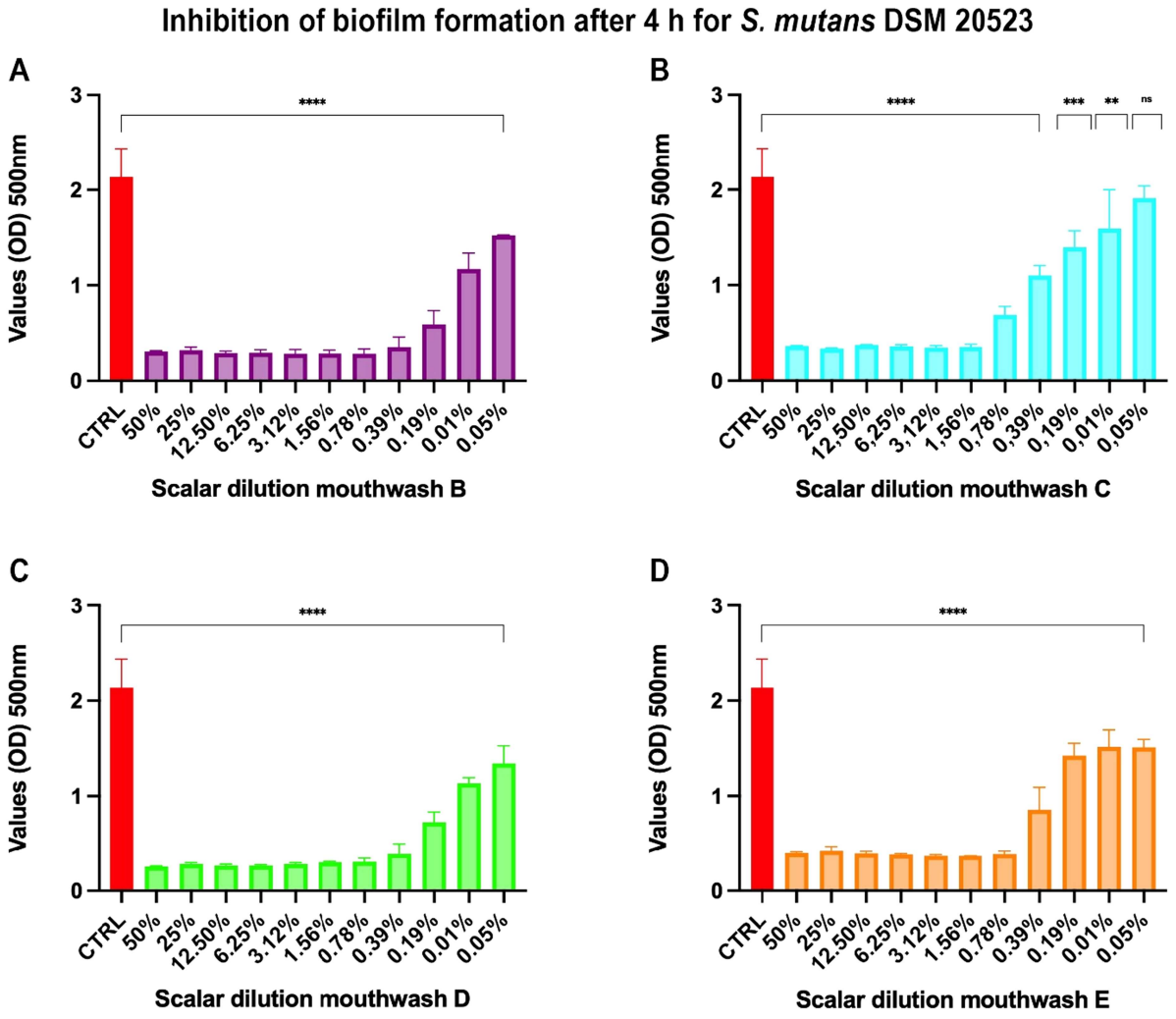

Figure 3. Inhibition of biofilm formation after 4 h for *S. mutans*DSM 20523. OD values of *S. mutans* biofilm, which was treated with mouthwash B, C, D and E after 4 h of biofilm formation. The mean (OD500) value for the control was 2.14 ± 0.30, while treated samples showed markedly reduced absorbance values ranging from: A) 0.31 ± 0.01 at 50% to 0.29 ± 0.02 at 1.56% for mouthwash B; B) 0.36 ± 0.01 (50%) to 1.91 ± 0.13 (0.05%) for mouthwash C; C) 0.26 ± 0.01 at 50% to 1.34 ± 0.18 at 0.05% for mouthwash D; D) 0.40 ± 0.01 at 50% to 1.51 ± 0.08 at 0.05% for mouthwash E. Statistical significance was analysed by ANOVA one-way and reported as *p*-value < 0.05 (*), *p*-value < 0.01 (**), *p*-value < 0.001 (***), *p*-value < 0.0001 (****), non-significative *p*-value > 0.05 (ns).

evaluated the presence or absence of typical *D. discoideum* colonies over the bacterial monolayer and the index of occurring phagocytosis. Figure 7 shows a similar number of colonies observed in the plates that were not treated and in those treated with placebo mouthwash A. Surprisingly, the number of colonies significantly decreases after treatment with mouthwashes B and C, with a maximum inhibitory effect in the presence of mouthwashes D and E, which do not contain sodium DNA. Fewer colonies were observed after treatment with mouthwash B (average = 5 in *E. coli*, average = 0 in *S. mutans*, respectively), compared to mouthwash C (average = 13 in *E. coli*, average = 16 in *S. mutans*). These differences were statistically significant ($p < 0.001$), indicating a clear dose-dependent effect related to the CHX concentration (0.2% in B vs. 0.12% in C). Moreover, comparing mouthwashes with identical CHX content (B vs. D at 0.2%, and C vs. E at 0.12%), those lacking sodium DNA (D and E) demonstrated significantly greater antibacterial activity, as reflected by lower colony counts (average = 1 in *E. coli*, 0 in *S. mutans*). These differences were not apparent when measuring optical density alone, suggesting that the *D. discoideum* model offers enhanced discrimination of bactericidal activity among formulations.

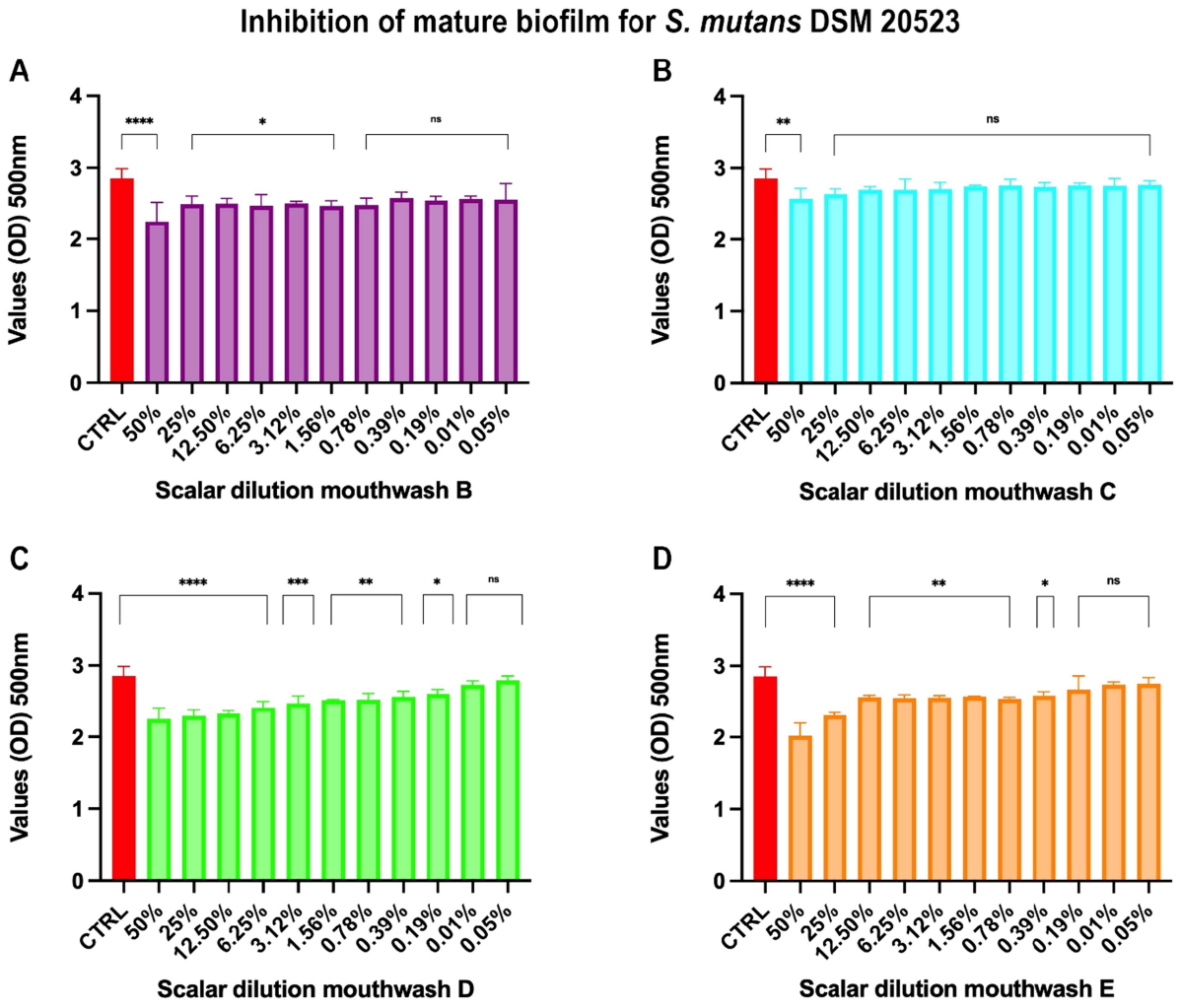

Figure 4. Inhibition of mature biofilm for *S. mutans*DSM 20523. OD values of *S. mutans* biofilm, treated with mouthwash B, C, D and E after 24 h of incubation to allow biofilm formation. The mean OD500 value of the control was 2.86 ± 0.13. A) For mouthwash B, the treatment with the highest concentration (50%) resulted in a reduction to 2.25, while lower concentrations (from 25 to 0.05%) resulted in progressively higher mean values (ranging from 2.49 to 2.56), approaching those of the untreated control. B) For mouthwash C, the treatment with the highest concentration (50%) slightly reduced the biofilm biomass to 2.58 ± 0.14, while lower concentrations (25–0.05%) exhibited OD values between 2.64 ± 0.07 and 2.77 ± 0.06, comparable to the control. C) For mouthwash D, the treatment with 50% mouthwash reduced biofilm formation to 2.25 ± 0.16, intermediate concentrations (25–0.78%) also showed lower OD values (from 2.29 to 2.53), whereas below ≤ 0.39% the inhibition progressively decreased. D) For mouthwash E, while exposure to 50% had a marked reduction of the biofilm biomass (2.06 ± 0.14), dilutions between 25 and 0.78% resulted in intermediate OD values (2.34 ± 0.09 to 2.58 ± 0.08). Statistical significance was analysed by ANOVA one-way and reported as p-value < 0.05 (*), p-value < 0.01 (**), p-value < 0.001 (***), p-value < 0.0001 (****), non-significative p-value > 0.05 (ns).

D. discoideum phagocytosis after treatment with mouthwashes

Given these findings, it could not be excluded that the tested mouthwashes also exerted direct effects on *D. discoideum* viability, beyond their bactericidal properties. To assess this, we performed a cell vitality assay to evaluate whether different mouthwash compositions interfered with *D. discoideum* growth (Figure 8A).

After 1 min of incubation followed by thorough washing, we conducted an XTT assay using 3×10^5 cells per 100 µL of medium. Cell vitality was significantly reduced by all tested mouthwashes ($p < 0.01$). Interestingly, mouthwash C exerted only a mild reduction in cell viability (86% of control), comparable to placebo A, whereas mouthwashes B, D, and E caused a marked cytotoxic effect, reducing viability to approximately 50%. These results indicate that the combination of sodium DNA and low CHX

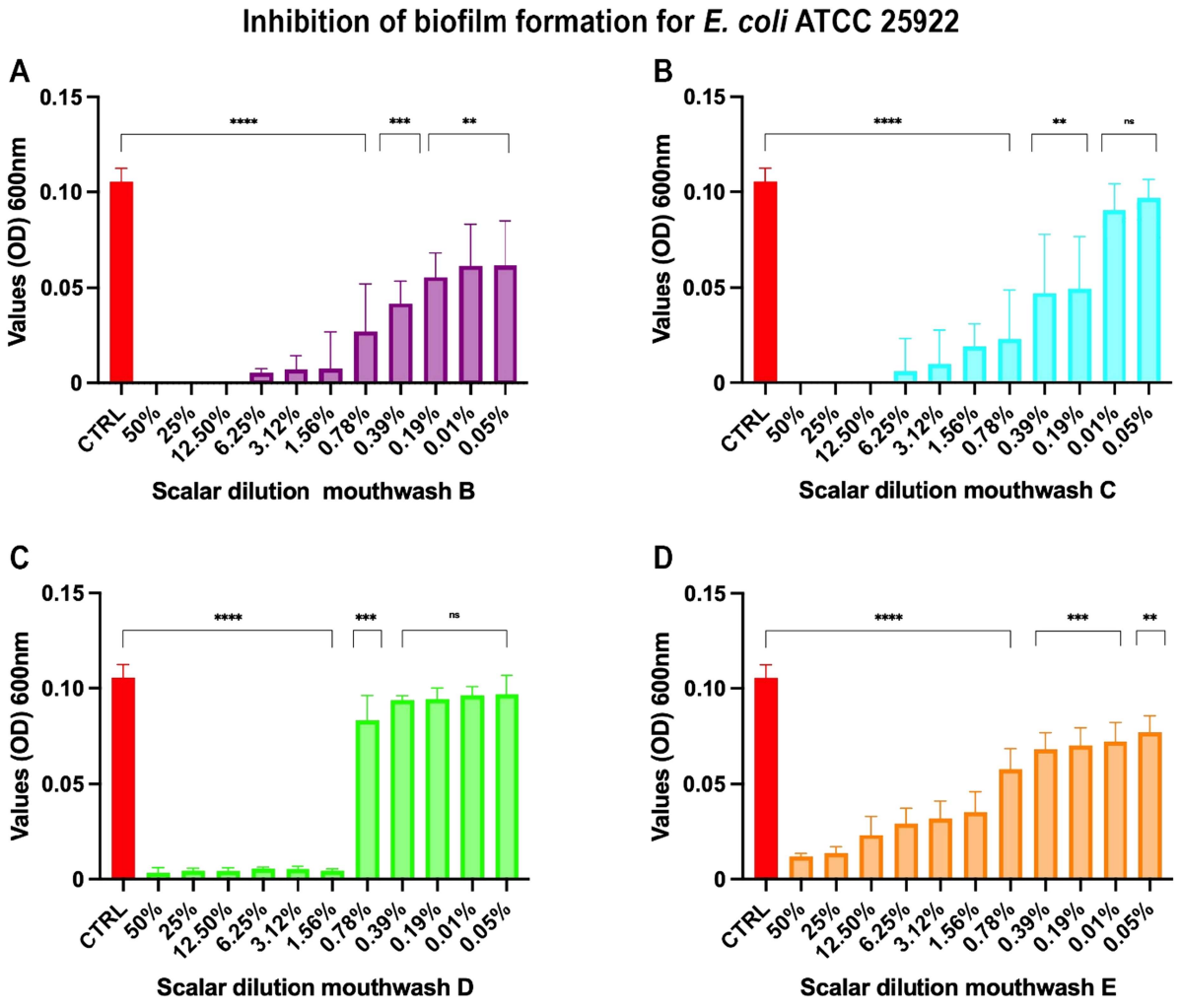

Figure 5. Inhibition of biofilm formation for *E. coli* ATCC 25922. OD values of *E. coli* biofilm treated with mouthwash B, C, D and E. The control group showed a mean OD600 of 0.105 ± 0.0069, while treated samples exhibited values ranging from: A) −0.0059 ± 0.0035 (50%) to 0.0618 ± 0.0232 (0.05%) for mouthwash B; B) −0.009 ± 0.007 (50%) to 0.063 ± 0.008 (0.05%) for mouthwash C; C) 0.008 ± 0.009 (50%) to 0.170 ± 0.001 (0.05%) for mouthwash D. D) Conversely, for mouthwash E, treated samples showed a marked reduction in biofilm biomass only at higher concentrations, with mean OD values of 0.012 ± 0.002 at 50% and 0.017 ± 0.003 at 25%, and a progressive increase in OD at lower concentrations (≤12.5%). Statistical significance was analysed by ANOVA one-way and reported as *p*-value < 0.05 (*), *p*-value < 0.01 (**), *p*-value < 0.001 (***), *p*-value < 0.0001 (****), non-significative *p*-value > 0.05 (ns).

concentration (0.12%) exerts the least cytotoxic effect on *D. discoideum*. To confirm these findings, *D. discoideum* was suspended for 1 min with each mouthwash, then washed and plated on agar containing a pre-grown *E. coli* or *S. mutans* monolayer to assess both phagocytosis and development (Figure 8B). Phagocytosis was more impaired in cells treated with mouthwashes lacking sodium DNA (D and E) compared to those containing it (B and C). Comparing formulations with identical CHX concentrations (B vs. D at 0.2%, and C vs. E at 0.12%), phagocytic activity was more severely disrupted when sodium DNA was absent. This effect was particularly evident for the 0.12% CHX formulations, where *D. discoideum* cells exhibited delayed aggregation, indicating compromised phagocytic function. Interestingly, mouthwash E showed a progressive loss of effectiveness over time, as *D. discoideum* cells eventually resumed aggregation and development, a phenomenon not observed with D. Measurements of phagocytosis area further supported these findings (Figure S1A). Fruiting bodies of *D. discoideum* were observed in all conditions after four days of incubation; however, their morphology was notably altered (small-fruiting-body phenotype) in samples treated with mouthwashes B, C, and D (Figure S1B). Overall, these

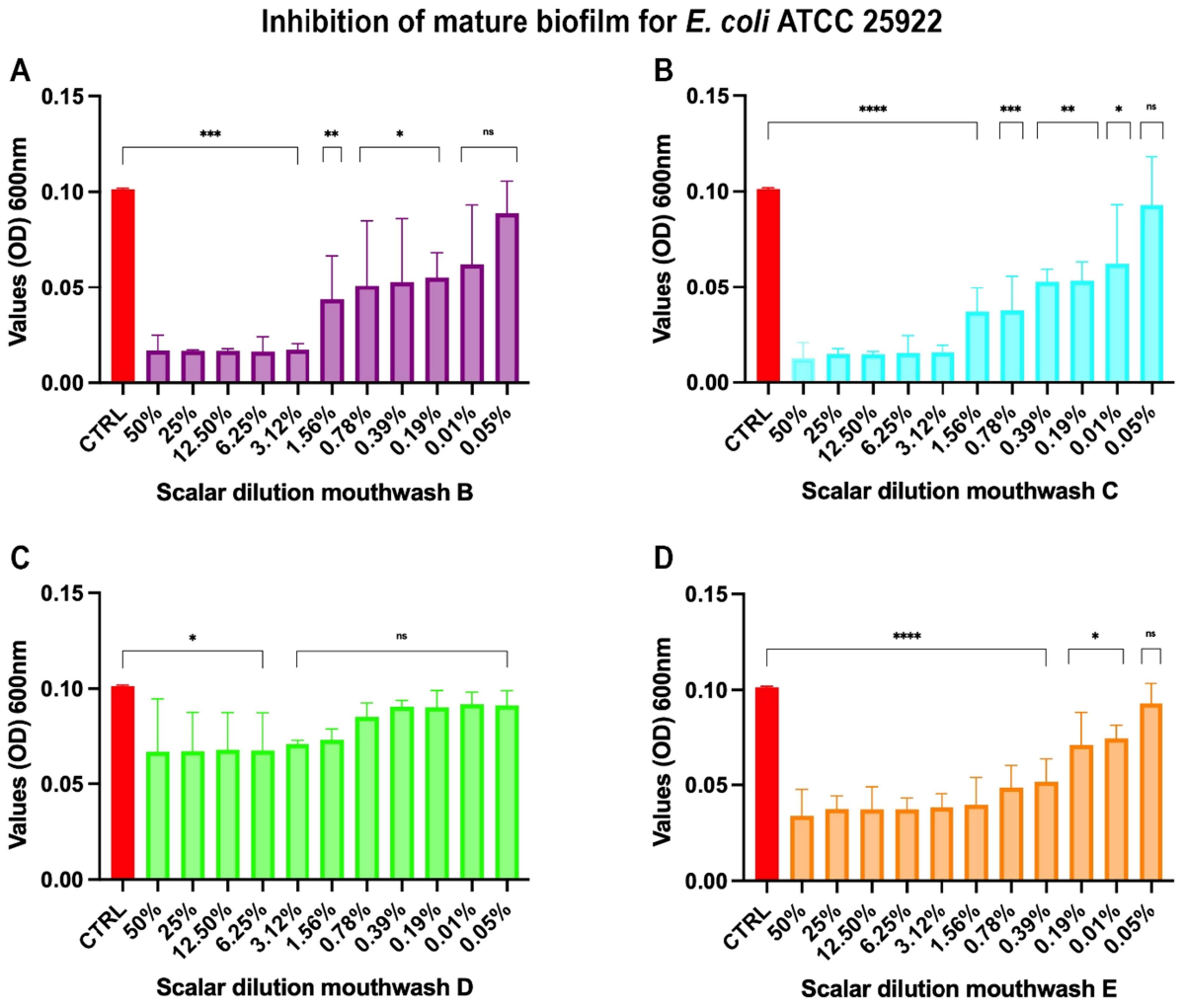

Figure 6. Inhibition of mature biofilm for *E. coli* ATCC 25922. OD values of *E. coli* biofilm, treated with mouthwash B, C, D and E after 24 h of incubation to allow biofilm formation. The mean OD600 value of the control was 0.101 ± 0.001. A) For mouthwash B, concentration from 50 to 3.12% maintained similar inhibitory levels (mean OD500 between 0.015 and 0.016); likewise B) mouthwash C showed a similar effect at the same concentrations. C) For mouthwash D from 50 to 6.25% had slightly lower mean values (0.067–0.068), while lower concentrations were similar to control values. D) For mouthwash E, the strongest inhibitory effect was recorded at higher concentrations (50–6.25%, $p < 0.0001$). Statistical significance was analysed by ANOVA one-way and reported as p-value < 0.05 (*), p-value < 0.01 (**), p-value < 0.001 (***), p-value < 0.0001 (****), non-significative p-value > 0.05 (ns).

results confirm that *D. discoideum* can discriminate the biological impact of different mouthwashes, and that sodium DNA mitigates CHX-induced cytotoxicity while supporting phagocytic function.

Metabolomic profiling of D. discoideum after exposure to CHX-based mouthwashes

Untargeted metabolomic profiling was performed to assess the cellular response of *D. discoideum* to placebo (A), mouthwash C (0.12% CHX + sodium DNA), and mouthwash E (0.12% CHX without sodium DNA). Mouthwashes C and E were selected because they share the same CHX concentration, differing only by the presence of sodium DNA. Among all formulations, mouthwash C displayed the lowest cytotoxic impact in preliminary assays, while mouthwash E, despite its higher toxicity, was included as a direct comparator to specifically evaluate the protective role of sodium DNA. Across all treatments, 541 metabolites were identified, with 293 showing statistically significant differences. Principal component analysis (PCA) demonstrated a clear separation of the three treatment groups (Figure 9B), with

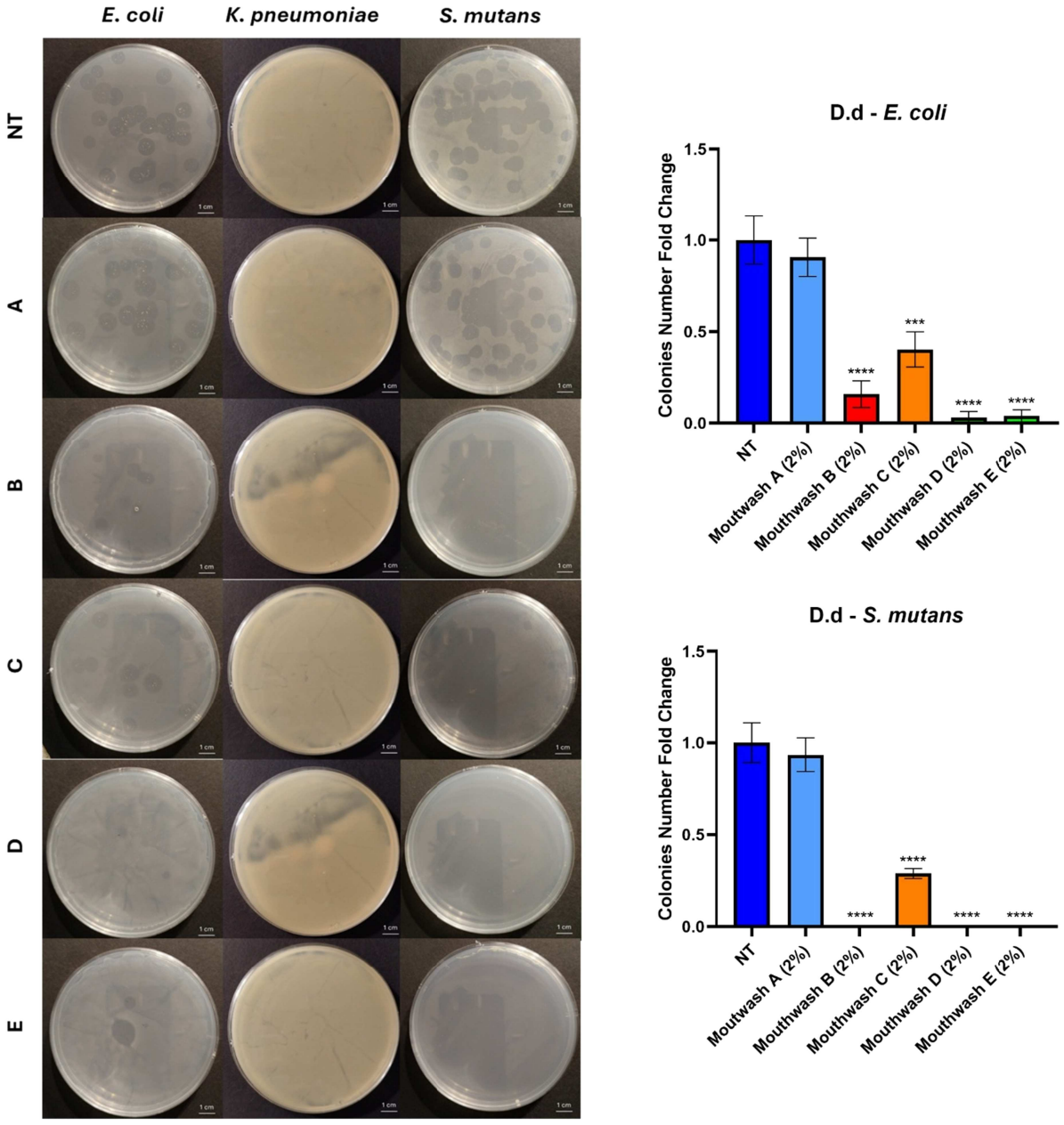

Figure 7. *D. discoideum*– bacteria co-culture assay after treatment with mouthwashes. *D. discoideum* and either *E. coli*, *S. mutans*, *K. pnumoniae* were co-cultured on appropriate agar plates in the presence of the previously described mouthwashes. *K. pneumoniae* was used as a negative control. The number of *D. discoideum* colonies was counted after an incubation of 3 days at 23 °C. The graphs in this figure represent the colonies' number fold change, where the not-treated sample was set as control (=1). Three replicates were performed for each bacterial strain, and standard deviations were calculated based on the means of each sample. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

mouthwash E-treated samples forming a distinct cluster, indicative of a marked shift in metabolic state compared with mouthwash C and placebo. Volcano plot analysis (Figure 9A) revealed 86 metabolites significantly downregulated and 7 upregulated in cells exposed to mouthwash E compared with mouthwash C, suggesting a pronounced metabolic imbalance in the absence of sodium DNA, despite identical CHX concentrations. Pathway enrichment analysis of these differentially expressed metabolites (Figure 9C) indicated that the most perturbed pathways were those related to amino acid metabolism (including glutamine, branched-chain amino acids, and histidine), lipid remodelling

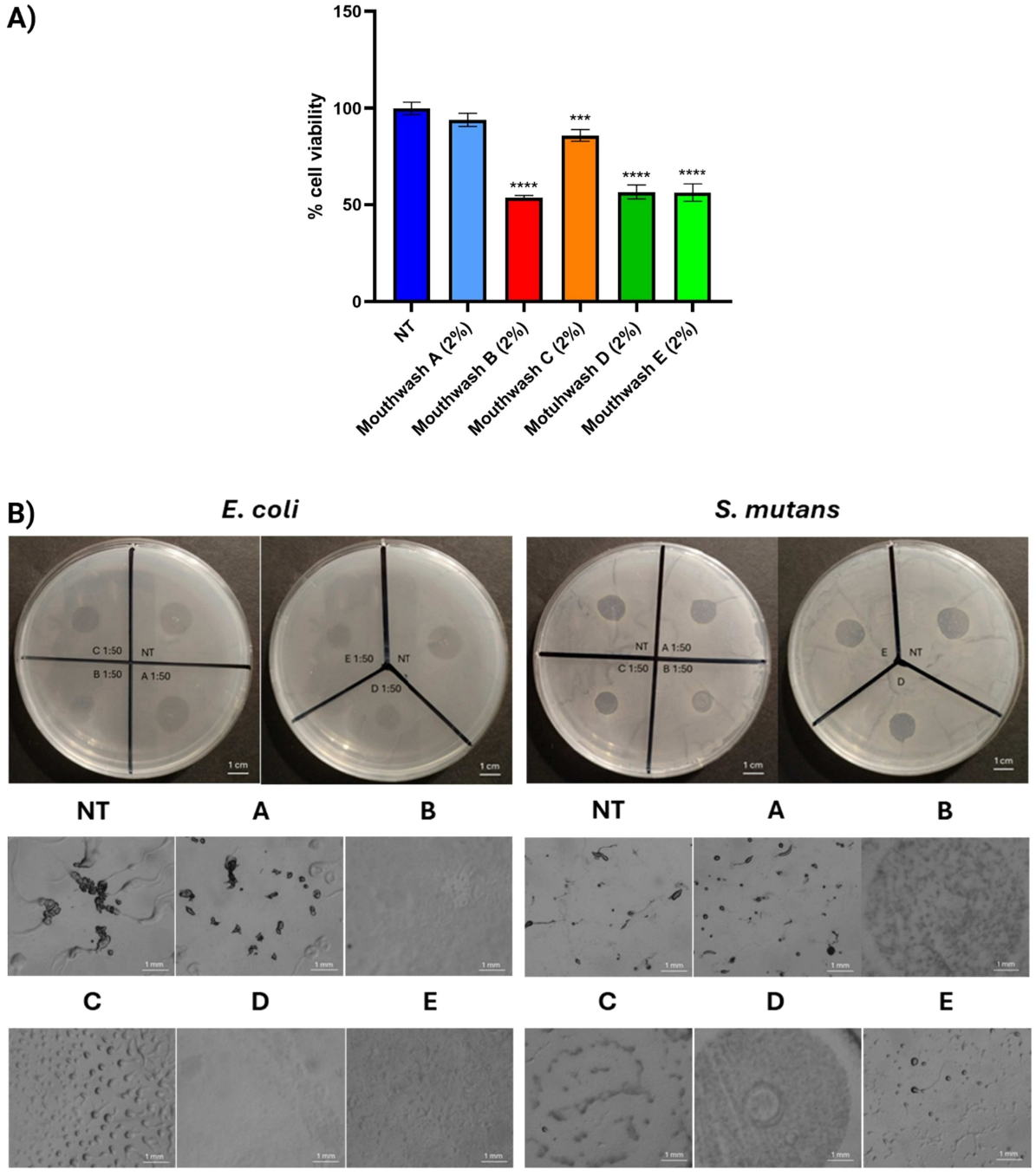

Figure 8. Phagocytosis assay after *D. discoideum*treatment with mouthwashes A) Viability assay was performed with XTT method. Not treated condition (NT) was considered 100% of vitality. B) *D. discoideum* cells were treated for 1 min with previously described mouthwashes at a final dilution of 2%. After that, small drops of cells were deposited on agar *N* plates with an already-grown monolayer of *E. coli*/*S. mutans*. The phagocytosis process was then monitored after 1–3 days of incubation at 23 °C. Pictures were taken using a digital microscope imager (Celestron), at a magnification of 6.5X. Three replicates have been performed and One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

(lysophosphatidylcholines), nucleotide metabolism (NAD, guanosine monophosphate), and cofactor/vitamin biosynthesis (pyridoxine, flavin derivatives). In particular, metabolites such as raffinose, stachyose, and hypoxanthine were strongly depleted in mouthwash E-treated cells, suggesting compromised energy homoeostasis and oxidative stress. Furthermore, pronounced alterations in lysophosphatidylcholine

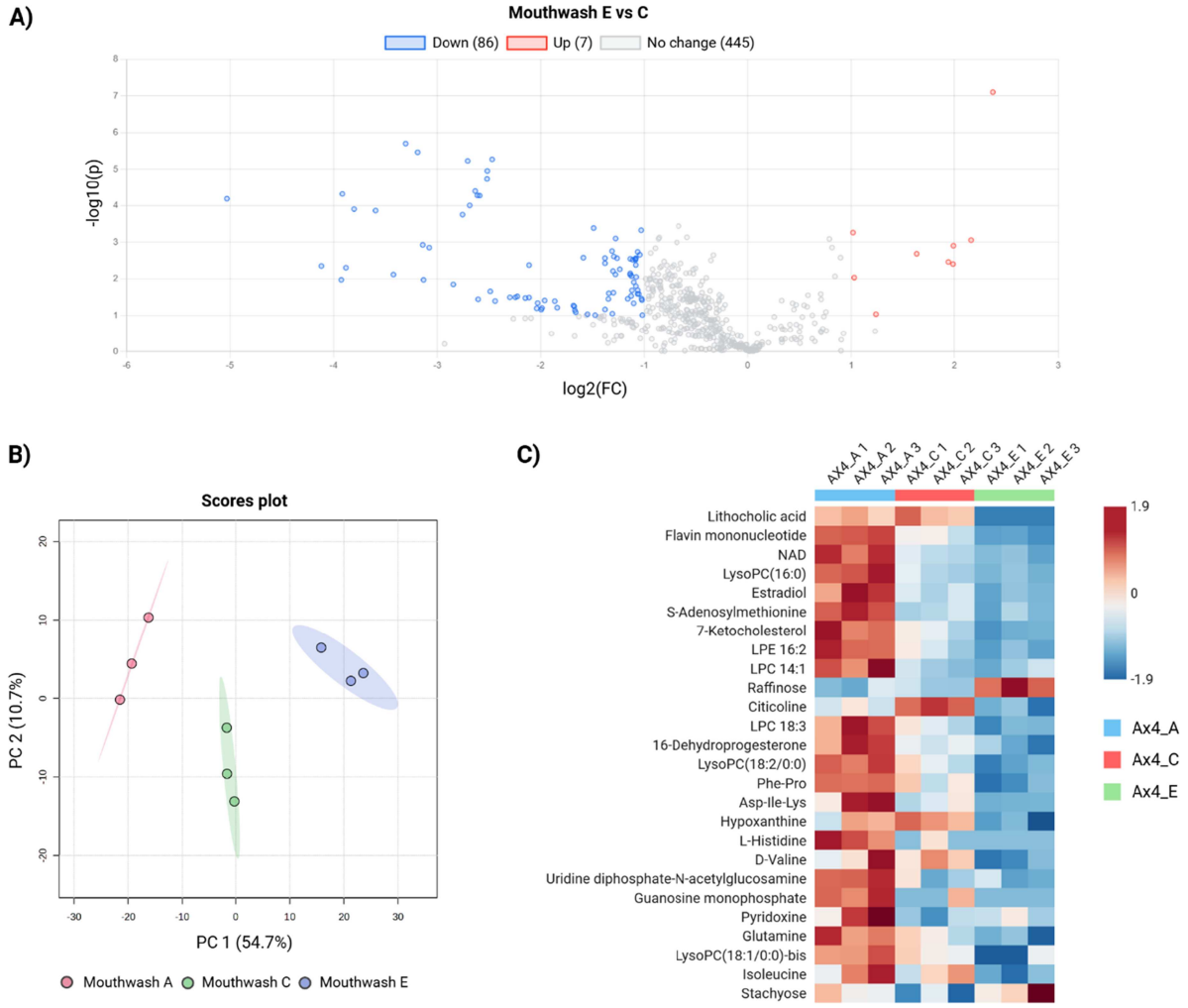

Figure 9. Untargeted metabolomic analysis of *D. discoideum*treated with mouthwashes. *D. discoideum* cells were treated for 1 min with mouthwashes A (placebo), C (0.12% CHX, w/sodium DNA) and E (0.12% CHX, w/o sodium DNA). Then, an untargeted metabolomic analysis has been performed. A total of 541 metabolites have been identified. A) PCA analysis was performed using MetaboAnalyst 6.0 tool. Groups were named after their correspondent mouthwash. PC 1 = 54.7%, PC 2 = 10.7%. B) Volcano-plot of comparison analysis between *D. discoideum* treated with mouthwash E versus C. Metabolites identified were subdivided into three categories: down-expressed (blue), up-expressed (red) and no change (grey). C) A further enrichment analysis was performed to correlate identified metabolites to cellular pathways using again MetaboAnalyst 6.0 tool. A total of 29 metabolites were differentially expressed between the three conditions, as reported in the heatmap in this figure. Three biological replicates have been performed. Statistical analysis was performed with the sequent conditions: FDR = 0.05 and One-way ANOVA with post-hoc Tukey's test.

profiles point to membrane destabilization, a hallmark of CHX cytotoxicity. By contrast, mouthwash C induced milder metabolic adjustments, consistent with a more preserved cellular physiology.

These metabolomic data collectively support the protective role of sodium DNA, as its presence appears to mitigate CHX-induced metabolic stress and preserve critical biosynthetic pathways in *D. discoideum*.

Discussion

The oral microbiome plays a crucial role in maintaining oral and systemic health homoeostasis, influencing the onset of various diseases. An imbalance in the oral microbiota can contribute to the development of conditions such as periodontitis, cardiovascular disease, and even diabetes. Drugs such as chemotherapy, antibiotics, and antiseptic agents in mouthwashes can significantly impact the composition of the

microbiome, potentially leading to dysbiosis, which may alter drug metabolism and efficacy. Over the past few decades, numerous antibacterial effectors and their biological significance have been explored. For the first time, in this study, we evaluated and compared the antibiofilm by crystal violet staining and antibacterial activity of different mouthwashes containing chlorhexidine (CHX) with or without adjunct sodium DNA through a time–killing assay following standardised guidelines. In addition, mouthwash activities were evaluated using a cellular model of *D. discoideum*, which is considered a valuable tool for studying phagocytosis.

Beyond evaluating the antibacterial activity of CHX-based formulation, this study primarily highlights the methodological innovation represented by the *D. discoideum* model combined with untargeted metabolomics. This approach provides a unique eukaryotic platform that a integrated microbial, cellular and metabolic readouts within the same experimental framework. *D. discoideum* functions as a professional phagocyte, sharing conserved molecular pathways with mammalian macrophages, including mechanism of chemotaxis, phagocytosis, vesicular trafficking, and oxidative stress responses. By coupling these functional assays with high-resolution metabolomic profiling, the model enables the identification of cytotoxic signatures and protective metabolic shifts induced by bioactive compounds. Therefore, rather than focusing exclusively on the antimicrobial properties of mounthwash formulations, this study emphasised the broader applicability of the *D. discoideum*-metabolomics system as a versatile and sensitive platform for screening antiseptic agents and assessing eukaryotic cell responses in a physiologically relevant context.

Primarly we demonstrated the antibacterial effectiveness of the tested mouthwashes against *Streptococcus mutans* and *E. coli*, although differences in the minimum concentrations required for inhibition and killing were observed. For *S. mutans*, mouthwash formulations containing 0.20 or 0.12% CHX plus sodium DNA (B, C) and 0.20% CHX (D) showed a strong bactericidal effect (99.9% killing), while the 0.12% CHX mouthwash (E), although bactericidal, reduced the initial inoculum by only 3 log10. Notably, mouthwashes containing 0.20 or 0.12% CHX plus sodium DNA (B, C) demonstrated faster bactericidal effects within 8 h, suggesting that sodium DNA may contribute to enhancing the bactericidal properties of CHX. Conversely, against *E. coli*, the addition of sodium DNA reduced the antimicrobial effect of CHX at both 0.20 and 0.12% concentrations (B, C) (3–2 log reduction, respectively), and the 0.12% CHX mouthwash (C) was not bactericidal even at 24 h, underscoring differential responses between Gram-positive (*S. mutans*) and Gram-negative (*E. coli*) species.

Moreover, the antibiofilm activity of these mouthwashes was investigated and highlighted different efficacy on the two models: a Gram positive, strong biofilm producer such as *S. mutans*, and a Gram negative model like *E. coli*, which can be considered a weak biofilm producer [27]. The different formulations of mouthwash B, C, D and E did not seem to affect their antibiofilm activity against *S. mutans*, as their efficacy was strong with or without sodium DNA and at different concentrations of CHX. Nevertheless, their efficacy was the strongest before biofilm formation or when the biofilm produced by *S. mutans* was only in an early, non-mature stage, indicating that these mouthwashes should be mostly used. The mouthwash may interfere with the early phases of biofilm in a preventive way as they cannot eradicate the mature biofilm. Conversely, for *E. coli* differences were found between mouthwashes, as those containing sodium DNA (mouthwash B and C) exerted a stronger inhibition of both biofilm formation and mature biofilm, suggesting that sodium DNA could facilitate CHX antibiofilm activity in this model.

These results are consistent with previous studies demonstrating the efficacy of chlorhexidine against *S. mutans* DSM20523 and *E. coli* ATCC25922, both in vitro and in clinical settings [35,36]. However, the use of CHX is well known to be associated with adverse effects, including microbiome imbalance, tooth staining, taste alteration, and cytotoxicity, highlighting the need for improved formulations. Sodium DNA has been reported to confer protection against oxidative stress and chemical injury, potentially mitigating the cytotoxic effects of CHX without compromising its antimicrobial activity. Studies suggest that sodium DNA may enhance membrane repair, modulate inflammatory signalling, and facilitate wound healing, thus offering additional clinical benefits [37].

In parallel, recent investigations have highlighted the utility of *D. discoideum* as a model organism to evaluate phagocytic responses and bactericidal activity [38]. Its ability to discriminate between Gram-positive and Gram-negative pathogens and its sensitivity to chemical stressors make it an attractive tool for screening oral antiseptics [39–41]. Co-culturing *D. discoideum* cells with *S. mutans* and *E. coli* in the

presence of various mouthwashes revealed significant alterations in colony formation. Mouthwashes B and C (0.2 and 0.12% CHX plus sodium DNA, respectively) reduced *D. discoideum* colonies relative to placebo (A), but the most pronounced inhibitory effects were observed with mouthwashes D and E (without sodium DNA). When comparing formulations with equivalent CHX concentrations (B vs. D and C vs. E), those containing sodium DNA consistently preserved phagocytic activity, suggesting that sodium DNA mitigates CHX-induced toxicity in *D. discoideum*.

Metabolomic profiling further elucidated the protective effect of sodium DNA. Untargeted metabolomics demonstrated that *D. discoideum* cells exposed to mouthwash E (0.12% CHX without sodium DNA) exhibited marked metabolic disruption compared to placebo (A) and sodium-DNA-containing mouthwash C (0.12% CHX). More specifically, E-treated cells showed depletion of glutamine, branched-chain amino acids, and histidine, all metabolites essential for protein synthesis, energy production, and redox balance. Such alterations are consistent with impaired cellular fitness and reduced phagocytosis. Similarly, downregulation of lysophosphatidylcholines and citicoline suggests defects in membrane remodelling and repair, likely compromising the cell's ability to counteract CHX-induced membrane damage. Citicoline (CDP-choline) is a central intermediate in the Kennedy pathway, required for phosphatidylcholine synthesis and membrane [42]. Experimental evidence shows that citicoline restores phospholipid levels and reduces oxidative injury in ischaemia and neuronal models, in part by preventing lipid peroxidation and preserving mitochondrial function [43,44]. Moreover, citicoline enhances glutathione synthesis and glutathione reductase activity, contributing to antioxidant defence mechanisms [45]. By contrast, C-treated cells (CHX + sodium DNA) exhibited only mild metabolic downregulation relative to placebo, and a significant increase of citicoline levels, suggesting that sodium DNA helps preserve pathways linked to energy production, membrane integrity, and redox homoeostasis. This aligns with emerging evidence that extracellular DNA fragments may act as cytoprotective signals, supporting mitochondrial metabolism, enhancing membrane repair, and buffering oxidative injury in eukaryotic cells [37]. Collectively, these metabolomic data confirm that CHX exerts antibacterial and cytotoxic effects, while sodium DNA attenuates the latter by preserving metabolic function, membrane integrity, and phagocytic capacity. This dual mechanism underscores the potential clinical benefit of CHX–sodium DNA formulations.

This study has some limitations that warrant consideration. The experiments were performed exclusively on reference strains from international microbiological banks (*S. mutans* DSM20523 and *E. coli* ATCC25922), which may not fully reflect the complexity of the oral microbiome, thereby limiting the external validity of our findings. Moreover, the regenerative and anti-inflammatory effects of sodium DNA should be further validated in fibroblast and keratinocyte models. Finally, longitudinal clinical studies are necessary to establish the efficacy and safety of these formulations in vivo. Finally, *D. discoideum* remains an evolutionarily distant, non-mammalian organism, lacking tissue organisation, adaptive immunity, and several signalling components specific to higher eukaryotes. These characteristics further constrain the external validity and limit direct extrapolation to complex, organism-level responses in humans. However, *D. discoideum* provides a powerful and ethically sustainable system to investigate conserved cellular mechanisms such as phagocytosis, oxidative stress responses, and cytotoxicity pathways, which are functionally relevant to mammalian innate immunity. Thus, while *D. discoideum* cannot fully recapitulate human biology, results obtained in this model offer mechanistic insights and serve as a translational bridge toward validation in mammalian systems.

Conclusions

This study is the first to integrate antimicrobial and biofilm assays, cellular models, and metabolomic profiling to demonstrate that CHX-containing mouthwashes exhibit potent antimicrobial activity, and that the inclusion of sodium DNA effectively reduces CHX-induced cytotoxicity and metabolic stress. Moreover, we highlight the sensitivity of the *D. discoideum*-metabolomics platform for simultaneous assessment of antimicrobial activity and host cell response. Indeed, even if higher concentrations of CHX (B and D) exhibit the maximal bactericidal activity, the combination of lower CHX with sodium DNA (C)

achieves the optimal balance between antimicrobial efficacy and host cell protection. Therefore, our findings support the use of *D. discoideum* in combination with antimicrobial testing as a robust and sensitive model for screening oral antiseptics and underscore the potential of CHX-sodium DNA formulations as adjunctive oral care agents. Future studies should focus on validating these findings in complex oral biofilms and clinical populations and further dissecting the molecular pathways by which sodium DNA confers cytoprotection.

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Author contributions

CRediT: **Simone Rocco** and **Adriana Antonina Tempesta:** Methodology, Resources; **Gaia Vertillo Aluisio:** Methodology; **Maria Lina Mezzatesta:** Conceptualisation; **Alessandra Romano:** Conceptualisation; **Valentina Schiavo:** Methodology; **Barbara Pergolizzi:** Writing – review & editing; **Maria Santagati:** Data curation, Formal analysis, Funding acquisition, Writing – review & editing; **Cristina Panuzzo** and **Gaetano Isola:** Conceptualisation, Data curation, Funding acquisition, Methodology, Supervision, Writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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