



Disruption and bactericidal indices depicted in polygonal graphs to show multiple outcome effects of root canal irrigant supplements on single- and dual-species biofilms

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Received: 17 June 2019 / Accepted: 7 January 2020
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Abstract

Objectives The aims of this study were to (1) investigate the relative time-dependent disruption and bactericidal effects of detergent-type surfactants on single- or dual-species biofilms of root canal isolates and (2) to examine the utility of polygonal graphs for depiction of biofilm disruption and cell killing.

Materials and methods Single-species biofilms of *Streptococcus sanguinis*, *Enterococcus faecalis*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* were grown on nitro-cellulose membranes for 72 h and immersed in Tween®80, cetyltrimethylammoniumbromide (CTAB), and sodium dodecyl sulphate (SDS) for 1-, 5- or 10-min ($n = 3$ per test). The number of viable and non-viable bacteria “disrupted” from the biofilm and those “remaining-attached” was determined using a viability stain in conjunction with fluorescence microscopy. The data were analysed using non-parametric Kruskal-Wallis test with 5% significance level.

Results Gram-negative obligate anaerobes were more susceptible to cell removal than gram-positive facultative anaerobes. The majority of cells were disrupted after 1-min of exposure; however, the extent varied according to the agent and species. CTAB and SDS were more effective than Tween 80™ at disrupting biofilms and killing cells but all agents failed to achieve 100% disruption/kill.

Conclusions Biofilm disruption and cell viability were influenced by the species, the test agent and the duration of exposure. CTAB and SDS were more effective in biofilm disruption than Tween 80™. Graphical depiction of biofilm disruption- and viability-outcomes provides an alternative means of simultaneously visualising and analysing relative efficacy in different domains.

Clinical relevance Surfactants were not as effective at biofilm disruption as NaOCl but may be added to other non-disruptive antibacterial agents to enhance this property.

Keywords Disruption · Bacterial killing · Irrigants · Root canal · Biofilm · Depiction of outcome measures

Introduction

Root canal treatment (RCT) fails to predictably eradicate bacteria in the apical anatomy even after using potent antibacterial irrigants [1, 2]. The encasement of bacteria within the root canal biofilm has been suggested as an explanation for this failure in therapy [3], emphasising the importance of biofilm disruption to facilitate subsequent and further anti-microbial action. Antibacterial agents’ actions on biofilms may yield diverse outcomes that include killing, either with- or without disruption, in a time-dependent and dose-dependent manner [4]. Investigation of the disruptive and bactericidal effects of common RCT irrigants on single- and dual-species biofilms [4] found NaOCl to be the most effective agent at both

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bacterial killing and biofilm disruption. CHX and iodine were effective at bacterial killing but not disruption, whilst EDTA showed biofilm disruption activity but exhibited lesser antimicrobial potential [4].

In other arenas of biofilm combat, combined chemical approaches have been proposed, including, dislodgement of cells from surfaces by dispersants and weakening of the biofilm structure by enzymes [5] or chelants of divalent cations [6]. Surfactants can act as dispersants and are classified into non-ionic, cationic and anionic. Polysorbate 80 (Tween 80™) is a non-ionic surfactant that is added to commercially available irrigants (MTAD [3% Doxycycline, 4.25% citric acid, 0.5% Tween 80™] [Dentsply Tulsa dental, Tulsa, OK]). Interestingly, the antibacterial effect of MTAD against *E. faecalis* biofilm was not affected by the elimination of Tween 80™ [7].

Cetyltrimethylammoniumbromide (CTAB), a cationic surfactant, is an additive in SmearClear® SybronEndo, Orange, CA, USA), Tetraclean® (1% doxycycline, 10% citric acid, 0.2% cetrimide; Ognalaboratori Farmaceutici), and QMiX® (Dentsply Tulsa Dental, Tulsa, OK, USA) and has been tested in experimental irrigants (MTAC 1 and MTAC-2) [7]. CTAB may affect the biofilm by forming electrostatic bonds that weaken the extracellular matrix (ECM) [8] and has been tested against *E. faecalis* or polymicrobial root canal biofilms as a single agent [9–11] as well as in combination with other agents (EDTA, maleic and citric acids, iodine potassium iodide, CHX) with promising results [10].

Sodium dodecyl sulphate (SDS), an anionic alkyl sulphate surfactant, may act by disrupting the hydrophobic interactions involved in cross-linking ECM [12] and exhibits a cell killing effect on salivary biofilms, maintained on hydroxyapatite discs [13].

Although multiple concurrent therapy approaches may eradicate biofilm more effectively [14], the selection of appropriate adjunct agents to traditional irrigants requires an easier approach to assimilate and compare anti-biofilm information on relative biofilm killing and disruption efficacies. The killing effects of both cationic (including CTAB) [7, 9, 15] and anionic surfactants (SDS) [16] have been investigated but the disruption/dispersion function has not been simultaneously investigated.

The present study investigated the relative time-dependent disruption and bactericidal effects of detergent-type surfactants on single- and dual-species biofilms of root canal isolates and explored the utility of the disruption/killing index to depict the outcomes simultaneously, as previously proposed [4].

Materials and methods

The bacterial isolates, from infected root canal systems, used in the study were *Streptococcus sanguinis* (*Ss*) E01-07-001,

Enterococcus faecalis (*Ef*) E01-07-048, *Porphyromonas gingivalis* (*Pg*) E01-07-035 and *Fusobacterium nucleatum* (*Fn*) E01-07-13. The isolates had been identified using partial 16S rRNA gene homology supplemented by biochemical analysis (API Strep32 system, Biomerieux, Basingstoke, UK), with purity assessed by Gram staining and microscopic examination.

The test solutions selected were Tween 80™ (Sigma®, Sigma Aldrich, St Louis, MO, USA), CTAB (10 mg/mL CTAB, Sigma®) and SDS (0.05 mg/100 mL SDS, Sigma®). Reduced transport fluid (RTF) [17] was used as a negative control. NaOCl (1%), iodometrically titrated (5% Teepol Bleach®, Teepol Products, Egham, UK) was used as a positive control.

Single-species biofilms were generated on nitro-cellulose membranes following a previously described protocol [18]. Briefly, 20 µL aliquots of a standardized culture (absorbance of 0.4 at 540 nm for all species) were inoculated onto sterile membrane filters (0.2 µm pore size, 5 mm diameter; Whatman International Ltd., Maidstone, UK) and placed on Blood Agar plates (BA; Oxoid, Basingstoke, UK). *S. sanguinis* and *E. faecalis* were incubated at 37 °C in air supplemented with 5% CO₂ for 48 h, while *P. gingivalis* and *F. nucleatum* were incubated in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, UK) at 37 °C for 72 h. Dual-species biofilms were generated using two of the test species (*S. sanguinis* and *F. nucleatum*). The ratio of the organisms was 1:2 (absorbance of 0.2 and 0.4 at 540 nm) for the *S. sanguinis* and *F. nucleatum*, respectively, and these were incubated anaerobically at 37 °C for 72 h.

Single- ($n = 15$ per agent) and dual-species ($n = 15$ per agent) biofilms were cultured and exposed to 1 mL of either the test agent or the control (RTF) within a validated disruptive assay protocol [19] at room temperature under aerobic conditions for 1-, 5- or 10-min, as previously described [4]. After the designated contact time, each membrane filter was removed and placed into 1 mL of neutralizing broth (NB; BD Difco, Oxford, UK). To determine the number of cells “disrupted” from the biofilm, the filter was vortexed at 200 rpm for 20 s to re-suspend the cells sufficiently loosened by the effect of the test agent from the biofilm. Each membrane filter was then transferred to a sterile 3-mL bottle containing 1 mL of RTF and vortexed at full speed for 1 min to remove the greater majority of bacteria “remaining-attached” to the membrane.

Five-point cell counts (live and dead cells) were conducted [20] using a microscope with a fluorescent light source (Olympus, Watford, UK) and viability stain (LIVE/DEAD® BacLight™, Invitrogen, Paisley, UK) to produce mean cell counts of both the “disrupted” and “remaining attached” cells. The control specimens ($n = 3$ per species) were only briefly exposed to RTF to enumerate base-line cell counts.

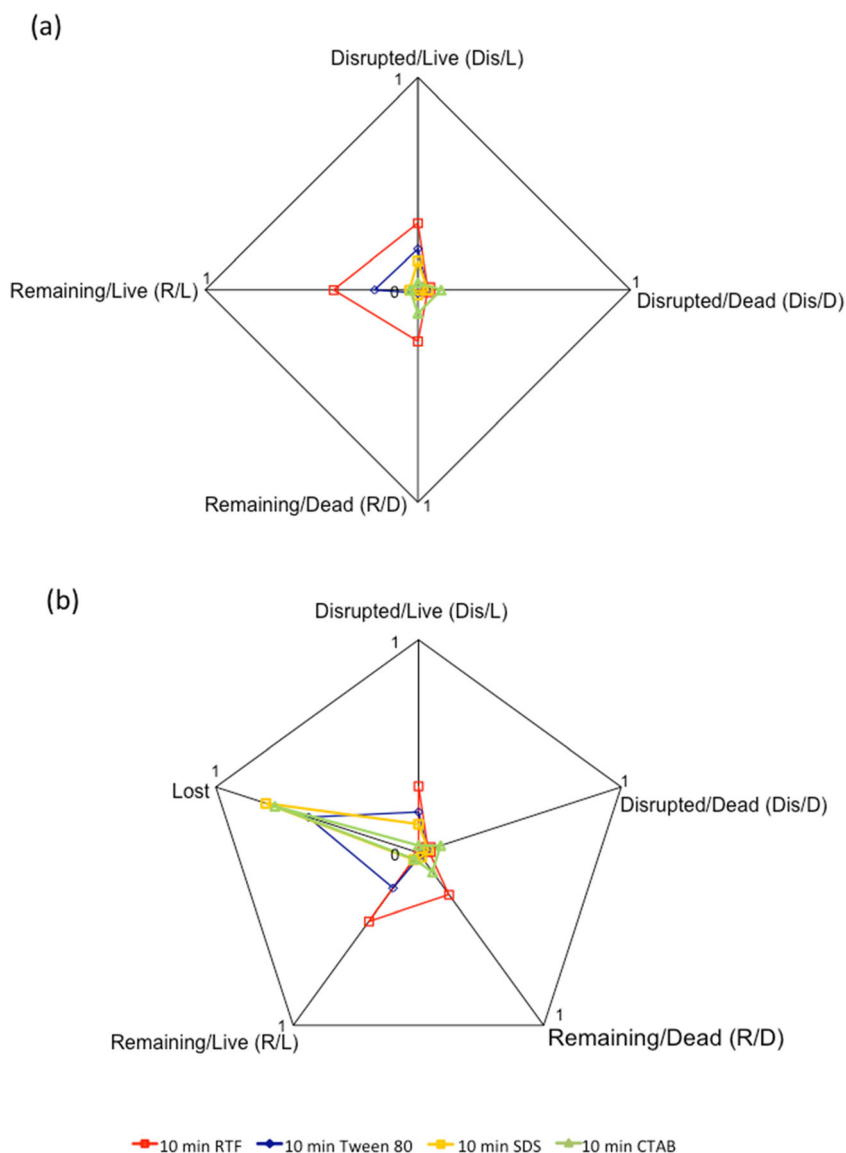
Each experiment was carried out in triplicate (3 technical and 5 biological repeats yielding 15 data sets per test agent/species), and the number of remaining-attached cells (live or dead) harvested from the single- or dual-species biofilms exposed to various test agents was compared with those exposed to RTF using the non-parametric Kruskal-Wallis test (STATA version 9.2, STATA Corporation, College Station, TX, USA). The significance level was set at 5%.

Four-axes polygraphs were used to present the proportions of “disrupted” and “remaining-attached” live/dead cells (Fig. 1a). A measure of cells lost by complete disintegration in the test solutions is inferred from the area under the plot (the smaller the area, the greater the disintegration, as represented by the data for SDS and CTAB), since this is not a real number. In an alternative approach to account numerically for cells lost by disintegration in the test/control agent, a five-axes disruption/killing polygraph (Fig. 1b) was derived. In this

approach, the baseline count was used as the denominator to calculate the proportion of live/dead cells amongst the disrupted and remaining-attached cells harvested; the fifth axis/point was annotated “lost cells”, the quantity of which was estimated as the difference between the baseline count and the total number of “disrupted” and “remaining-attached” cells harvested. Figure 1b shows a large proportion of “lost cells” after exposure to SDS or CTAB, which is consistent with the small plot area displayed in Fig. 1a.

In addition to the quantitative analyses, qualitative Confocal laser scanning microscopy (CLSM) was also carried out using a Bio-Rad MRC 2000® (Hemel Hempstead, UK). Briefly, membrane filters containing biofilms were attached to the base of a 5-cm sterilin dish using a small drop of glue (Loctite®, Winsford, Cheshire, UK). The filters were then covered with 5 mL of BacLight™ viability stain and left to incubate for 10 min and viewed with a water immersion

Fig. 1 Disruption index polygraphs with **a** four- or **b** five-axes



objective (Olympus® × 40). These images were then constructed and manipulated using a software programme (Image J®, <http://rsb.info.nih.gov/ij/>).

Results

Single-species biofilms

The mean number (and standard deviations) of “remaining-attached live” (R/L)-, “remaining attached dead” (R/D)-, “disrupted live” (Dis/L)- or “disrupted dead” (Dis/D)- cells per slide view (5 fields) from single species biofilms ($n = 3$) at baseline and following exposure to test- or control-agents are presented in Table 1. The base-line data demonstrated the validity of the definition of “disrupted” versus “remaining-attached”, in that without immersion for the designated time in a test or control agent, none of the species showed removal of either live or dead cells. Even at baseline, there were relatively high proportions of R/D cells in the *Fn* and *Pg* groups.

All agents, including RTF, disrupted the single-species biofilms. After RTF exposure, the “disrupted” and “remaining-attached” *Ss* cells were all live, whilst *Ef*, *Fn* and *Pg* biofilms contained a population of both live (larger fraction) and dead (smaller fraction) cells. There were also reduced numbers of “remaining-attached” cells recovered from *Pg* biofilms after 5- or 10-min exposure to RTF, when compared with 1-min exposure or the baseline. After NaOCl exposure, minimal dead cells (disrupted and “remaining-attached”) could be harvested, after 5- or 10-min exposure.

Table 2 shows that the number of R/L and R/D cells harvested from the single-species biofilms exposed to test agents were lower than the number of cells harvested after exposure to RTF, with exceptions of *Ss* biofilms exposed to Tween 80™ and *Pg* biofilms exposed to CTAB.

There was no significant difference in *Ss* biofilm disruption by the different test agents compared with RTF, regardless of duration of exposure (Table 2). However, after 10-min exposure to Tween 80™ or CTAB, a large proportion of “remaining-attached” cells harvested were dead (Table 1).

Exposure of *Ef* biofilms to the test agents for 1- or 5-min (except for 1-min exposure to Tween 80™) resulted in fewer “remaining-attached” cells harvested when compared with RTF (Table 1). Exposure of *Ef* biofilms to Tween 80™ or CTAB for 10 min resulted in a small fraction of recovered R/D cells (Table 1). Tween 80™, over time, resulted in increased “lost” *Ef* cells but stable proportions of R/L cells. A large proportion of cells were “lost” after exposure to SDS or CTAB with the former being slightly more effective in disruption and killing.

There were no R/L or D/L cells recovered after 10-min exposure (Table 1) from the *Fn* and *Pg* biofilms. After 5- or 10-min exposures of *Fn* biofilms to various agents, the

number of “remaining-attached” cells was significantly ($p < 0.01$) less than with RTF (Table 2), although this was not replicated within the *Pg* group.

Dual-species biofilm

In the dual-species tests (Table 3), substantially more *Ss* cells were recovered compared to *Fn* cells. The majority of *Ss* cells remained attached to the membrane, whereas smaller proportions of *Fn* cells remained attached after exposure to RTF when compared with the baseline count (Table 3). In general, SDS was the most effective agent at disrupting both *Ss* and *Fn* contents of the dual species biofilm; it was followed by CTAB and then Tween 80™. The number of “remaining-attached” *Ss* or *Fn* cells exposed to SDS was significantly ($p < 0.05$) less than those exposed to RTF (Table 3). After exposure to CTAB, the *Ss* “remaining-attached” cells were significantly ($p = 0.02$) less than those exposed to RTF but this was not observed amongst the *Fn* cells.

A graphical approach to improve visualisation of the complex data depicts the data sets in two polygonal graph formats (4-axes and 5-axes) to compare the outcomes for the test/control agents (Fig. 2).

Discussion

The three selected test agents represented each of the three types of surfactants and had reported biofilm disruptive capability [4, 11, 21]. RTF was employed as a negative control as it was expected to exert a minimal effect on the biofilm because of its transport medium role. The control confirmed that the disruption protocol incurred cell death and was therefore used to gauge the relative rate of cell death across the test agents. Positive control data using NaOCl and test model parameters were published previously [4]; the model allowed evaluation of the relative bactericidal and biofilm disruption effects of the test agents.

Root canal irrigants have been tested in vitro using a variety of biofilm models [7, 22–24], with perceived advantages and disadvantages attributed to each. Generation of biofilms on dentine discs, or in root canals, may have greater proximity to the clinical scenario but carry other confounding risks, including variable dentinal tubule structure, root canal geometry, buffering effect, residual pulp- or organic tissue, inclusion of stagnation planes and vapour lock effects [25, 26]. The well-established nitrocellulose disc model was therefore chosen as the substrate for the present study to exclude such confounding effects. Multi-species biofilms grown for at least 3 weeks show increased maturity and survival, when exposed to anti-microbial agents, compared to those grown with more limited durations [27]. The mono- and dual-species biofilms

Table 1 Mean values and standard deviations of live or dead cells per view ($n = 5$) of disrupted (Dis) and remaining-attached (R) cells harvested from single-species biofilms ($n = 3$) at baseline and following exposure to test agents for various durations.

Agent	1 min												5 min												10 min											
	Ss				Ef				Fn				Pg				Ss				Ef				Fn				Pg							
	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R	Dis	R		
*Baseline Live	Mean	0	428.7	0	898.5	0	48.0	0	343.3	0	428.7	0	898.5	0	48.0	0	343.3	0	428.7	0	898.5	0	48.0	0	343.3	0	428.7	0	898.5	0	48.0	0	343.3			
SD		0	376.5	0	493.8	0	292.4	0	545.2	0	376.5	0	493.8	0	292.4	0	545.2	0	376.5	0	493.8	0	292.4	0	545.2	0	376.5	0	493.8	0	292.4	0	545.2			
Dead	Mean	0	15.0	0	66.5	0	141.5	0	708.9	0	15.0	0	66.5	0	141.5	0	708.9	0	15.0	0	66.5	0	141.5	0	708.9	0	15.0	0	66.5	0	141.5	0	708.9			
SD		0	31.7	0	61.0	0	140.7	0	510.3	0	31.7	0	61.0	0	140.7	0	510.3	0	31.7	0	61.0	0	140.7	0	510.3	0	31.7	0	61.0	0	140.7	0	510.3			
NaOCl Live	Mean	19.3	18.0	4.0	0	6.0	4.0	4.0	0.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
SD		19.7	4.0	3.5	0	7.2	5.3	6.9	1.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
Dead	Mean	68.0	28.7	201.3	31.3	42.0	81.3	42.7	94.7	40.7	21.3	4.7	16.7	6.7	44.7	48.0	90.0	6.7	9.3	0.7	10.0	9.3	36.0	12.7	26.7											
SD		45.4	11.5	83.5	9.5	12.2	31.9	21.4	31.0	16.0	3.1	4.2	5.0	8.1	15.5	18.0	50.0	7.0	5.8	1.2	5.3	4.6	26.2	5.0	7.6											
RTF Live	Mean	65.3	227.3	352.7	446.7	32.0	141.3	3.3	43.3	76.0	232.7	331.3	453.3	15.3	100.7	0.7	2.7	54.7	155.3	240.0	453.3	56.7	104.7	2.0	0.6											
SD		15.5	45.7	226.4	230.1	24.6	123.7	4.2	38.8	36.2	96.7	118.9	83.3	21.6	59.0	1.2	4.6	24.1	78.6	87.2	458.8	88.0	56.9	3.5	7.0											
Dead	Mean	0	0	53.3	273.3	14.0	160.0	51.3	113.3	0	0	126.7	206.7	38.0	305.3	56.0	47.0	0	0	146.7	113.3	52.8	310.7	54.0	61.3											
SD		0	0	20.1	80.8	7.2	63.9	8.3	26.6	0	0	130.1	64.3	24.2	88.1	31.0	24.2	0	0	102.6	23.1	42.7	48.4	19.7	26.9											
Tween 80 Live	Mean	28.0	81.3	216.7	229.3	0	2.7	0	52.0	8.0	15.3	18.7	238.0	0	0.7	67.3	2.0	9.3	28.0	0.1	228.7	0	0	0	0											
SD		17.8	27.2	101.0	100.6	0	1.2	0	76.2	6.9	6.1	22.0	161.3	0	1.2	27.2	3.5	11.0	10.6	0.2	41.7	0	0	0	0											
Dead	Mean	31.3	222.0	50.7	17.3	2.7	34.7	61.3	66.7	123.3	183.3	21.3	22.7	2.0	30.0	67.0	20.7	145.3	176.7	2.7	40.0	2.7	32.7	70.0	38.0											
SD		13.3	189.2	25.5	10.1	1.2	11.0	15.5	31.9	48.1	99.5	19.6	11.0	0	6.9	27.2	27.2	66.0	102.5	3.1	6.9	3.1	14.0	41.0	14.0											
SDS Live	Mean	23.3	79.3	152.0	44.7	4.0	17.3	0	4.0	30.7	48.0	104.7	29.3	0	4.7	0	0	19.3	14.0	25.3	28.7	0	0.6	0	0											
SD		9.9	37.9	36.5	23.0	2.0	4.6	0	2.0	8.1	33.0	96.8	4.6	0	3.1	0	0	11.0	5.3	12.1	27.2	0	1.2	0	0											
Dead	Mean	29.3	44.0	53.3	27.3	20.7	42.7	50.7	33.3	40.7	72.7	12.7	18.7	19.3	42.0	41.3	8.0	24.7	43.3	14.7	11.3	7.3	56.7	54.0	31.3											
SD		12.1	4.0	20.1	8.1	28.9	8.1	3.1	5.0	34.1	52.1	13.0	7.0	11.7	13.1	6.1	7.2	17.5	26.9	10.3	2.3	3.1	30.6	14.4	13.6											
CTAB Live	Mean	44.7	30.0	38.7	40.7	0	0.7	0	16.7	51.3	62.7	7.3	6.0	0	0	0	0.6	24.0	14.0	1.3	0	0	0	0	0											
SD		49.8	12.2	19.7	37.5	0	1.2	0	16.0	25.2	81.1	6.4	7.2	0	0	0	1.2	15.9	8.7	2.3	0	0	0	0	0											
Dead	Mean	42.7	44.0	122.7	124.7	0.7	37.3	54.0	66.7	22.0	54.0	154.7	108.0	0	6.7	60.0	55.3	89.3	100.0	163.3	99.3	0.7	0	78.0	79.3											
SD		16.8	22.3	68.2	12.2	1.2	59.2	12.5	31.9	21.2	29.6	78.3	61.0	0	8.1	38.1	22.0	63.9	115.7	25.2	8.1	1.2	0	14.0	60.5											

*Not exposed by indicated timings

Ss *S. sanguinis*, Ef *E. faecalis*, Fn *F. gingivalis*, RTF Reduced transport fluid, SDS sodium dodecyl sulphate, CTAB cetyl trimethyl ammonium bromide

Table 2 Comparisons of “remaining-attached” cells of *S. sanguinis*, *E. faecalis*, *F. nucleatum* and *P. gingivalis* after exposure to test agents or RTF for various durations

	1 min	5 min	10 min
<i>S. sanguinis</i>			
*RTF	227.3 ± 26.4	232.7 ± 55.8	155.3 ± 45.4
RTF-Tween 80	-76.0 ± 116.7	34.0 ± 82.6	-49.3 ± 75.6
RTF-SDS	104.0 ± 35.7	112.0 ± 61.0	98.0 ± 47.3
RTF-CTAB	153.3 ± 32.3	116.0 ± 78.4	41.3 ± 79.9
<i>E. faecalis</i>			
*RTF	720.0 ± 179.3	660.0 ± 20.0	566.7 ± 257.3
RTF-Tween 80	473.3 ± 186.8	399.3 ± 95.5**	298.0 ± 258.8
RTF-SDS	648.0 ± 180.2**	612.0 ± 20.1**	526.7 ± 257.7
RTF-CTAB	554.7 ± 181.5**	546.0 ± 41.2**	467.3 ± 257.3
<i>F. nucleatum</i>			
*RTF	301.3 ± 99.7	406.0 ± 83.1	415.3 ± 40.7
RTF-Tween 80	264.0 ± 99.9	375.3 ± 83.2**	382.7 ± 41.5**
RTF-SDS	241.3 ± 99.9	359.3 ± 83.3**	358.0 ± 44.2**
RTF-CTAB	263.3 ± 105.6	399.3 ± 83.2**	415.3 ± 40.7**
<i>P. gingivalis</i>			
*RTF	156.7 ± 37.7	50.0 ± 15.0	68.0 ± 19.4
RTF-Tween 80	38.0 ± 68.1	27.3 ± 23.2	30.0 ± 21.0
RTF-SDS	119.3 ± 37.9	42.0 ± 15.6	36.7 ± 21.0
RTF-CTAB	73.3 ± 39.8	-6.0 ± 19.9	-11.3 ± 40.0

*The mean (±standard error) of attached cells recovered following exposure to RTF

**The difference between RTF and test agent was significant at the 1% level

were tested at 48 and 72 h in the present study and so may yield comparatively different cellular-killing rates.

Although the biofilms were grown anaerobically, the disruption assays could practically only be performed under normal atmospheric conditions and may have negatively affected the biofilms, explaining the substantial killing and disruption efficacy of the RTF on *Pg*. However, clinical treatment also incurs aerobiosis and so arguably the test provides realism in that sense [22].

The Gram-negative anaerobic biofilms were more susceptible to the test surfactants than their Gram-positive facultative counterparts, an observation consistent with previous disruption assays [4] and in vivo findings [28]. The similarity between the disruption and killing of *Ss* and *Fn* grown in single-versus dual-species biofilms was in keeping with a study that found no difference in CHX efficacy against *Ss* grown as single-species biofilms and within plaque microcosms [29]. In contrast, other species became more resistant to hydrogen peroxide and tetracycline when grown within multispecies biofilms [30], with the inference that enhanced resistance resulted from increased biofilm depth and, as yet to be substantiated factors such as, quorum sensing [30].

Table 3 Mean values and standard deviations of live or dead cells per view ($n = 5$) of disrupted (Dis) or “remaining-attached” (R) cells harvested from dual species biofilms ($n = 3$) at baseline and following exposure to test agents for 1 min. *Ss* *S. sanguinis*, *Fn* *F. nucleatum*

Agent		Ss		Fn		
		Dis	R	Dis	R	
*Baseline	Live	Mean	0	784.7	0	360.0
		SD	0	830.6	0	163.9
	Dead	Mean	0	200.0	0	60.0
		SD	0	185.2	0	63.2
NaOCl	Live	Mean	3.3	9.3	0.7	0
		SD	7.2	16.7	2.6	0
	Dead	Mean	70.7	28.7	14.0	0
		SD	111.0	26.4	14.5	16.8
RTF	Live	Mean	54.0	630.7	0	8.7
		SD	120.1	625.5	0	20.0
	Dead	Mean	29.3	37.3	36.0	12.0
		SD	30.1	35.8	59.6	18.2
Tween 80	Live	Mean	6.0	118.0	0	4.0
		SD	18.4	142.8	0	7.4
	Dead	Mean	39.3	187.3	2.7	9.3
		SD	52.6	239.9	8.0	12.8
				** $p = 0.1$		** $p = 0.8$
SDS	Live	Mean	34.7	43.3	0	0
		SD	55.1	72.8	0	0
	Dead	Mean	21.3	46.0	0	0
		SD	24.5	54.0	0	0
				** $p = 0.001$		** $p = 0.003$
CTAB	Live	Mean	30.7	98.7	0	2.0
		SD	40.3	268.5	0	4.1
	Dead	Mean	242.7	218.7	24.0	26.0
		SD	725.5	434.2	63.8	63.8
				** $p = 0.02$		** $p = 0.7$

** p values for Kruskal-Wallis test comparing the total number of remaining-attached (live or dead) cells exposed to the test agent against those exposed to RTF

CTAB disruptive efficacy against *Ef* biofilm was consistent with other studies [9]. SDS showed similar disruptive capabilities against all tested biofilms and was not less effective at eliminating Gram-negative species than its cationic counterparts, as previously reported [31].

The inherent positive or negative charges in CTAB and SDS may explain their more pronounced biofilm disruption and killing properties than the non-ionic surfactant, Tween 80™. CTAB is rapidly adsorbed to bacterial cell surfaces due to its positive charge, causing cell membrane disorganisation and potentially rendering the bacteria more negatively charged and hydrophobic [32].

The proposed 4-axes disruption polygraph [4] was modified to depict and facilitate comparison of the relative killing

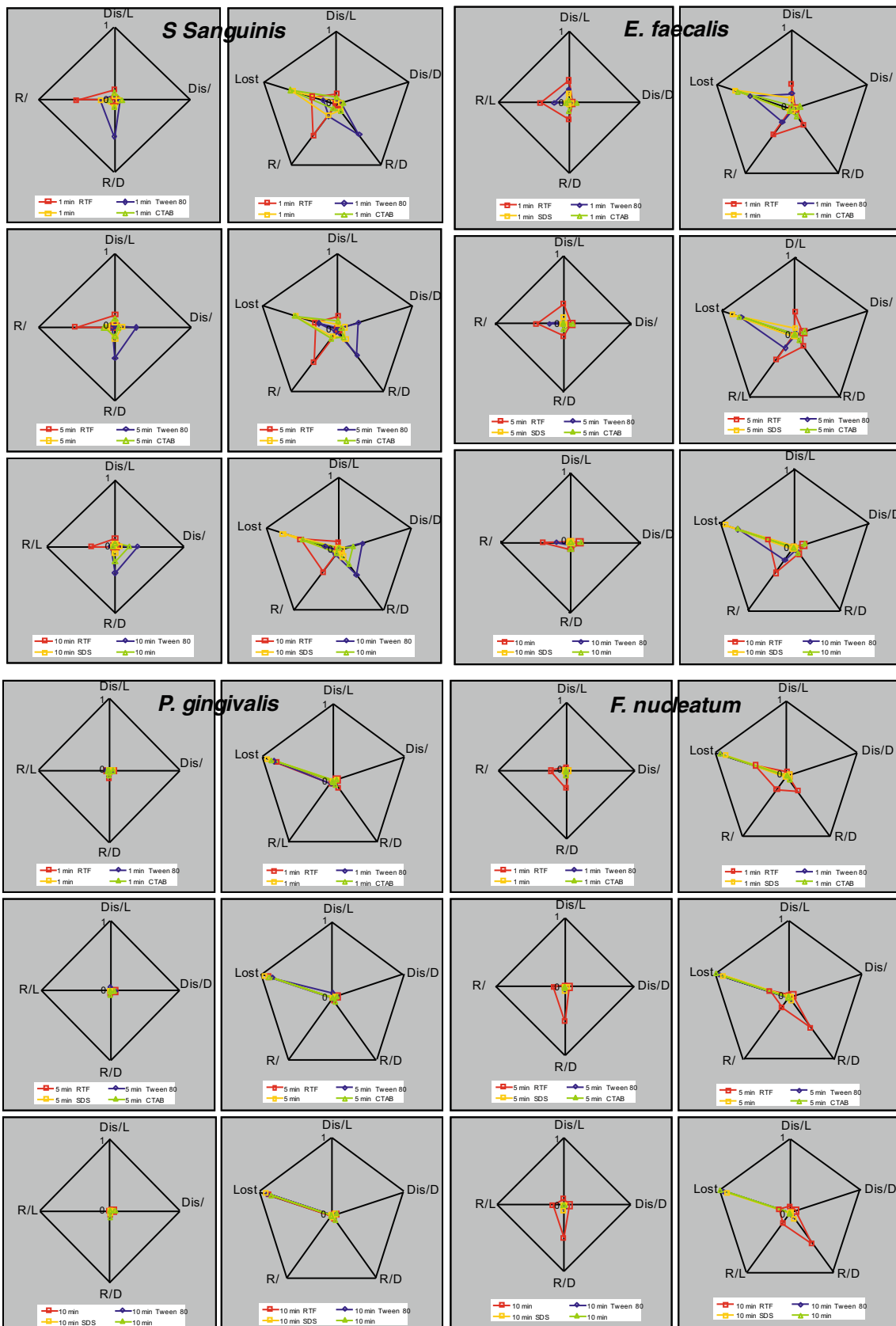
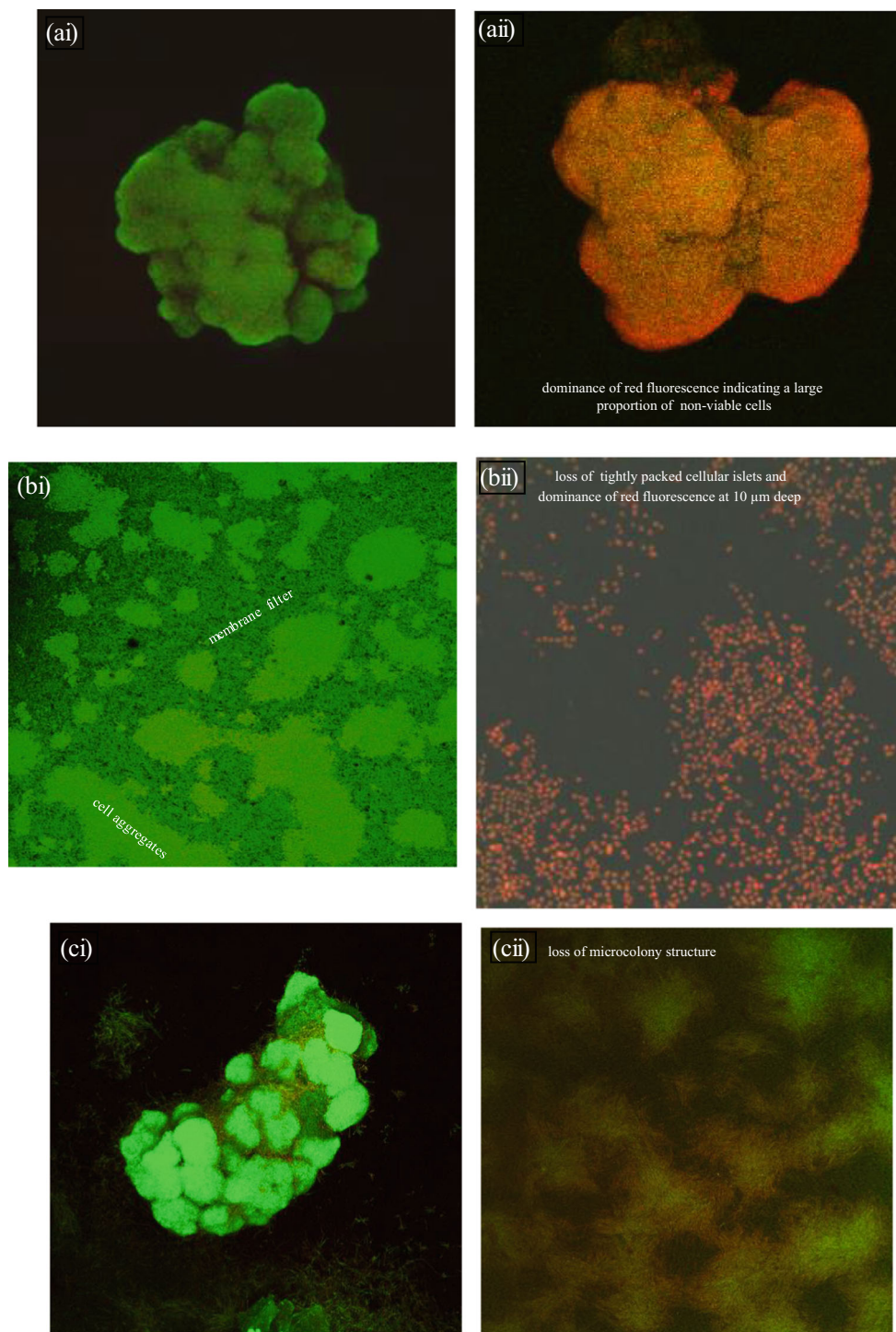


Fig. 2 Four-axes (left columns) and five-axes (right columns) disruption/bactericidal indices depicting the relative proportion of biofilm disruption and bacterial killing by test agents and test species. (Dis, disrupted; R, remaining; L, live; D, dead)

Fig. 3 Confocal micrographs of **a** *S. sanguinis* ($300 \times 300 \mu\text{m}$), **b** *E. faecalis* ($400 \times 100 \mu\text{m}$) and **c** *S. sanguinis* and *F. nucleatum* dual species ($400 \times 100 \mu\text{m}$). (i) control biofilm and (ii) after 1-min immersion in CTAB



and disruption effects of the test agents against the single-species biofilms (Fig. 2). A shortcoming of the 4-axes polygraph is that it does not provide a direct measure of the disintegrated cells but depicts inferred “loss” of cells by virtue of fewer quantifiable cells giving a smaller area under the plot.

In the alternative approach, the disintegrated biofilm component is estimated from the discrepancy between number of

cells at baseline compared with those recovered after test agent exposure, presented as the fifth axis (Fig. 2).

Confocal laser scanning microscopy (CLSM; Bio-Rad MRC 2000®, Hemel Hempstead, UK) was used to characterise the disruptive effect of CTAB, using a previously reported method [4]. Although the structural integrity of the *Ss* biofilm appeared relatively intact, the dominating red fluorescence

indicated a large proportion of dead cells. In contrast, the *Ef* biofilm showed only a single layer of dead cells. The absence of cellular structures in the *Fn* and *Pg* biofilms (Fig. 3) was in keeping with the disruption assay results.

Synergism or potentiation of antimicrobial effect by combining agents is a valid tactic [9, 10, 14] and the use of an ionic surfactant, such as SDS or CTAB, either as a mixture or an additional step when using non-disruptive conventional irrigants and may be considered in future research and clinical practice. The multiple outcome measure depiction in polygraphs offers a simple, visual comparison to assess antagonism, synergism or potentiation.

Conclusions

The multiple outcome measures of antimicrobial effect on biofilms, depicted in the 4- or 5-axes polygonal graphs, facilitate an alternative means of visualizing complex data for comparison of selected variables. Biofilm disruption and cell viability were influenced by species, test agents and duration of exposure. SDS and CTAB generally showed good cell disruption and killing properties but failed to achieve 100% effect.

Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

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