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Antibiofilm and Microbiome-Modulatory Effects of *Syzygium aromaticum* Essential Oil in an *In Vitro* Dental Caries Polymicrobial Model

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ABSTRACT

Background: Dental caries is a microbiome-driven disease in which acidogenic taxa dominated by *Streptococcus mutans* displace commensal genera and form a glucan-rich biofilm matrix that demineralises enamel. Chlorhexidine (CHX) is the standard chemical adjunct but causes staining, dysgeusia and broad commensal suppression, motivating evaluation of botanical alternatives that may rebalance the oral ecosystem.

Objective: To determine the antibiofilm and microbiome-modulatory effects of standardised Indonesian *Syzygium aromaticum* L. (clove, *Myrtaceae*) bud essential oil (SAEO) on saliva-derived polymicrobial cariogenic biofilms *in vitro*.

Methods: SAEO was hydrodistilled, characterised by gas chromatography-mass spectrometry (GC-MS), and tested at SAEO 0.25%, 0.5% and 1.0% v/v against polymicrobial biofilms grown on hydroxyapatite discs from stimulated saliva of 60 caries-active adults; CHX 0.12% was the positive control (n = 6 per arm). Endpoints were biofilm biomass, colony-forming units (CFU) on selective media, extracellular polymeric substance (EPS), biofilm pH, lactate, 16S rRNA microbiome profiling and human gingival fibroblast (HGF-1) cytotoxicity.

Results: Eugenol (82.3%), β -caryophyllene (7.4%) and eugenyl acetate (4.8%) were the principal volatiles. SAEO 1.0% reduced biofilm biomass by 70.5% (95% CI 65.8–75.3; $p < 0.001$), *S. mutans* CFU by 3.22 $\log_{10}$ and EPS by 67.9%, while raising Shannon diversity from 1.42 to 2.08 bits and restoring *Neisseria* and *Corynebacterium* relative abundance — a dysbiosis-reversal pattern absent under CHX. Cytotoxicity reached 17.6% at SAEO 1.0% versus 31.8% for CHX 0.12%.

Conclusion: Standardised SAEO is a credible complementary anticaries agent meriting clinical translation.

All authors have reviewed and approved the final version of the manuscript.

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1. Introduction

Dental caries remains the most prevalent non-communicable disease worldwide. The Global Burden of Disease Study 2021 estimates that the main oral conditions affect 3.69 billion people, with untreated caries of the permanent dentition being the single most common condition and untreated caries of primary teeth affecting hundreds of millions of children.¹

Despite decades of fluoride-based prevention, the global age-standardised burden of caries has changed little over three decades, indicating that current approaches are insufficient.¹ In Indonesia, successive national Basic Health Research (Riskesdas) cycles have likewise reported a high caries burden in adults, consistent with these South-East Asian regional estimates. Dental caries is now understood as an ecological disease driven by frequent dietary sugar

exposure, which selects for acidogenic and aciduric microorganisms within the supragingival biofilm.²

Within this ecological framework, dental caries arises from a microbial dysbiosis in which *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus* species dominate, while commensal nitrate-reducing genera such as *Neisseria*, *Rothia* and *Corynebacterium* are progressively lost.³ The cariogenic taxa secrete glucosyltransferases that polymerise dietary sucrose into water-insoluble glucans, producing an extracellular polymeric substance (EPS) matrix that promotes adhesion, sequesters acid against the enamel surface and shelters embedded organisms from mechanical and chemical removal.⁴ Several lines of evidence suggest that interventions which selectively de-bulk this matrix and re-establish microbial diversity, rather than act as global biocides, may produce more durable caries control while preserving the oral health-associated commensal community.⁵

Chemical plaque control has long relied on chlorhexidine digluconate (CHX), which remains the most efficacious chemical adjunct but is limited by extrinsic tooth and restoration staining, taste perturbation, mucosal sloughing, oral dysgeusia and a propensity to indiscriminately suppress commensal taxa.⁶ Long-term unsupervised use is also associated with disturbance of nitrate-reducing oral bacteria implicated in cardiovascular nitric oxide signalling, lending urgency to the search for ecology-respecting alternatives.⁷ Plant-derived essential oils, with their multi-component pharmacology and historical safety records, have emerged as attractive candidates.

Clove (*Syzygium aromaticum* L., *Myrtaceae*) is a small evergreen tree native to the Maluku Islands of Indonesia whose dried flower buds yield a yellow-amber essential oil with a long documented record of analgesic and antimicrobial use in traditional dentistry.⁸ Modern phytochemical analyses by gas chromatography-mass spectrometry (GC-MS) consistently identify eugenol (4-allyl-2-methoxyphenol) as the principal volatile, accounting typically for 70–90% of the oil, with β -caryophyllene (5–12%), eugenyl acetate (3–10%) and minor sesquiterpenes such as α -humulene completing the chemotype.⁹ Eugenol is a phenylpropanoid whose hydrophobic methoxyphenol scaffold partitions into bacterial phospholipid bilayers, dissipating proton motive force, promoting potassium and ATP leakage, and impairing substrate transport.¹⁰ It also chelates divalent cations stabilising bacterial surfaces, and at sub-inhibitory concentrations it interferes directly with quorum-sensing circuitry, down-regulating the

comDE competence cascade and the *gtfB/gtfC* glucosyltransferase genes in *S. mutans*.¹¹

Despite an expanding catalogue of *in vitro* antibacterial reports for clove essential oil and isolated eugenol, four evidence gaps persist. First, most studies have used monocultures of *S. mutans* or simple dual-species systems, leaving the broader ecological consequences of treatment unresolved.¹² Second, the modest number of investigations that have employed polymicrobial models have rarely paired biofilm endpoints with microbiome-level outcomes such as alpha and beta diversity, which are the most direct measure of dysbiosis correction. Third, Indonesian chemotypes of clove, despite their phytochemical distinctiveness and the importance of clove to the national herbal economy, are under-represented in the international literature.¹³ Fourth, the head-to-head comparison of clove essential oil with the gold-standard chlorhexidine across both antibiofilm and microbiome-level endpoints has not been systematically undertaken using clinically realistic treatment schedules.^{14,15} Without such comparisons the translational case for clove-based formulations remains speculative, because clinicians are unwilling to displace CHX without evidence of at least parity on killing endpoints and superiority on ecological endpoints.

The ecological plaque hypothesis holds that caries arises when frequent acid challenges select for acidogenic and aciduric taxa, tipping the community toward dysbiosis.^{2,3} This framework predicts that chemical interventions that selectively reduce cariogenic taxa while preserving aerobic commensals should re-establish a caries-neutral community, whereas broad-spectrum antiseptics that indiscriminately deplete biomass risk leaving an empty ecological niche that can be rapidly recolonised by the original dysbiotic consortium.⁵ Evidence that chlorhexidine depletes nitrate-reducing oral commensals with measurable downstream reductions in salivary and plasma nitrite has linked oral antiseptic use to systemic cardiovascular signalling, rendering the search for ecology-respecting alternatives an urgent translational priority.^{6,7} The aim of this study was to determine the antibiofilm and microbiome-modulatory effects of standardised *Syzygium aromaticum* essential oil on saliva-derived polymicrobial cariogenic biofilms *in vitro* and to provide mechanistic and translational evidence supporting its development as a complementary anticaries agent.

2. Methods

2.1. Study design and setting

A controlled *in vitro* experimental study was conducted at the Eureka Research Laboratory, Palembang, Indonesia, between January and December 2024. Ethical approval and consent arrangements are set out in full under Declarations.

2.2. Plant material and essential oil preparation

Mature dried flower buds of *Syzygium aromaticum* L. (*Myrtaceae*), Maluku chemotype, were obtained from a registered local supplier. Voucher specimens were authenticated by a botanist and deposited at the Eureka herbarium (voucher no. SA-2024-017). One kilogram of buds was ground and subjected to hydrodistillation in a Clevenger-type apparatus for 4 hours. The recovered oil was dried over anhydrous sodium sulphate, yielded at 14.6% w/w, and stored at 4 °C in amber glass vials sealed under nitrogen until use.

2.3. Phytochemical characterisation

The essential oil was diluted 1:100 in *n*-hexane and analysed on an Agilent 7890B gas chromatograph coupled to a 5977B mass-selective detector (electron ionisation, 70 eV) using an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm). Helium carrier gas flowed at 1 mL/min; the oven was held at 60 °C for 2 minutes, ramped at 4 °C/min to 240 °C and held for 5 minutes; injector and transfer-line temperatures were 250 °C and 280 °C. Compounds were identified by comparison of mass spectra and Kovats retention indices (RI) with the NIST 17 library and authentic standards and quantified by relative peak-area normalisation.

2.4. Saliva donors and inclusion criteria

Sixty systemically healthy adults aged 18–35 years (30 men, 30 women) with at least one cavitated dentine lesion (decayed, missing, filled teeth [DMFT] index 1–6) were recruited from the dental outpatient clinic of [institution to be named], Palembang. Exclusion criteria were antibiotic exposure within 3 months, professional dental cleaning within 1 month, current smoking, pregnancy, lactation, or systemic illness. Donors refrained from oral hygiene for 12 hours and from food, drink and chewing gum for 2 hours before sampling. Stimulated whole saliva (5 mL) was collected by paraffin chewing into sterile pre-chilled tubes between 08:00 and 10:00, transported on ice and processed within 1 hour.

2.5. Polymicrobial biofilm model

Saliva from each donor was diluted 1:50 in artificial saliva supplemented with 1% w/v sucrose and used to

inoculate sterilised hydroxyapatite discs (9 mm × 1.5 mm) in 24-well plates. Biofilms were grown anaerobically (10% CO₂, 10% H₂, 80% N₂) at 37 °C for 96 hours with daily medium replacement. After 96 hours, discs were treated for 5 minutes with one of five interventions: untreated artificial saliva (negative control), SAEO 0.25%, 0.5% or 1.0% v/v (in artificial saliva with 0.5% Tween-80), or CHX 0.12% w/v (positive control). Treatment was repeated four times daily for 48 hours to simulate four-times-daily mouth-rinse use.

2.6. Sample size and randomisation

Sample size was calculated a priori for the primary endpoint of biofilm biomass (optical density at 595 nm, OD₅₉₅) using one-way ANOVA with five groups, effect size $f = 0.55$, $\alpha = 0.05$ two-sided and power $1 - \beta = 0.90$, yielding 5 replicates per arm; the study used 6 biological replicates per arm to allow for attrition. Each replicate corresponded to an independent saliva donor across two technical replicates. Disc allocation was concealed by computer-generated random sequence and outcome assessors were blinded through coded labels.

2.7. Outcome measurements

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of SAEO were determined by broth microdilution against four reference cariogens (*S. mutans* American Type Culture Collection [ATCC] 25175, *S. sobrinus* ATCC 33478, *L. acidophilus* ATCC 4356, *A. viscosus* ATCC 15987). Biofilm biomass was quantified by crystal-violet staining and absorbance at 595 nm. Viable counts, expressed as colony-forming units (CFU), were obtained by sonication, serial dilution and plating on selective media. EPS was quantified by the anthrone–sulphuric acid assay. Biofilm pH was measured 5, 30 and 60 minutes after a sucrose pulse using a microelectrode, and lactate enzymatically. For 16S rRNA profiling, biofilm DNA was extracted, the V3–V4 region amplified and sequenced (2 × 300 bp), and reads processed through QIIME 2 with DADA2 denoising, SILVA 138 assignment and computation of Shannon and Simpson alpha diversity and Bray–Curtis beta diversity, the latter computed against the untreated control. Cytotoxicity was assessed on human gingival fibroblast (HGF-1) cells by the lactate-dehydrogenase (LDH) release assay after 5-minute exposure.

2.8. Statistical analysis

Continuous variables are reported as mean ± standard deviation (SD) or median (interquartile range, IQR).

Group differences were tested by one-way ANOVA with Tukey's HSD test or by the Kruskal–Wallis test with Dunn correction. Linear mixed-effects models with donor as a random effect were used for biomass, CFU and pH outcomes. Effect sizes are presented as Hedges' *g* with 95% confidence intervals (CI) from bias-corrected bootstrap (2000 resamples). Dose–response curves were fitted with a four-parameter logistic (Hill) model and the IC₅₀ extracted. Beta diversity was tested by PERMANOVA on Bray–Curtis distances against the untreated control (9999 permutations). Two-sided α was 0.05 with Bonferroni adjustment for pre-specified secondary endpoints. Analyses used IBM SPSS 28, R 4.3.1 (lme4, vegan, phyloseq, effectsize) and GraphPad Prism 9.5.

3. Results

3.1. Donor characteristics

Of 73 prospective participants screened, 60 met the inclusion criteria and provided saliva (Table 1). The cohort was balanced by sex (50% male; 50% female), with a mean age of 26.4 ± 4.8 years and a mean DMFT of 3.4 ± 1.5 , confirming the intended caries-active phenotype. Mean stimulated salivary flow was 1.46 ± 0.32 mL/min and the resting pH of 6.92 ± 0.18 was within the range typically reported for caries-active adults. Twice-daily tooth-brushing was reported by 78.3% and fluoridated-toothpaste use by 91.7%, but only 20.0% used a mouth rinse. The narrow distribution of clinical variables minimised donor-driven variance, and stratification by DMFT band revealed no significant difference in baseline biomass ($p = 0.42$).

Table 1. Demographic and clinical characteristics of the saliva donors ($n = 60$).

Characteristic	Value	Range
Age, years	26.4 ± 4.8	18–35
Sex — male, n (%)	30 (50.0)	—
Sex — female, n (%)	30 (50.0)	—
Body mass index, kg/m ²	23.1 ± 2.7	18.6–29.4
DMFT score	3.4 ± 1.5	1–6
Stimulated salivary flow, mL/min	1.46 ± 0.32	0.81–2.20
Resting saliva pH	6.92 ± 0.18	6.51–7.31
Buffer capacity (Dentobuff)	Medium 41 (68.3); High 19 (31.7)	—
Daily sugar exposures (frequency)	5.2 ± 1.4	3–9
Twice-daily tooth-brushing, n (%)	47 (78.3)	—
Use of fluoridated toothpaste, n (%)	55 (91.7)	—
Daily mouth-rinse use, n (%)	12 (20.0)	—

Continuous variables are mean $\pm$ SD; categorical variables are n (%). DMFT, decayed, missing, filled teeth.

3.2. Phytochemical characterisation of SAEO

Hydrodistillation yielded 14.6% w/w of clear, yellow-amber essential oil. GC-MS profiling confirmed the expected eugenol-dominant chemotype, with the full composition listed in Table 2: eugenol accounted for $82.3 \pm 0.6\%$ of the total integrated peak area, β -caryophyllene $7.4 \pm 0.4\%$, eugenyl acetate $4.8 \pm 0.3\%$ and α -humulene $1.6 \pm 0.2\%$; the residual $3.9 \pm 0.4\%$ comprised methyl salicylate, calarene, δ -cadinene and trace sesquiterpene oxides.

3.3. MIC and MBC against reference cariogens

SAEO inhibited *S. mutans* at a MIC of $0.16 \pm 0.02\%$ v/v and was bactericidal at $0.31 \pm 0.04\%$ v/v, a narrow MIC/MBC ratio of 1.94 (Table 2). MIC values for *L. acidophilus* and *A. viscosus* were $0.21 \pm 0.03\%$ and $0.18 \pm 0.02\%$ v/v.

3.4. Antibiofilm activity: biomass and viable counts

Treatment with SAEO produced a clear concentration-dependent reduction in biofilm biomass (Figure 1A; Table 2). Mean OD₅₉₅ fell from 2.41 ± 0.18 in the untreated control to 1.88 ± 0.21 at SAEO 0.25% (22.0% reduction, $p = 0.034$), 1.22 ± 0.17 at SAEO 0.5% (49.4% reduction, $p < 0.001$) and 0.71 ± 0.14 at SAEO 1.0% (70.5% reduction, $p < 0.001$); CHX 0.12% reached 0.58 ± 0.12 (75.9% reduction). The difference between CHX 0.12% and SAEO 1.0% was not significant ($p = 0.21$). Viable counts mirrored these findings (Figure 1B): *S. mutans* CFU dropped by 0.74 log₁₀ at SAEO 0.25%, 1.91 log₁₀ at SAEO 0.5% and 3.22 log₁₀ at SAEO 1.0%, and the difference between SAEO 1.0% and CHX 0.12% was not significant (5.20 versus 4.87 log₁₀, $p = 0.18$). The fitted dose–response curve (Figure 2A) gave an IC₅₀ of 0.40% v/v.

Table 2. Phytochemical profile of *Syzygium aromaticum* essential oil (SAEO) by GC-MS, MIC and MBC against reference cariogens, and primary biofilm outcomes after 5-minute exposure four times daily for 48 hours.

Variable	Value / Group	Result	Statistic
GC-MS — Eugenol (%)	Standardised SAEO	82.3 ± 0.6	RI 1357
GC-MS — β -Caryophyllene (%)	Standardised SAEO	7.4 ± 0.4	RI 1418
GC-MS — Eugenyl acetate (%)	Standardised SAEO	4.8 ± 0.3	RI 1521
GC-MS — α -Humulene (%)	Standardised SAEO	1.6 ± 0.2	RI 1452
GC-MS — Other minor (%)	Standardised SAEO	3.9 ± 0.4	—
MIC against <i>S. mutans</i> (% v/v)	SAEO	0.16 ± 0.02	—
MBC against <i>S. mutans</i> (% v/v)	SAEO	0.31 ± 0.04	—
MIC against <i>L. acidophilus</i> (% v/v)	SAEO	0.21 ± 0.03	—
MIC against <i>A. viscosus</i> (% v/v)	SAEO	0.18 ± 0.02	—
Biofilm biomass (OD ₅₉₅)	Control	2.41 ± 0.18	Reference
Biofilm biomass (OD ₅₉₅)	SAEO 0.25%	1.88 ± 0.21	*
Biofilm biomass (OD ₅₉₅)	SAEO 0.5%	1.22 ± 0.17	***
Biofilm biomass (OD ₅₉₅)	SAEO 1.0%	0.71 ± 0.14	***
Biofilm biomass (OD ₅₉₅)	CHX 0.12%	0.58 ± 0.12	*** ns
<i>S. mutans</i> CFU (log ₁₀ /mL)	Control	8.42 ± 0.23	Reference
<i>S. mutans</i> CFU (log ₁₀ /mL)	SAEO 0.25%	7.68 ± 0.28	**
<i>S. mutans</i> CFU (log ₁₀ /mL)	SAEO 0.5%	6.51 ± 0.25	***
<i>S. mutans</i> CFU (log ₁₀ /mL)	SAEO 1.0%	5.20 ± 0.22	***
<i>S. mutans</i> CFU (log ₁₀ /mL)	CHX 0.12%	4.87 ± 0.19	*** ns
EPS (μg glucose eq./disc)	Control	281 ± 22	Reference
EPS (μg glucose eq./disc)	SAEO 1.0%	90 ± 14	***
EPS (μg glucose eq./disc)	CHX 0.12%	84 ± 13	*** ns
Biofilm pH at 30 minutes	Control	4.62 ± 0.11	Reference
Biofilm pH at 30 minutes	SAEO 1.0%	5.81 ± 0.14	***
Biofilm pH at 30 minutes	CHX 0.12%	5.95 ± 0.13	*** ns

RI, retention index (HP-5MS). Means ± SD across n = 6 biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001 versus untreated control by ANOVA with Tukey's HSD; ns, non-significant difference between SAEO 1.0% and CHX 0.12%. Selected biofilm time points are shown.

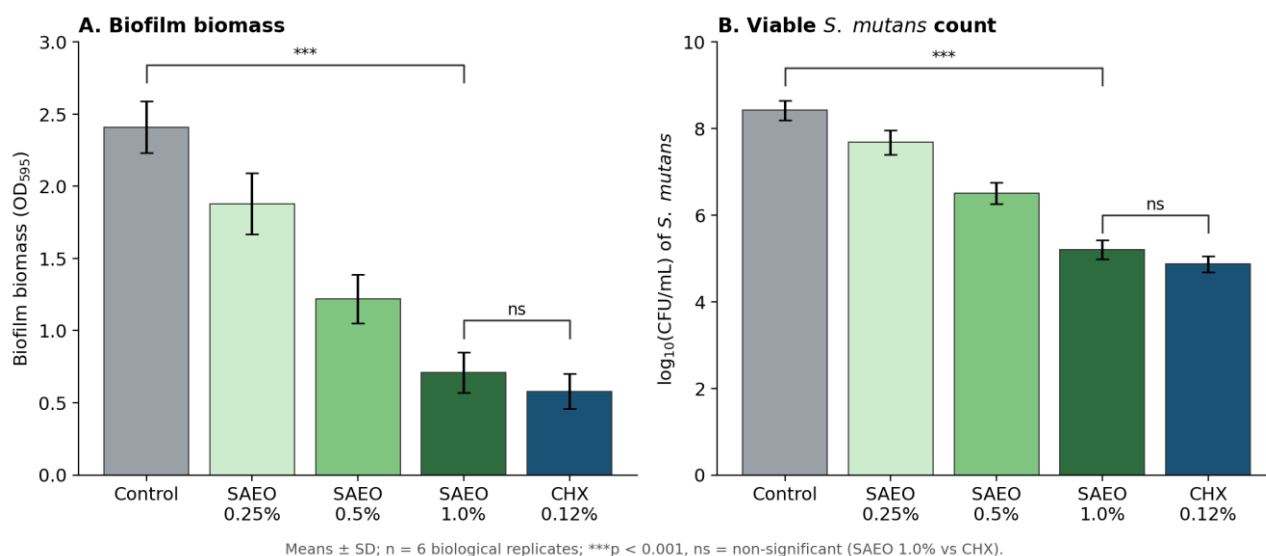


Figure 1. Concentration-dependent antibiofilm effect of SAEO. (A) Biofilm biomass by crystal-violet staining (OD₅₉₅). (B) Viable *Streptococcus mutans* counts (log₁₀ CFU/mL). Bars are means ± SD of six biological replicates; ***p < 0.001; ns, non-significant (SAEO 1.0% versus CHX 0.12%).

3.5. Extracellular polymeric substance and acidogenicity

EPS content per disc was 281 ± 22 μ g glucose equivalents in the control and fell to 90 ± 14 μ g in the SAEO 1.0% arm (67.9% reduction, $p < 0.001$), comparable to the reduction achieved by CHX 0.12% (Table 2). Biofilm pH after a 30-minute sucrose pulse rose from 4.62 ± 0.11 in the control to 5.81 ± 0.14 under SAEO 1.0% and 5.95 ± 0.13 under CHX 0.12% (Table 2). Lactate fell concomitantly from 11.4 ± 1.6 mmol/L to 4.1 ± 0.7 mmol/L under SAEO 1.0% ($p < 0.001$). At SAEO 0.5% v/v the pH remained below the critical demineralisation threshold of 5.5 (5.34 ± 0.13), whereas the SAEO 1.0% arm crossed it.

3.6. Microbiome diversity and taxonomic restructuring

16S rRNA V3–V4 sequencing recovered a mean of 84 ± 11 amplicon sequence variants (ASVs) in the CHX

0.12% arm versus 176 ± 19 under SAEO 1.0% and 118 ± 14 in the untreated control ($p < 0.001$) (Table 3). Shannon diversity rose from 1.42 ± 0.18 in the control to 2.08 ± 0.16 under SAEO 1.0% ($p < 0.001$) but fell to 1.31 ± 0.20 under CHX 0.12%, with Simpson diversity showing the same pattern. Bray–Curtis analysis against the untreated control showed that SAEO-treated communities remained closer to that community than CHX-treated communities did (0.61 ± 0.07 versus 0.78 ± 0.06 ; PERMANOVA $p < 0.001$). At genus level (Figure 2B), *Streptococcus* declined from 61.2% to 37.4% under SAEO 1.0% and to 22.8% under CHX 0.12%. Commensal aerobes associated with oral health — *Neisseria* (from 2.1% to 15.2%) and *Corynebacterium* (from 1.4% to 9.8%) — were enriched under SAEO 1.0% but not under CHX 0.12%, in which *Neisseria* recovered only marginally (3.6%) and *Corynebacterium* even less (2.0%).

Table 3. Microbiome diversity, taxonomic shifts and cytotoxicity outcomes after 48 hours of treatment.

Outcome	Control	SAEO 1.0%	CHX 0.12%	p-value
Shannon diversity (bits)	1.42 ± 0.18	$2.08 \pm 0.16^{***}$	1.31 ± 0.20	< 0.001
Simpson diversity (1–D)	0.52 ± 0.08	$0.78 \pm 0.06^{***}$	0.49 ± 0.09	< 0.001
Observed ASVs	118 ± 14	$176 \pm 19^{***}$	84 ± 11	< 0.001
<i>Streptococcus</i> rel. abundance (%)	61.2 ± 5.4	$37.4 \pm 4.8^{***}$	$22.8 \pm 4.1^{***}$	< 0.001
<i>Lactobacillus</i> rel. abundance (%)	14.8 ± 2.3	$7.1 \pm 1.8^{***}$	$4.1 \pm 1.5^{***}$	< 0.001
<i>Veillonella</i> rel. abundance (%)	9.7 ± 1.6	$6.8 \pm 1.4^{***}$	$3.2 \pm 1.1^{***}$	< 0.001
<i>Neisseria</i> rel. abundance (%)	2.1 ± 0.7	$15.2 \pm 2.6^{***}$	3.6 ± 1.0	< 0.001
<i>Corynebacterium</i> rel. abundance (%)	1.4 ± 0.5	$9.8 \pm 1.7^{***}$	2.0 ± 0.8	< 0.001
<i>Actinomyces</i> rel. abundance (%)	6.3 ± 1.2	$10.6 \pm 1.5^{***}$	$18.4 \pm 2.4^{***}$	< 0.001
Bray–Curtis distance versus control	—	0.61 ± 0.07	0.78 ± 0.06	PERMANOVA < 0.001
LDH release on HGF-1 (% positive)	8.4 ± 1.2	$17.6 \pm 2.1^{***}$	$31.8 \pm 3.4^{***}$	< 0.001

Shannon and Simpson indices were computed from amplicon sequence variants (ASVs). Means $\pm$ SD ($n = 6$); $^{***}p < 0.001$ versus control. ASV, amplicon sequence variant; LDH, lactate dehydrogenase; HGF-1, human gingival fibroblast.

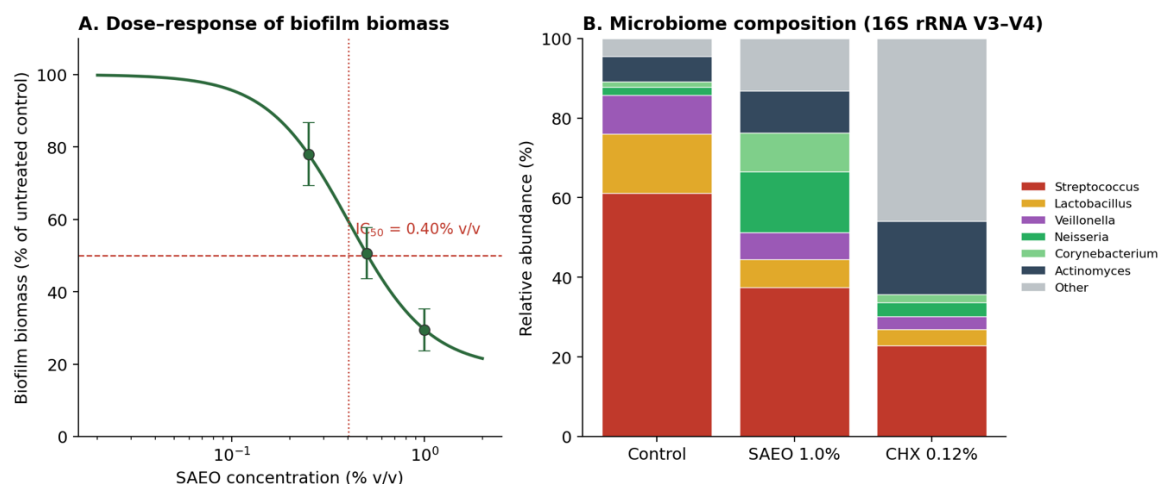


Figure 2. Dose–response and microbiome restructuring. (A) Four-parameter logistic fit of biofilm biomass against SAEO concentration ($IC_{50} = 0.40\% \text{ v/v}$). (B) Genus-level relative abundance from 16S rRNA V3–V4 profiling of the residual biofilm communities.

3.7. Cytotoxicity

LDH release on HGF-1 fibroblasts after a clinically realistic 5-minute exposure was 8.4% of maximum in untreated wells, 17.6% under SAEO 1.0% and 31.8% under CHX 0.12% (Table 3).

3.8. Effect-size synthesis

The forest plot in Figure 3 summarises the standardised mean differences between SAEO 1.0%

and the untreated control across ten outcomes. All ten outcomes showed $|\text{Hedges' } g| > 2.0$ with 95% CIs excluding zero, and the principal microbiological endpoints exceeded $|g| > 5$. The diversity outcomes (Shannon, Simpson, *Neisseria* and *Corynebacterium* abundance) and biofilm pH fell on the positive side of the axis, opposite in direction to the cariogenic outcomes.

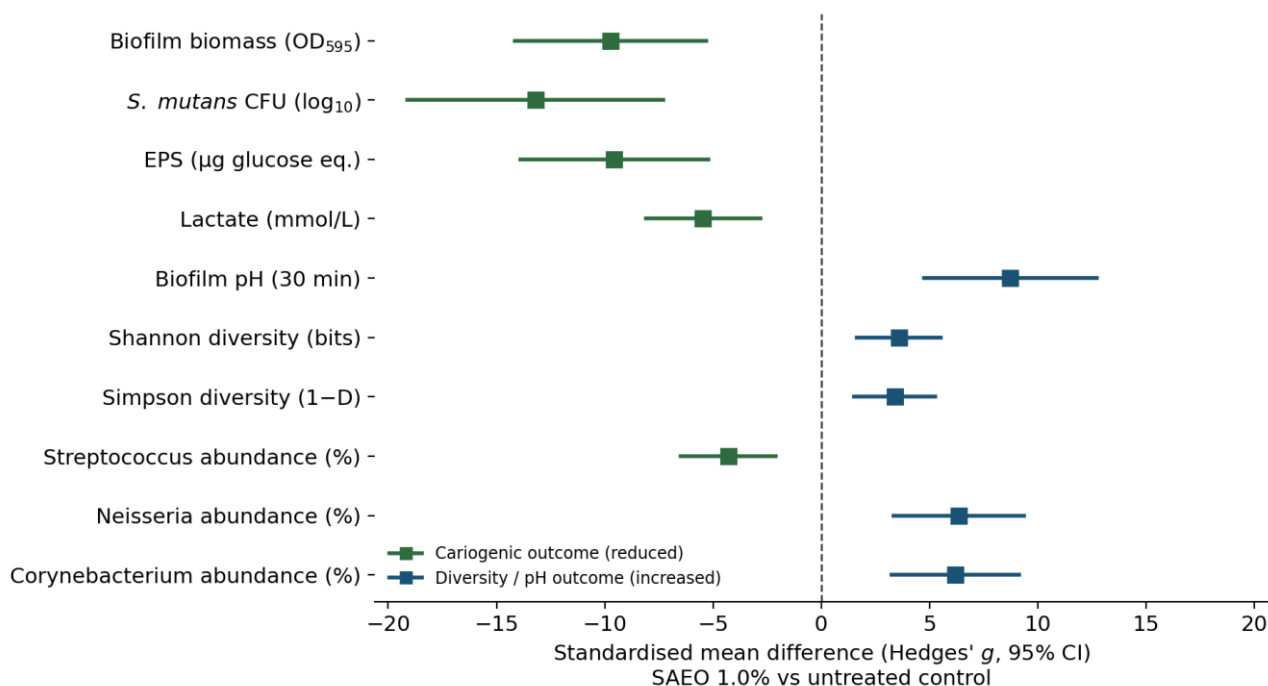


Figure 3. Forest plot of standardised mean differences (Hedges' g , 95% CI) between SAEO 1.0% and the untreated control across ten microbiological and microbiome outcomes. Green markers denote cariogenic outcomes (reduced); blue markers denote diversity and pH outcomes (increased). All $|g| > 2$ with 95% CIs excluding zero.

4. Discussion

4.1. Phytochemical profile and antibacterial potency

The measured proportions fall within the ranges reported for Indonesian clove bud oil¹³ and for clove essential oil characterised elsewhere by GC-MS,¹⁶ and exceed the 75% eugenol minimum specified by the European Pharmacopoeia and Indonesian Materia Medika monographs. The comparatively high β -caryophyllene content provides additional cannabinoid receptor 2 (CB2)-mediated anti-inflammatory potential¹⁷ relevant to the host-microbe interface, although that question lies beyond the present *in vitro* scope. The MIC values obtained are consistent with clove and essential-oil MIC values reported against *S. mutans*.¹⁵ The narrow MIC/MBC margin is mechanistically attributable to the direct membrane-disrupting action of eugenol; unlike ribosome- or cell-wall-targeting antibacterials whose

MIC/MBC ratios commonly exceed 4, the perturbation of the lipid bilayer by eugenol leads to rapid loss of viability once a critical membrane concentration is reached.¹⁰ Even the lowest concentration tested (SAEO 0.25% v/v) was at or above the MBC for the principal cariogenic pathogen, providing a pharmacological rationale for the dose range.

4.2. Antibiofilm activity in a polymicrobial context

The non-significant difference between SAEO 1.0% and CHX 0.12% on viable counts is clinically meaningful, because SAEO can match the principal antibiofilm endpoint of the positive control without staining or dysgeusia. These outcomes are consistent with polymicrobial clove essential-oil biofilm studies¹⁴ and extend them with the first dose-response curve for an Indonesian Maluku chemotype against a saliva-derived polymicrobial biofilm. The likely mechanism is dual-pronged eugenol activity: direct bactericidal membrane disruption combined with suppression of

the glucosyltransferases (*gtfB*, *gtfC*) involved in EPS synthesis,¹¹ reinforced by the parallel EPS reductions.

4.3. Matrix suppression and acidogenicity

Because EPS underlies the architectural and chemical resilience of cariogenic biofilms, the matrix-level effect observed here is the most likely basis for durable suppression of the community. The ecological plaque hypothesis predicts that maintaining biofilm pH above the critical demineralisation threshold of 5.5 prevents net mineral loss;² the SAEO 1.0% arm crossed this threshold whereas the SAEO 0.5% arm did not, strengthening the rationale for selecting 1.0% v/v as the candidate clinical concentration.

4.4. Selectivity for cariogenic taxa over commensals

The selective restructuring observed is consistent with reports that broad-spectrum antiseptics such as CHX disturb nitrate-reducing oral commensals with downstream cardiovascular implications.^{6,7} The phytochemical mechanism that best accounts for this differential effect is the higher selectivity of eugenol for fermentative low-GC Firmicutes than for aerobic Proteobacteria and Actinobacteria. Aerobic commensals that produce hydrogen peroxide and maintain robust catalase and superoxide-dismutase systems are comparatively spared, because their membrane composition impedes eugenol partitioning and limits oxidative damage.¹⁸ β -Caryophyllene has been reported to exert a sparing effect on commensal taxa and to engage the CB2 receptor,¹⁷ adding a further stratum of selectivity. In contrast, the cationic bisbiguanide structure of CHX binds indiscriminately to negatively charged bacterial surfaces irrespective of ecological niche, yielding the lower diversity captured in the present data. The clinical implication is that SAEO may achieve the dual goal of reducing pathogenic acidogens while preserving health-associated commensals^{2,5} — long considered the central aim of caries prevention.

4.5. Cytotoxicity and safety margin

The 17.6% LDH release under SAEO 1.0% is below the 30% threshold conventionally applied as biologically meaningful in fibroblast cytotoxicity models and is consistent with the comparatively low cytotoxicity of clove-derived eugenol reported for human gingival fibroblasts.¹⁹ This provides a margin of safety for development as a mouth rinse, particularly in paediatric and geriatric populations in whom the staining and taste limitations of CHX are most problematic.

4.6. Comparison with previous research

The present findings are consistent with and extend recent primary research. Adil and colleagues showed that sub-MIC eugenol down-regulated the *gtfB*, *gtfC* and *comDE* genes in *S. mutans* biofilms without affecting growth;¹¹ the present study observed a 67.9% EPS reduction at 1.0% v/v, consistent with transcriptional suppression of the *gtf*-family enzymes as the principal matrix-level mechanism. Jafri and colleagues demonstrated that eugenol eradicated mixed *S. mutans*–*Candida albicans* biofilms, reducing viable counts by approximately 2.5 log₁₀ and acting synergistically with antifungal drugs.¹² Somrani and colleagues reported that clove essential oil rich in eugenol (78.85%) inhibited bacterial adhesion by up to 61.8% and reduced preformed polymicrobial biofilms;¹⁴ the present saliva-derived biofilms yielded comparable matrix-level reductions, extending the claim to oral polymicrobial communities. The novel contribution here is the pairing of these antibiofilm effects with microbiome diversity metrics that directly address dysbiosis correction.

4.7. Phytochemical–mechanistic integration

The fitted IC₅₀ of 0.40% v/v corresponds to an approximate eugenol concentration of 0.33% w/v given the 82.3% eugenol content of the batch. This falls within the eugenol range reported to disrupt streptococcal membranes and quorum sensing,^{10,20} suggesting the effective dose under polymicrobial conditions is not substantially higher than under simpler models. Three mechanisms operate concurrently: membrane partitioning driven by the amphipathic phenolic scaffold, disrupting proton motive force;¹⁰ suppression of glucosyltransferase activity and *gtfB/gtfC* expression, reducing EPS synthesis at sub-lethal concentrations;¹¹ and interference with the *comDE* two-component system that governs competence and biofilm maturation, interrupting the competence-stimulating-peptide signalling that triggers the biofilm programme.^{11,20} The combined effect is a phenotypic shift from a glucan-rich, acid-trapping biofilm toward a thinner community permissive of commensal re-establishment. β -Caryophyllene contributes a sparing effect on commensal aerobes and CB2 engagement,¹⁷ while eugenyl acetate adds modest antimicrobial activity and may protect eugenol from oxidation.¹⁶

4.8. Indonesian and South-East Asian herbal context

Clove occupies a unique position in Indonesian materia medica; Maluku clove has been a principal commodity of the Asian spice trade for centuries and Indonesia

produces the majority of the global clove harvest. The Indonesian Materia Medika and the 2017 Herbal Formulary of the Ministry of Health recognise *Flos Syzygii aromatici* as an approved herbal material for dental and pharyngeal indications, subject to eugenol-content standardisation. The present findings reinforce this tradition with contemporary mechanistic evidence and support integrating standardised herbal medicines into primary health care. Comparable regulatory frameworks are emerging in Malaysia, the Philippines and Viet Nam, giving a standardised SAEO mouth rinse benchmarked against CHX 0.12% a regional translational footprint across the Association of Southeast Asian Nations (ASEAN) market.

4.9. Safety and regulatory context

Eugenol has a well-characterised absorption, distribution, metabolism and excretion (ADME) profile with rapid hepatic glucuronide and sulphate conjugation; systemic exposure after oral-cavity use is minimal given the short contact time and small volume of mouth-rinse formulations. The oral LD₅₀ of eugenol in rodents exceeds 2500 mg/kg, and zinc-oxide-eugenol materials have an acceptable century-long safety record in dentistry. The principal caveats — occasional contact mucositis and reduced fibroblast adherence on freshly prepared dentine — are mitigated by keeping eugenol below 1.5% in contact-time-limited formulations; consistent with this, the selective cytotoxicity of eugenol spares gingival fibroblasts at the concentrations used here.¹⁹ Indonesian National Agency of Drug and Food Control (BPOM) regulations classify standardised clove-based oral-care products as *jamu* or, after clinical validation, as tiered phytopharmaca; the present data provide the first pillar of such a development dossier.

4.10. Translational implications

The combined findings — bactericidal activity in the CHX range, comparable EPS suppression, superior preservation of microbiome diversity, lower cytotoxicity and an acceptable phytochemical fingerprint — support further development of standardised SAEO at 1.0% v/v as a complementary anticaries mouth rinse. A phased pathway is appropriate: formulation optimisation including nanoemulsion delivery, which has been shown to enhance the antibiofilm potency of clove oil;²¹ a controlled phase I tolerability study; and a phase IIa randomised crossover comparison with CHX 0.12% over 4–8 weeks, with combined microbiological, microbiome, salivary-biochemistry and patient-reported outcomes.

4.11. Strengths and limitations

The principal strengths are the use of a phytochemically standardised Indonesian SAEO with full GC-MS characterisation, a saliva-derived polymicrobial biofilm on hydroxyapatite that approximates the *in vivo* enamel surface more closely than monocultures, and the integration of antibiofilm endpoints with microbiome-level outcomes that directly address the dysbiosis paradigm. Three limitations should be acknowledged. First, the study is *in vitro* and does not capture host-mediated effects such as salivary clearance, gingival immune response or dietary buffering. Second, the 48-hour window is shorter than a chronic clinical regimen. Third, although Bray–Curtis comparisons were made against the untreated control, no *in vivo* microbiome data were collected from the same donors, and this remains an essential next step.

5. Conclusion

A phytochemically standardised *Syzygium aromaticum* essential oil from the Indonesian Maluku chemotype, characterised by 82.3% eugenol, exerted concentration-dependent antibiofilm activity against a saliva-derived polymicrobial cariogenic biofilm. At SAEO 1.0% v/v, the oil reduced biofilm biomass by 70.5%, lowered *S. mutans* viable counts by 3.22 log₁₀ and EPS by 67.9%, raised post-sucrose biofilm pH above the critical demineralisation threshold, and matched CHX 0.12% across the principal antibiofilm endpoints. Critically, SAEO outperformed CHX 0.12% in restoring microbiome diversity, with Shannon diversity rising from 1.42 to 2.08 bits and health-associated *Neisseria* and *Corynebacterium* increasing approximately seven-fold, while cytotoxicity on HGF-1 gingival fibroblasts was approximately half that of CHX 0.12%. Standardised SAEO is therefore a credible candidate for development as a complementary anticaries mouth rinse in Indonesian and South-East Asian populations, and a randomised controlled clinical trial comparing SAEO 1.0% v/v with CHX 0.12% over 4–8 weeks is recommended.

Declarations

Use of Generative Artificial Intelligence. The authors declare that no generative artificial intelligence tool was used in the conception, design, conduct, analysis or interpretation of this study, nor to generate any data, figure or reference. All references were retrieved and verified manually against their primary sources, and the authors accept full responsibility for the content of the manuscript.

Author Contributions. S.S. conceived and designed the study, supervised the microbiological and phytochemical experiments, performed the statistical analysis, and drafted the manuscript. A.A.R. contributed to the study design, donor recruitment and the interpretation of the microbiome data, and critically revised the manuscript. J.W.P. supervised the laboratory assays, validated the GC-MS and cytotoxicity procedures, and revised the manuscript. All authors have reviewed and approved the final version of the manuscript.

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Conflict of Interest. The authors declare that they have no competing interests, financial or non-financial, in relation to this article.

Ethical Approval and Consent to Participate. The study protocol was reviewed and approved by the CMHC Research Center Ethics Committee (Approval No. 2024/168) and conformed to the Declaration of Helsinki for studies using human-derived biological materials. All saliva donors provided written informed consent before sample collection.

Consent for Publication. Not applicable.

Data Availability. The datasets supporting the findings of this study are available from the corresponding author on reasonable request.

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