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## Antimicrobial Efficacy of an Innovative Emulsion of Medium Chain Triglycerides against Canine and Feline Periodontopathogens

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1 **Antimicrobial Efficacy of an Innovative Emulsion of Medium Chain Triglycerides against**  
2 **Canine and Feline Periodontopathogens**

3

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12

13 **Conflict of interest statement**

14 *G. Lavery was supported by an InterTradeIreland Fusion grant supporting collaborative research*  
15 *between Ward Research & Development Ltd and Queen's University Belfast; L. Coyle is an*  
16 *employee of Ward Research & Development Ltd and R. Breathnach, B. Gilmore and D.Jones have*  
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18

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22 *aspects of this manuscript.*

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29 **Structured Summary**

30 *Objectives*

31 To test the *in vitro* antimicrobial efficacy of a non-toxic emulsion of free fatty acids against clinically  
32 relevant canine and feline periodontopathogens for the prevention of periodontitis and gingivitis in  
33 cats and dogs.

34 *Methods*

35 Antimicrobial kill kinetics were established utilising an alamarBlue® viability assay against ten strains  
36 of canine and feline periodontopathogens, in the biofilm mode of growth, at a concentration of 0.125%  
37 v/v medium chain triglyceride (ML:8) emulsion. The results were compared with 0.12% v/v  
38 chlorhexidine digluconate and a commercially available xylitol-containing dental formulation (Virbac  
39 Vet Aquadent®). Mammalian cellular cytotoxicity was also investigated for both the ML:8 emulsion  
40 and chlorhexidine digluconate (0.25 to 0.0625% v/v) using *in vitro* tissue culture techniques.

41 *Results*

42 No statistically significant difference was observed in the antimicrobial activity of 0.125% v/v ML:8  
43 emulsion and 0.12% v/v chlorhexidine digluconate against all ten periodontopathogens tested; a high  
44 percentage kill rate (> 70%) was achieved within 5 minutes of exposure and at subsequent time points  
45 investigated. A statistically significant improvement in antibiofilm activity was seen with 0.125% v/v  
46 ML:8 emulsion when compared with a currently available xylitol containing drinking water additive  
47 (Virbac Vet Aquadent®). The ML:8 emulsion possessed a significantly lower (P<0.001) toxicity  
48 profile when compared to 0.12% v/v chlorhexidine digluconate in cytotoxicity assays.

49 *Clinical Significance*

50 The ML:8 emulsion exhibited significant potential as a putative effective antimicrobial alternative to  
51 chlorhexidine- and xylitol- based products for the prevention of periodontal disease, which, when  
52 compared to chlorhexidine at equivalent concentration, exhibited significantly reduced cytotoxic  
53 characteristics.

54

55 *Keywords:* Periodontitis, antimicrobial, medium chain triglycerides, biofilm bacteria, fatty acids.

56

57 **Introduction**

58 Periodontitis is the most common described progressive inflammatory disease in companion animal  
59 practice, affecting more than 80-85% of dogs and cats above three years of age (Watson 2006).

60 Within the oral cavity, the condition refers to inflammation of the tooth support structures leading to  
61 damage and loss of the periodontal membrane, alveolar bone and adjacent soft tissues; the resulting  
62 damage may potentially result in tooth loss. The severity and prognosis of dental disease is dependent  
63 on multiple factors including species, age, breed, genetics, nutritional status, the presence of irritants,  
64 chewing activity, co-morbidities, dental crowding, occlusion and oral microbial profile (Harvey and  
65 Emily 1993). The prospect for systemic and chronic diseases to develop subsequent to periodontal  
66 disease is high due to the dense vascular network of the gum tissue (DeBowes *et al.* 1996).

67

68 The tooth and its supporting structures provide an optimum environment for the growth and  
69 replication of transient microorganisms within the mouth (Wiggs and Lobprise 1997). Food particles  
70 collect between the teeth to provide a nutrient source for the development of a bacterial biofilm  
71 community (plaque) at the tooth's surface. Bacteria, growing as biofilms, are notoriously difficult to  
72 eradicate, often requiring bactericidal concentrations of 10-1000 times that of free-floating, planktonic  
73 bacteria in suspension. Exposure to sub-optimal or sub-therapeutic antimicrobial concentrations in the  
74 biofilm thereby increases the potential for antimicrobial resistance development (Stewart and  
75 Costerton 2001). Within days minerals in the saliva, such as calcium, combine with plaque to form  
76 calculus material (tartar) and an immune response is initiated by the host resulting in the inflammatory  
77 signs of gingivitis and periodontitis, indirect periodontal destruction, pain, halitosis and loss of  
78 appetite (Wiggs and Lobprise 1997).

79

80 The microbial ecology of the oral cavity of cats and dogs is vastly diverse with aerobic bacteria  
81 predominating in the early phase of gingivitis, followed by a predominantly anaerobic and Gram-  
82 negative bacterial profile when periodontitis becomes established (Hennet and Harvey 1991) (Harvey  
83 *et al.* 1995). The process of bacterial biofilm formation begins on the tooth surface immediately after  
84 successful scaling. Initially, Gram-positive cocci, including *Streptococci* (Leonhardt *et al.* 1992)

85 (Radice *et al.* 2006) become attached to the surface. Further growth and maturation of this aerobic or  
86 facultative flora leads to depletion of locally available oxygen and anaerobes such as *Porphyromonas*  
87 *gingivalis* and *Fusobacterium nucleatum* become more predominant (Cleland 2001). As gingival  
88 inflammation develops in response to the presence of bacteria, metabolic and inflammatory products  
89 such as endotoxins become constitute major components of the gingival fluid, contributing to local  
90 oral and systemic tissue destruction and dental bone loss (Holmstrom and others 2004). Invasive  
91 pathogens capable of active spread through the systemic blood supply may colonise other highly  
92 vascularised tissues including the kidneys, liver and heart. These micro-abscesses reduce overall long-  
93 term health for the animal, increasing risk factors associated with heart disease, hypertension and  
94 kidney disease (Glickman *et al.* 2011).

95  
96 For both cats and dogs, treatment of established infection centres on the use of antibiotics and anti-  
97 inflammatory agents, along with dental scaling and polishing (physical removal of calculus)  
98 performed by a veterinary practitioner. Scaling and removal of subgingival plaque is particularly  
99 problematic, often requiring administration of a general anaesthetic with the procedure repeated  
100 regularly throughout the animal's lifetime (Harvey 2005). Preventative therapy is typically centred on  
101 mechanical removal of adhered bacteria with a routine of regular toothbrushing aided by veterinary  
102 toothpastes containing antimicrobials ranging from chlorhexidine digluconate and cetylpyridium  
103 chloride to enzyme-based formulations. However, in addition to pet compliance issues, the effective  
104 removal of plaque requires the pet owner to be manually dexterous and patient (Iacono *et al.* 1998). In  
105 real-life practice, these factors often limit successful compliance.

106  
107 This study describes the formulation of an antimicrobial emulsion, intended for buccal application in  
108 companion animals, comprising of a medium chain triglyceride (ML:8) oil phase dispersed in water  
109 that displays *in vitro* efficacy at a low concentration (0.125% v/v) against resistant biofilm forms of  
110 ten periodontopathogens clinically implicated in canine and feline dental disease (Elliot *et al.* 2005)  
111 (Kolenbrander *et al.* 2002). This antimicrobial emulsion has the potential to be utilised as a drinking  
112 water additive to increase ease of use for the pet owner and subsequent aid compliance, with the

113 overall aims of reducing long-term oral bacterial bioburden and the incidence of periodontal disease in  
114 both cats and dogs.

115

## 116 **Materials and Methods**

### 117 **Formulation of ML:8 Emulsion**

118 The antimicrobial composition of ML:8 consists of an oil in water emulsion. A mixture of free fatty  
119 acids solubilised in water is promoted by the addition of membrane lipids, in this case lecithin. Lower  
120 melting point fatty acids such as caprylic and oleic acid were utilised to a final concentration of  
121 6.375% w/w. The ratio of free fatty acids to membrane lipid was 1.275:1. The final formulation was  
122 freshly diluted 1 in 51 (0.125% v/v free fatty acids) in sterile water before analysis.

123

### 124 **Comparator Substances**

125 Chlorhexidine digluconate was obtained from Sigma-Aldrich (Dorset, UK). A commercially available  
126 drinking water additive (Virbac Vet Aquadent®) containing xylitol 0.5% concentration (with  
127 chlorhexidine <0.01%) was obtained from Virbac (Bury St Edmonds, UK).

128

### 129 **Microbial Isolates Investigated**

130 *Haemophilus actinomycetemcomitans* (NCTC 10979), *Streptococcus sanguinis* (NCTC 10904) were  
131 obtained from HPA Culture Collections (Salisbury, UK). *Porphyromonas cangingivalis* (VPB 4874),  
132 *Porphyromonas salivosa* (VPB 3313), *Porphyromonas gingivalis* (VPB 5089), *Fusobacterium*  
133 *nucleatum* (VPB 4888), *Eikenella corrodens* (VPB 3935), *Bacteroides fragilis* (VPB 3371), *Prevotella*  
134 *intermedia* (VPB 3321) and *Tannerella forsythesis* (VPB 4947) were obtained from Dr Denise  
135 Wigney, Faculty of Veterinary Science, University of Sydney, Australia. All isolates of  
136 microorganisms were stored at -80°C in 10% glycerol.

137

### 138 **Rate of Reduction in Viability of Periodontal Biofilms using an alamarBlue® Assay**

139 Microorganisms under investigation were grown over 48-72 hours at 37°C in Tryptone Soya broth in a  
140 Biomat Class II Microbiological anaerobic Safety Cabinet (Don Whitely Scientific Ltd., Shipley, UK).

141 Upon visual confirmation of growth, the inoculum and adjusted to an optical density of 0.3 at 550nm  
142 in QSR5 solution, which was equivalent to  $1 \times 10^8$  colony forming units per milliliter (CFU/mL). This  
143 suspension was further diluted in Tryptone Soya broth (TSB) (1 in 50) and dispensed aseptically in  
144 100 $\mu$ L aliquots to each well of the microtitre plate. The inoculated plates were placed in Anaerogen  
145 sachets (Oxoid, Hampshire, UK) and the air tight sachets containing the inoculated plates were  
146 removed from the cabinet and biofilm were formed on the surface of the well under shear stress  
147 provided by a Gallenkamp gyratory incubator at 37°C. After an inoculation period of 48 hours, the  
148 Anaerogen sachets containing the biofilm growth plates were transferred to the anaerobic cabinet,  
149 cultures were decanted and the plates irrigated twice with 200 $\mu$ L of sterile autoclaved 0.9% w/v  
150 sodium chloride (NaCl) in each well. Washed plates were tapped gently upside down on a sterile  
151 paper towel to remove residual wash. The prepared biofilm was treated for evaluation of viability  
152 using alamarBlue® in a method similar to that used by Pettit *et al.* (2005). The viability of established  
153 biofilms was assessed by re-charging wells from above immediately after washing and without drying,  
154 with 0.1mL fresh TSB broth containing 20% v/v alamarBlue® (AbD Serotec, Oxford, UK), incubating  
155 the plate at 37°C for one hour followed by spectrophotometric measurement of absorbance at a  
156 wavelength of 570nm. AlamarBlue® is resazurin, a redox indicator which is reduced by metabolic  
157 activity of viable microbial cells to pink fluorescent resorufin. The reduction of viability (killing) of  
158 established biofilm with the test formulations was evaluated by loading wells containing washed  
159 biofilm from above immediately after washing with 0.1mL of 0.125% v/v ML:8 emulsion, 0.12% v/v  
160 chlorhexidine digluconate or xylitol followed by incubation at 37°C. Time points selected for analysis  
161 of ML:8 emulsion/chlorhexidine digluconate/xylitol activity on established biofilms were 0, 5, 10, 20,  
162 30, 40, 50 and 60 minutes, followed by 2, 4 and 24 hours. Each control well had 8 replicates at each  
163 time point. Blank determinations (100% viability: positive control) were conducted using sterile  
164 distilled water. Immediately following incubation, the exposed biofilms were washed twice by  
165 irrigation with sterile 0.9% w/v NaCl and recharged with fresh TSB containing 20% v/v alamarBlue®  
166 (sterilized by passage twice through a syringe fitted with a 0.22 $\mu$ m membrane filter) and 3% w/v  
167 Tween 80 (polysorbate), incubated at 37°C for 1 hour and the development of UV absorbance was then  
168 measured spectrophotometrically at 570nm. A positive control (100% microbicidal effect) was also

169 included in the assay using 2% chlorhexidine digluconate. Reduction in viability of biofilm following  
170 exposure to the test formulations was expressed as a percentage based on the percentage reduction  
171 between untreated (blank) wells and treated wells using the following equation:

172

$$173 \quad \% \text{reduction in viability} = \frac{\text{Abs}_{570\text{nm}} \text{ ML:8 Emulsion/ Chlorhexidine digluconate/ Virbac Vet Aquadent®} - \text{Abs}_{570\text{nm}} \text{ Blank}}{\text{Abs}_{570\text{nm}} \text{ Chlorhexidine digluconate 2\%} - \text{Abs}_{570\text{nm}} \text{ Blank}} \times 100$$

174

### 175 **Cell Survival Analysis (cytotoxicity assays)**

176 Two individual mammalian cell lines were selected for cytotoxicity analysis following exposure to the  
177 test formulations. These were Het-1A (ATCC CRL-2692) human oesophageal cells and NCTC Clone  
178 929 (ATCC CCL 1) murine fibroblast subcutaneous connective tissue cells; areolar and adipose  
179 International Standard cell lines. Both cell lines were obtained from LGC Standards (London, UK).  
180 NCTC Clone 929 (ATCC CCL 1) cell line was cultured in Minimum Essential Medium (MEM)  
181 containing phenol red with Earle's Salts, L-Glutamine, supplemented with 10% Horse  
182 Serum, 100iu/mL penicillin and 100µg/mL streptomycin supplied by Invitrogen (Paisley, UK). The  
183 Het-1A (ATCC CRL-2692) cell line was cultured in Bronchial Epithelial cell Basal Medium (BEBM)  
184 supplemented by Bronchial Epithelial Cell Growth Medium (BEGM) SingleQuot Kit and Growth  
185 Factors (Lonza, Basle, Switzerland). Cells were grown at 37°C and 5% CO<sub>2</sub> and subcultured at 80 –  
186 90 % confluency. Subculturing consisted of removal of spent medium, rinsing of the adherent cell  
187 surface with sterile autoclaved phosphate buffered saline (PBS) before treatment with a 0.05%  
188 Trypsin/0.53mM EDTA·4Na solution (Invitrogen, Paisley, UK) for cell monolayer detachment. For  
189 the Het-1A (ATCC CRL-2692) cell line Trypsin/EDTA required inclusion of 0.5%  
190 polyvinylpyrrolidone (Sigma-Aldrich, Dorset, UK) and preparation flasks were required to be  
191 precoated with 0.01mg/mL fibronectin, 0.03mg/mL bovine collagen type I and 0.01mg/mL bovine  
192 serum albumin (all supplied by Invitrogen, Paisley, UK) to facilitate attachment.

193

194 Cell viability was assessed by means of a quantitative alamarBlue® assay, using a modification of the  
195 method of O'Brien *et al.* (2000). Cells were cultured (until at least third passage) and inoculated into  
196 96-well tissue culture treated microtitre plates at a concentration of 1 x 10<sup>4</sup> cells/well and incubated at



197 37°C and 5% CO<sub>2</sub> for 24 ± 1 hour, until approximately 90% confluency as described above. After this  
198 time, the medium was removed and replaced with required fresh growth medium, containing doubling  
199 dilutions of ML:8 emulsion at final concentrations of 0.25-0.0625% v/v with eight replicates at each  
200 concentration. Chlorhexidine digluconate was tested over the same concentration range and acted as a  
201 comparative control. Time points selected for analysis of ML:8 emulsion/chlorhexidine digluconate  
202 activity on established cell lines were 5, 30 and 60 minutes. Absorption was measured at 570nm in a  
203 Tecan Sunrise® plate reader after a development time of 10 hours for NCTC Clone 929 (ATCC CCL  
204 1) cell line and 4 hours for Het-1A (ATCC CRL-2692) cell line. A positive control (100% reduction  
205 in viability) was also included in the assay using 90% ethanol (Sigma-Aldrich, Dorset, UK); the  
206 negative control consisted of untreated cell line wells percentage cell viability was calculated relative  
207 to untreated control wells after subtraction of the blank value corresponding to untreated cells in the  
208 absence of alamarBlue® reagent.

209 
$$\% \text{ reduction in viability} \equiv \frac{\text{Abs}_{570\text{nm}} \text{ ML:8 Emulsion / Chlorhexidine digluconate} - \text{Abs}_{570\text{nm}} \text{ Blank}}{\text{Abs}_{570\text{nm}} \text{ ethanol } 90\% - \text{Abs}_{570\text{nm}} \text{ Blank}} \times 100$$

210

211

## 212 **Statistical Analysis**

213 Statistical analyses were performed using GraphPad InStat 3. Standard deviations were obtained at  
214 each concentration/timepoint of antimicrobials tested based on eight replicates for both quantitative  
215 biofilm and cell cytotoxicity viability assays and mean values obtained. Further statistical analysis  
216 was employed using a one way Analysis of Variance (ANOVA), with a Tukey-Kramer multiple  
217 comparisons test used to identify individual differences between the antibiofilm activity of 0.125% v/v  
218 ML:8 emulsion and 0.12% v/v chlorhexidine digluconate, and also the antibiofilm activity of 0.125%  
219 v/v ML:8 emulsion and 2.4% v/v Virbac Vet Aquadent® (as directed by the manufacturer), at relative  
220 timepoints. ANOVA with a Tukey-Kramer multiple comparisons test was also utilised for statistical  
221 analysis of cytotoxicity data of ML:8 emulsion and chlorhexidine digluconate at the same  
222 concentrations (0.25-0.06125% v/v) and relative timepoints. ANOVA assumes that the data is  
223 sampled from populations that follow Gaussian distributions. Data was shown to be normally

224 distributed using the Kolmogorov and Smirnov method. In all cases a probability of  $P \leq 0.05$  denoted  
225 significance.

226

## 227 **Results**

228 The 0.125% v/v ML:8 emulsion displayed significant activity against biofilm forms of the 10  
229 periodontopathogens investigated within 5 to 10 minutes exposure. Antibiofilm efficacy was  
230 significantly greater than the 2.4% v/v Virbac Vet Aquadent® (xylitol containing formulation) and  
231 statistically similar to 0.12% v/v chlorhexidine digluconate. Antibiofilm activity for 0.125% v/v ML:8  
232 emulsion, 0.12% v/v chlorhexidine digluconate and xylitol containing formulation are displayed in  
233 Figures 1-10. ML:8 emulsion was significantly less cytotoxic than chlorhexidine digluconate at  
234 similar concentrations ( $P < 0.001$  for all assays). Cytotoxicity of ML:8 emulsion and chlorhexidine  
235 digluconate at the same concentrations (0.25-0.06125% v/v) against CCL 1 (NCTC Clone 929) murine  
236 fibroblast subcutaneous connective tissue monolayer cells and human oesophageal tissue monolayer  
237 cells (ATCC CRL-2692) are shown in Figures 11 and 12, respectively.

238

## 239 **Discussion**

240 The results of the current study demonstrate that ML:8 emulsion displayed a high degree of potency  
241 against 48 hour biofilm forms of the 10 periodontopathogens investigated. High percentage kill rates  
242 ( $> 70\%$ ) were achieved against the majority of test organisms within 5 minutes of exposure, and at all  
243 subsequent time points. The selection of biofilm forms of bacteria was purposeful in order to test the  
244 ability of the formulation to eradicate this more resistant bacterial phenotype present within the oral  
245 cavity (Hojo *et al.* 2009). The majority of previously reported dental-related studies have centred on  
246 human plaque and less resistant liquid planktonic forms of bacteria (Stanley *et al.* 1989) (McBain *et*  
247 *al.* 2004), and whilst contributing valuable information, their clinical relevance may be limited in  
248 comparison to biofilm-based data. The bacteria selected for assessment in the current study were  
249 derived from an extensive literature search for relevant canine and feline periodontopathogens, and as  
250 such, have direct relevance to the clinical microbiota encountered in canine/feline periodontal disease

251 (Syed and Svanberg 1981)(Svanberg *et al.* 1982)(Hennet and Harvey 1991)(Leonhardt *et al.*  
252 1992)(Okuda and Harvey 1992)(Harvey *et al.* 1995)(Harvey 1998)(Harvey 2005).

253

254 The antimicrobial activity of free fatty acids has been widely reported previously in the literature  
255 (Kabara *et al.* 1972). Research conducted by Sun *et al.* (2002) concluded that caprylic (C<sub>8</sub>), capric  
256 (C<sub>10</sub>) and lauric acid (C<sub>12</sub>) displayed antimicrobial activity with lauric and caprylic acid shown to be  
257 most efficacious against Gram-positive and Gram-negative bacteria, respectively. The ML:8 emulsion  
258 formulation described here displayed rapid antimicrobial efficacy, showing high potential to be an  
259 effective drinking water additive for periodontal disease prevention at low concentrations (0.125%  
260 v/v), despite the limited exposure times that can be achieved within the oral cavity. To test this  
261 hypothesis further, we compared the anti-biofilm activity of the ML:8 emulsion with the gold standard  
262 in human/veterinary dental hygiene (0.12% chlorhexidine digluconate) and another commercially  
263 available veterinary dental formulation containing 0.5% xylitol (prediluted). With the exception of the  
264 0 minute timepoints for *Porphyromonas gingivalis* (VPB 5089), *Eikenella corrodens* (VPB 3935) and  
265 *Tannerella forsythensis* (VPB 4947), there was no significant difference in the antibiofilm activity of  
266 0.125% v/v ML:8 emulsion and 0.12% v/v chlorhexidine digluconate (P>0.05). However, although  
267 chlorhexidine has been a mainstay in the control and treatment of dental pathogens in human health  
268 (Roberts *et al.* 2002), similar effective concentrations cannot be employed in canine drinking water  
269 formulations as the ingestion/swallowing of solutions containing 0.12% v/v chlorhexidine on a daily  
270 basis is likely to be associated with significant cytotoxicity, as demonstrated by the results generated  
271 in this study (*see later*). At concentrations significantly below the traditional 0.12% v/v threshold  
272 employed in human products, the range of chlorhexidine-based drinking water additives available on  
273 the veterinary market have debatable *in vitro* and *in vivo* efficacy (Roudebush *et al.* 2005).

274

275 Virbac Vet Aquadent® contains xylitol and <0.05% chlorhexidine digluconate (the chlorhexidine  
276 component is not included as an active ingredient). Our results show Aquadent® to have limited  
277 efficacy against biofilm forms of periodontal bacteria tested up to 24 hours under the conditions of this  
278 assay. At the 24 hour timepoint, the reduction in viable biofilm reached a maximum of 53% against

279 *Eikenella corrodens* (VPB 3935). The majority of bacteria/timepoints studied showed a mean  
280 reduction of biofilm of less than 10%. Overall, the 0.125% v/v ML:8 emulsion showed a statistically  
281 significant increase in biofilm reduction when compared with Aquadent® in 93.6% of the comparative  
282 time points/bacteria tested (103 out of 110 sample points;  $P < 0.001$ ). Although issues have been  
283 raised in relation to the potential toxic effects of xylitol ingestion in dogs (Murphy *et al.* 2012), the  
284 dose levels employed in Aquadent® have not been associated with any reported toxic effects in the  
285 published literature.

286

287 Figures 11 and 12 show that at therapeutic concentrations (0.12% v/v) chlorhexidine digluconate  
288 demonstrated toxicity against mammalian cell lines; therefore, its long-term use or suitability as a  
289 drinking water additive may be limited by potential gastrointestinal and oral mucosal damage. At the  
290 same concentrations and correlating to the same exposure times and cell lines, the toxicity  
291 demonstrated by ML:8 emulsion was significantly lower ( $P < 0.001$ ) than for chlorhexidine  
292 digluconate. After up to 60 minutes exposure to varying concentrations of ML:8 emulsion (0.25 to  
293 0.0625% v/v), the relative percentage kill of CCL 1 (NCTC Clone 929) murine fibroblast cells was  
294 absent (0%). The results obtained for chlorhexidine digluconate against both human oesophageal  
295 tissue (ATCC CRL-2692) and the International cytotoxicity standard CCL 1 (NCTC Clone 929)  
296 murine fibroblast cells showed a statistically significant increase ( $P < 0.001$ ) in the cytotoxic activity of  
297 chlorhexidine digluconate relative to ML:8 at all time points studied. Quantitative evaluations such as  
298 the alamarBlue® assay determine that a reduction of cell viability by more than 30% is indicative of  
299 cytotoxicity (International Standard ISO10993-5). Selection of the International cytotoxicity standard  
300 CCL 1 (NCTC Clone 929) murine fibroblast cell line allowed this novel formulation to be assessed for  
301 cytotoxicity in general, whilst selection of a mammalian oesophageal cell line also allowed the toxicity  
302 of ML:8 to be compared to a clinically relevant cell line.

303

### 304 **Conclusions**

305 The formulation and 1 in 51 dilution of this novel 6.375% v/v ML:8 emulsion to drinking water  
306 allows the active free fatty acids to be present at an antimicrobially active and non-cytotoxic 0.125 %

307 v/v final concentration. Such a product can increase compliance and ease of use allowing daily  
308 administration to help prevent periodontal disease, with superior *in vitro* results compared with a  
309 currently available xylitol-based drinking water additive. The findings of this study validate the use of  
310 the ML:8 emulsion as part of an ever increasing evidence-based approach to biofilm control in  
311 veterinary dental applications (Hamp and others 1973). Although *in vivo* clinical trials are now  
312 indicated to corroborate these findings, our initial laboratory results show large scope and promise for  
313 continuing research in this area.

314

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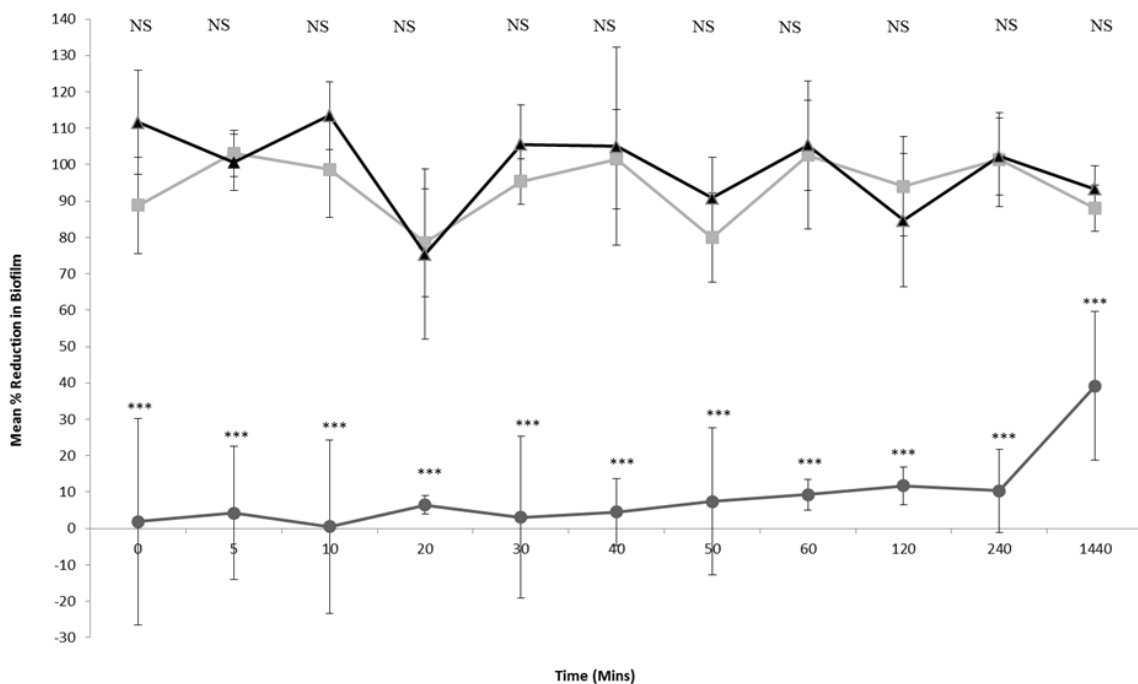
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400 **Figure/Legends**



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402 **Fig. 1. Mean percentage reduction in 48 hour biofilm of *Haemophilus actinomycetemcomitans***

403 **(NCTC 10979) over a period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion,**

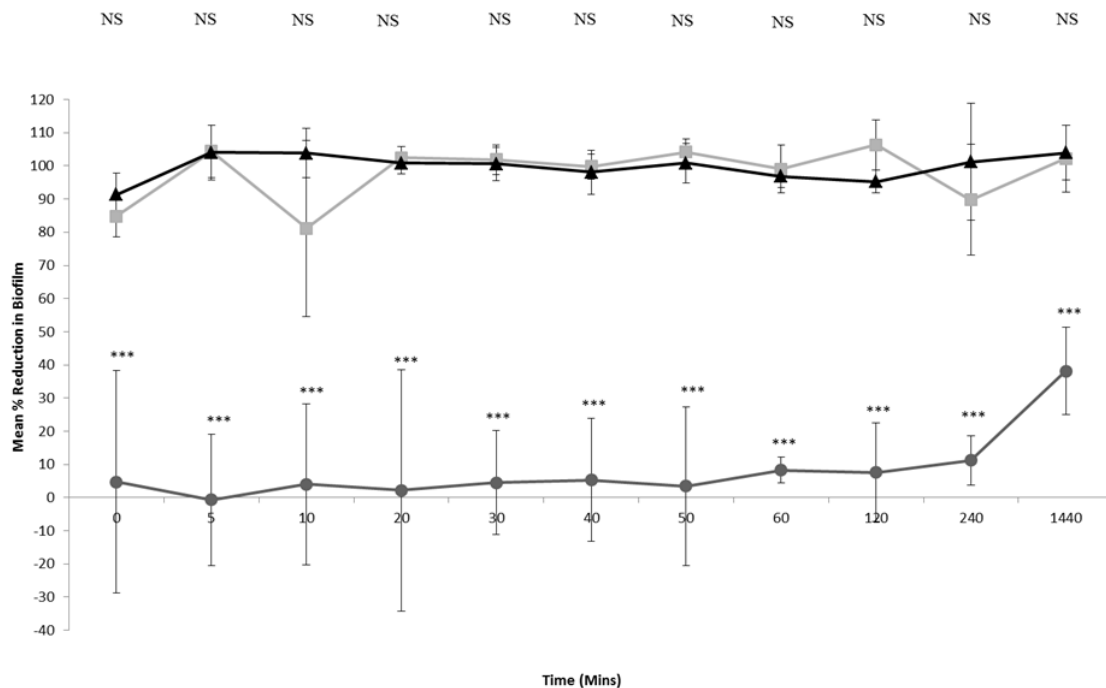
404 **0.12%v/v Chorhexidine digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an**

405 **alamarBlue® assay. Results are displayed as the mean of 8 replicates. Key: ■: 0.12%v/v**



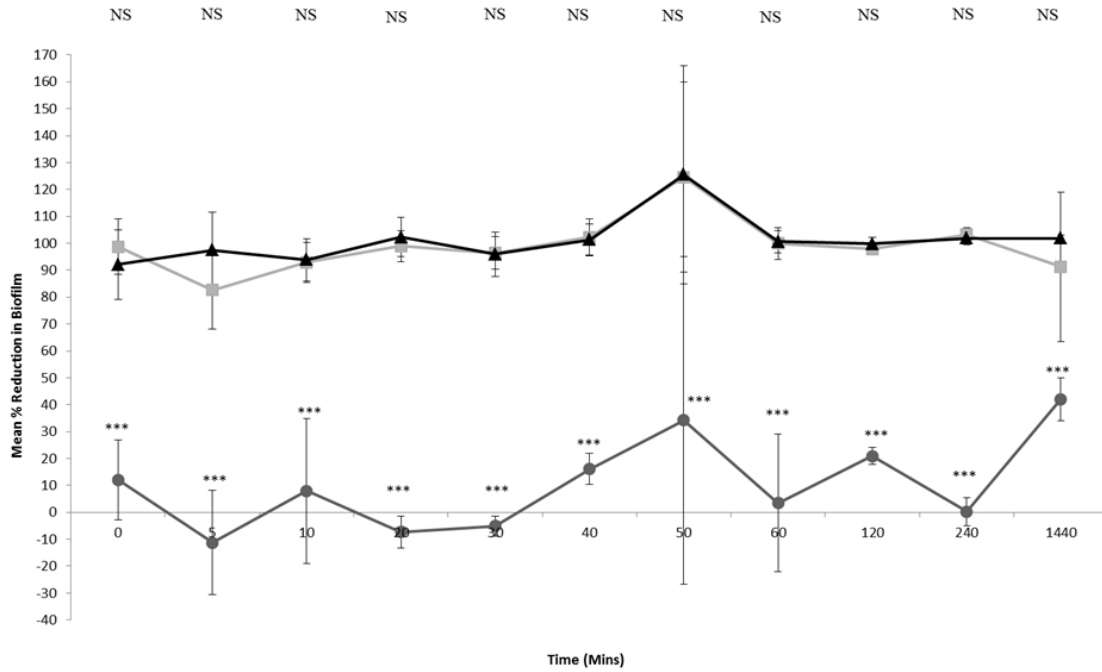
406 Chlorhexidine digluconate, ▲: 0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®,  
 407 ns: no significant difference (P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference  
 408 between efficacy of 0.125%v/v ML:8 Emulsion and 0.12%v/v Chlorhexidine digluconate or  
 409 2.4%v/v Virbac Vet Aquadent® at same timepoint.

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 413 **Fig. 2. Mean percentage reduction in 48 hour biofilm of *Streptococcus sanguinis* (NCTC 10904)**  
 414 **over a period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion, 0.12%v/v**  
 415 **Chorhexidine digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an alamarBlue® assay.**  
 416 **Results are displayed as the mean of 8 replicates. Key: ■: 0.12%v/v Chlorhexidine digluconate,**  
 417 **▲: 0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®, ns: no significant difference**  
 418 **(P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference between efficacy of**  
 419 **0.125%v/v ML:8 Emulsion and 0.12%v/v Chlorhexidine digluconate or 2.4%v/v Virbac Vet**  
 420 **Aquadent® at same timepoint.**

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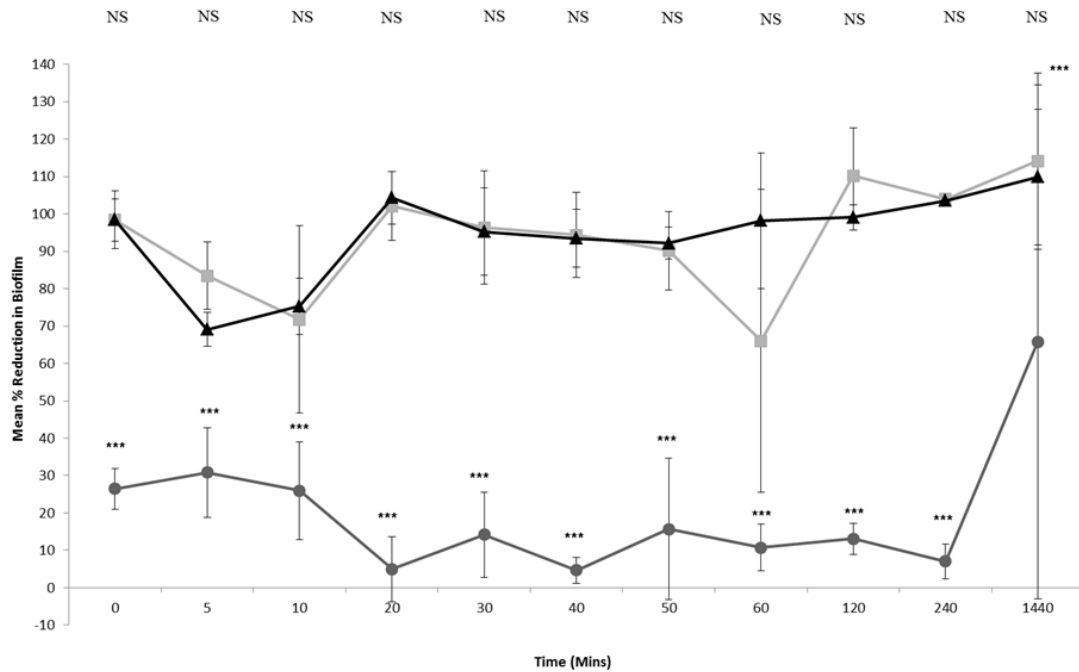


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**Fig. 3. Mean percentage reduction in 48 hour biofilm of *Porphyromonas cangingivalis* (VPB 4874) over a period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion, 0.12%v/v Chlorhexidine digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an alamarBlue® assay. Results are displayed as the mean of 8 replicates. Key: ■: 0.12%v/v Chlorhexidine digluconate, ▲: 0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®, ns: no significant difference (P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference between efficacy of 0.125%v/v ML:8 Emulsion and 0.12%v/v Chlorhexidine digluconate or 2.4%v/v Virbac Vet Aquadent® at same timepoint.**

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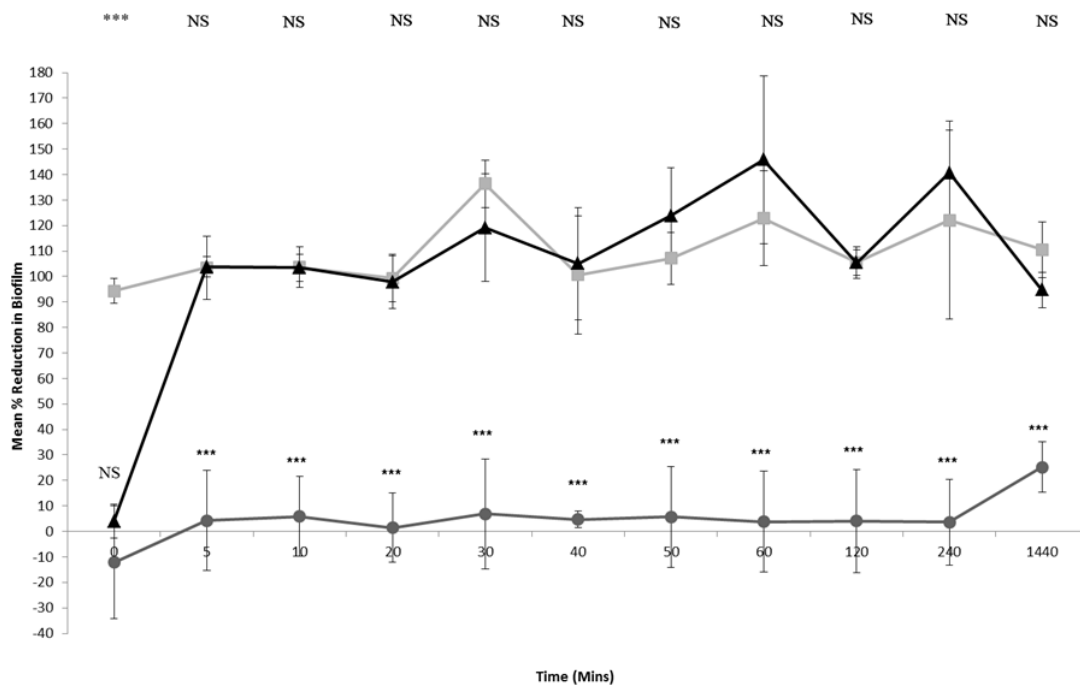
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439 **Fig. 4. Mean percentage reduction in 48 hour biofilm of *Porphyromonas salivosa* (VPB 3313)**  
440 **over a period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion, 0.12%v/v**  
441 **Chorhexidine digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an alamarBlue® assay.**  
442 **Results are displayed as the mean of 8 replicates. Key: ■: 0.12%v/v Chlorhexidine digluconate,**  
443 **▲ : 0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®, ns: no significant difference**  
444 **(P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference between efficacy of**  
445 **0.125%v/v ML:8 Emulsion and 0.12%v/v Chlorhexidine digluconate or 2.4%v/v Virbac Vet**  
446 **Aquadent® at same timepoint.**

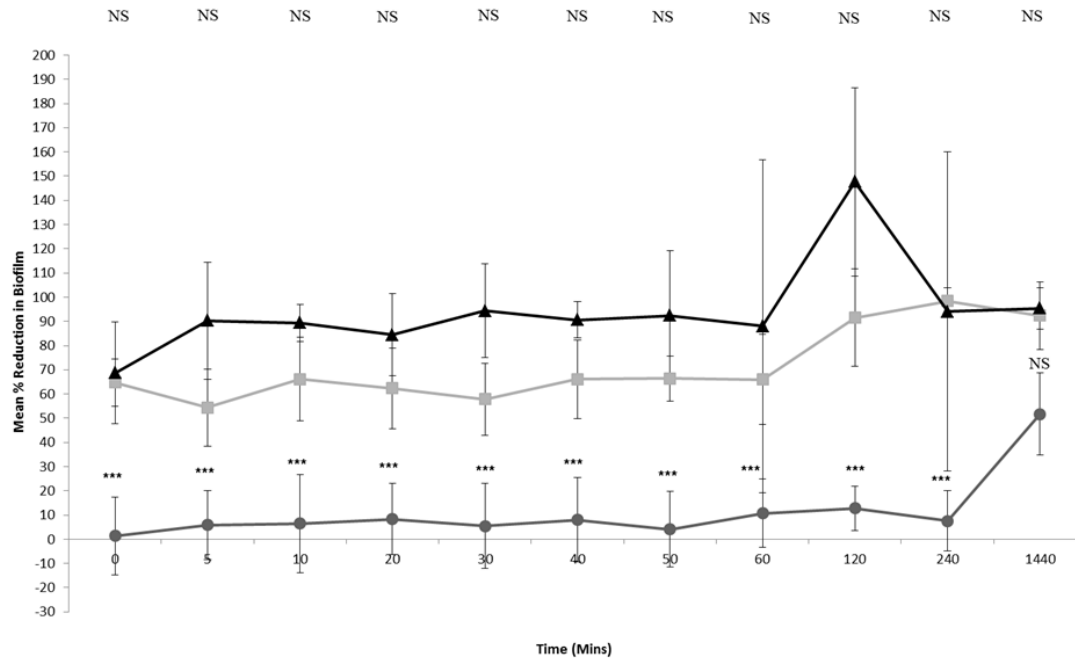
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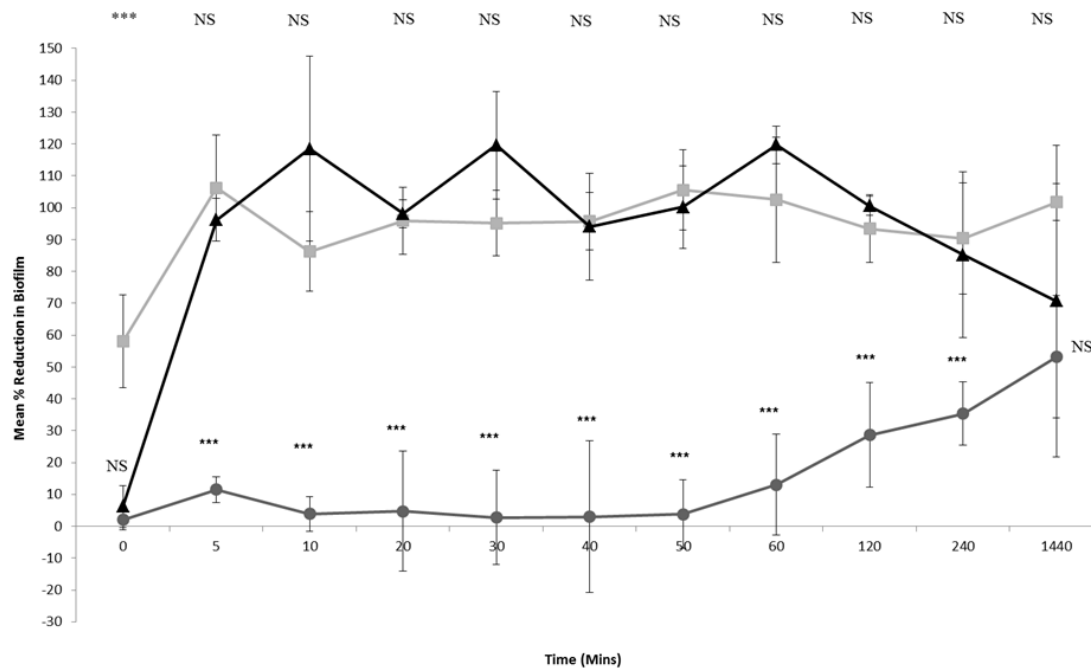
449 **Fig. 5. Mean percentage reduction in 48 hour biofilm of *Porphyromonas gingivalis* (VPB 5089)**  
 450 **over a period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion, 0.12%v/v**  
 451 **Chorhexidine digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an alamarBlue® assay.**  
 452 **Results are displayed as the mean of 8 replicates. Key: ■: 0.12%v/v Chlorhexidine digluconate,**  
 453 **▲: 0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®, ns: no significant difference**  
 454 **(P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference between efficacy of**  
 455 **0.125%v/v ML:8 Emulsion and 0.12%v/v Chlorhexidine digluconate or 2.4%v/v Virbac Vet**  
 456 **Aquadent® at same timepoint.**

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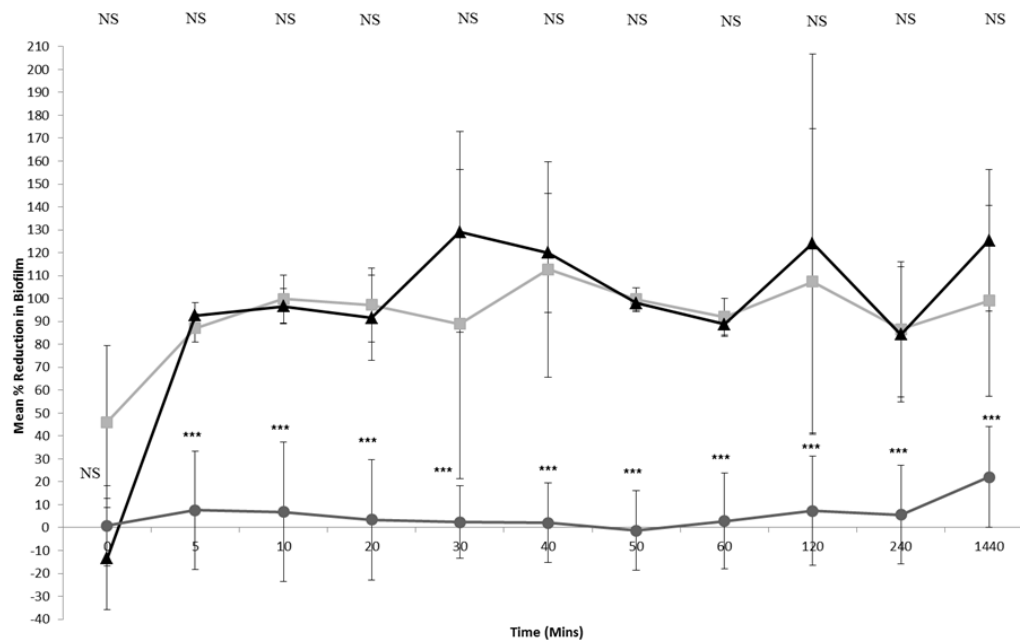
459 **Fig. 6. Mean percentage reduction in 48 hour biofilm of *Fusobacterium nucleatum* (VPB 4888)**  
 460 **over a period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion, 0.12%v/v**  
 461 **Chorhexidine digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an alamarBlue® assay.**  
 462 **Results are displayed as the mean of 8 replicates. Key: ■: 0.12%v/v Chlorhexidine digluconate,**  
 463 **▲: 0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®, ns: no significant difference**  
 464 **(P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference between efficacy of**  
 465 **0.125%v/v ML:8 Emulsion and 0.12%v/v Chorhexidine digluconate or 2.4%v/v Virbac Vet**  
 466 **Aquadent® at same timepoint.**



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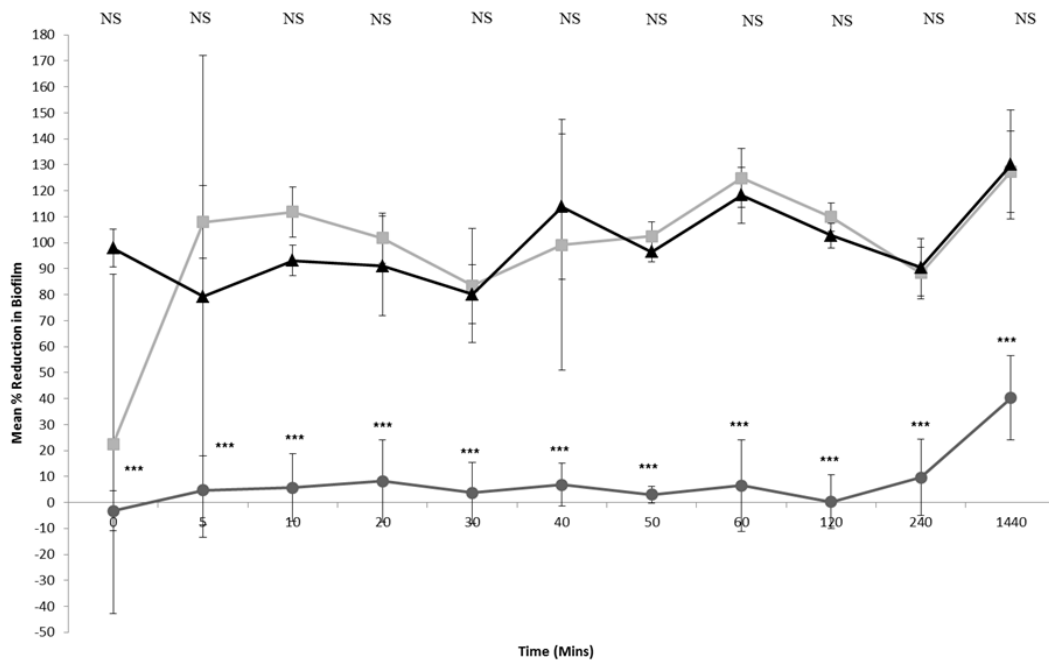
468 **Fig. 7. Mean percentage reduction in 48 hour biofilm of *Eikenella corrodens* (VPB 3935) over a**  
 469 **period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion, 0.12%v/v Chlorhexidine**  
 470 **digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an alamarBlue® assay. Results are**  
 471 **displayed as the mean of 8 replicates. Key: ■: 0.12%v/v Chlorhexidine digluconate, ▲:**  
 472 **0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®, ns: no significant difference**  
 473 **(P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference between efficacy of**  
 474 **0.125%v/v ML:8 Emulsion and 0.12%v/v Chlorhexidine digluconate or 2.4%v/v Virbac Vet**  
 475 **Aquadent® at same timepoint.**

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478 **Fig. 8. Mean percentage reduction in 48 hour biofilm of *Bacteroides fragilis* (VPB 3371) over a**  
 479 **period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion, 0.12%v/v Chlorhexidine**  
 480 **digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an alamarBlue® assay. Results are**  
 481 **displayed as the mean of 8 replicates. Key: ■: 0.12%v/v Chlorhexidine digluconate, ▲:**  
 482 **0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®, ns: no significant difference**  
 483 **(P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference between efficacy of**  
 484 **0.125%v/v ML:8 Emulsion and 0.12%v/v Chlorhexidine digluconate or 2.4%v/v Virbac Vet**  
 485 **Aquadent® at same timepoint.**

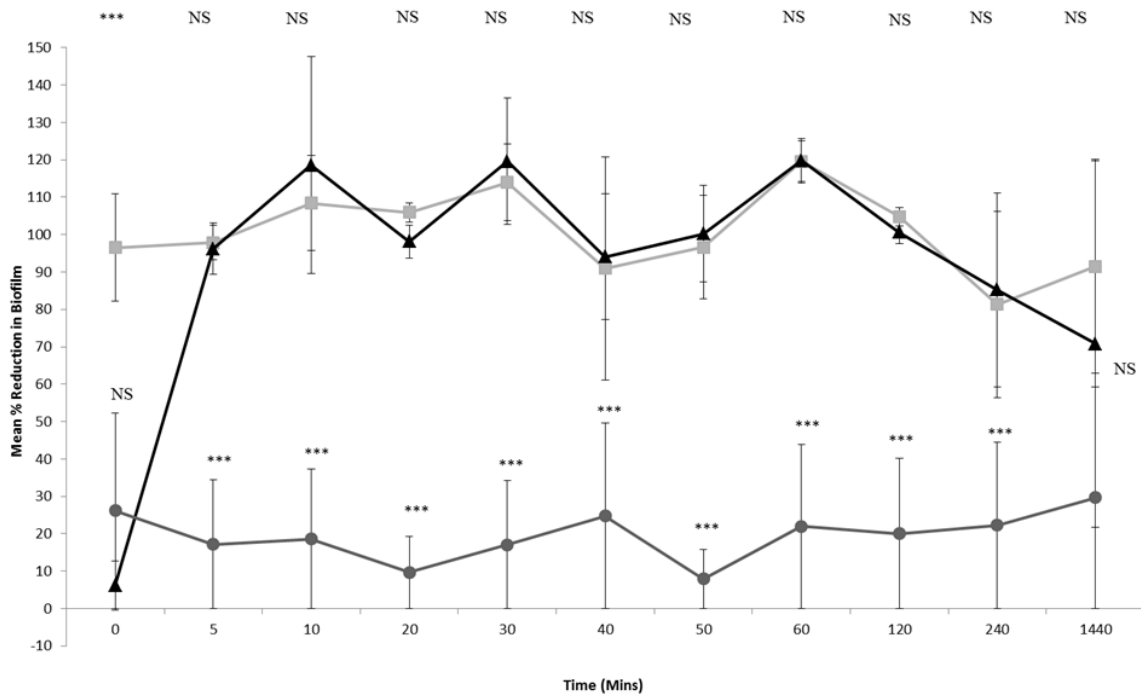


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487 **Fig. 9. Mean percentage reduction in 48 hour biofilm of *Prevotella intermedia* (VPB 3321) over a**  
 488 **period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion, 0.12%v/v Chlorhexidine**  
 489 **digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an alamarBlue® assay. Results are**  
 490 **displayed as the mean of 8 replicates. Key: ■: 0.12%v/v Chlorhexidine digluconate, ▲:**  
 491 **0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®, ns: no significant difference**  
 492 **(P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference between efficacy of**  
 493 **0.125%v/v ML:8 Emulsion and 0.12%v/v Chlorhexidine digluconate or 2.4%v/v Virbac Vet**  
 494 **Aquadent® at same timepoint.**

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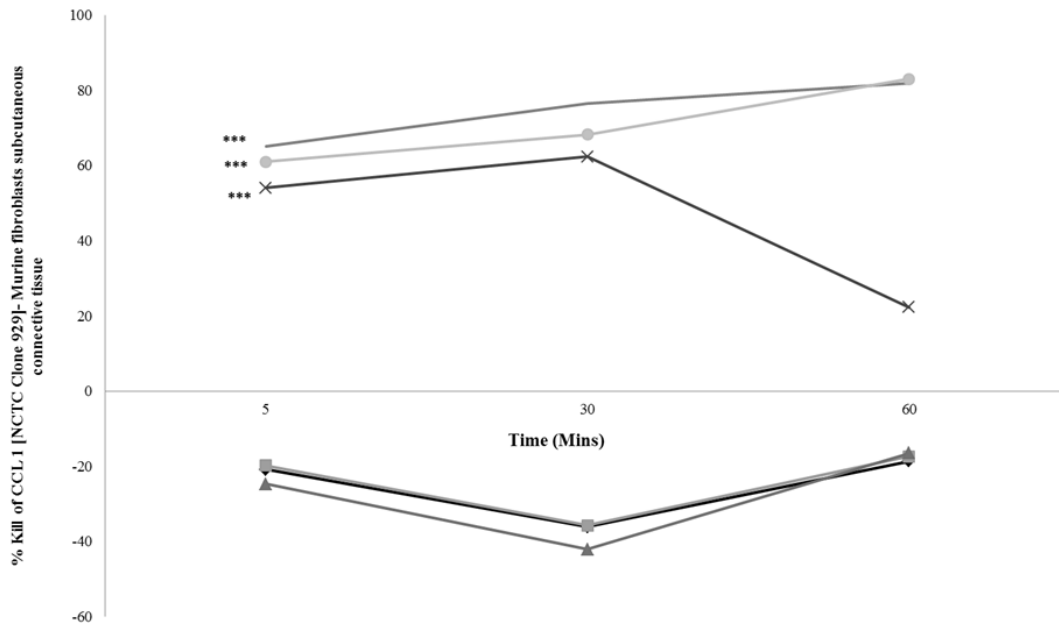
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497 **Fig. 10. Mean percentage reduction in 48 hour biofilm of *Tanerrella forsythesis* (VPB 4947) over**  
 498 **a period of 24 hour exposure to food grade 0.125%v/v ML:8 Emulsion, 0.12%v/v Chorhexidine**  
 499 **digluconate and 2.4%v/v Virbac Vet Aquadent® utilising an alamarBlue® assay. Results are**  
 500 **displayed as the mean of 8 replicates. Key: ■: 0.12%v/v Chlorhexidine digluconate, ▲:**  
 501 **0.125%v/v ML:8 Emulsion, ●: 2.4%v/v Virbac Vet Aquadent®, ns: no significant difference**  
 502 **(P>0.05) \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference between efficacy of**  
 503 **0.125%v/v ML:8 Emulsion and 0.12%v/v Chorhexidine digluconate or 2.4%v/v Virbac Vet**  
 504 **Aquadent® at same timepoint.**

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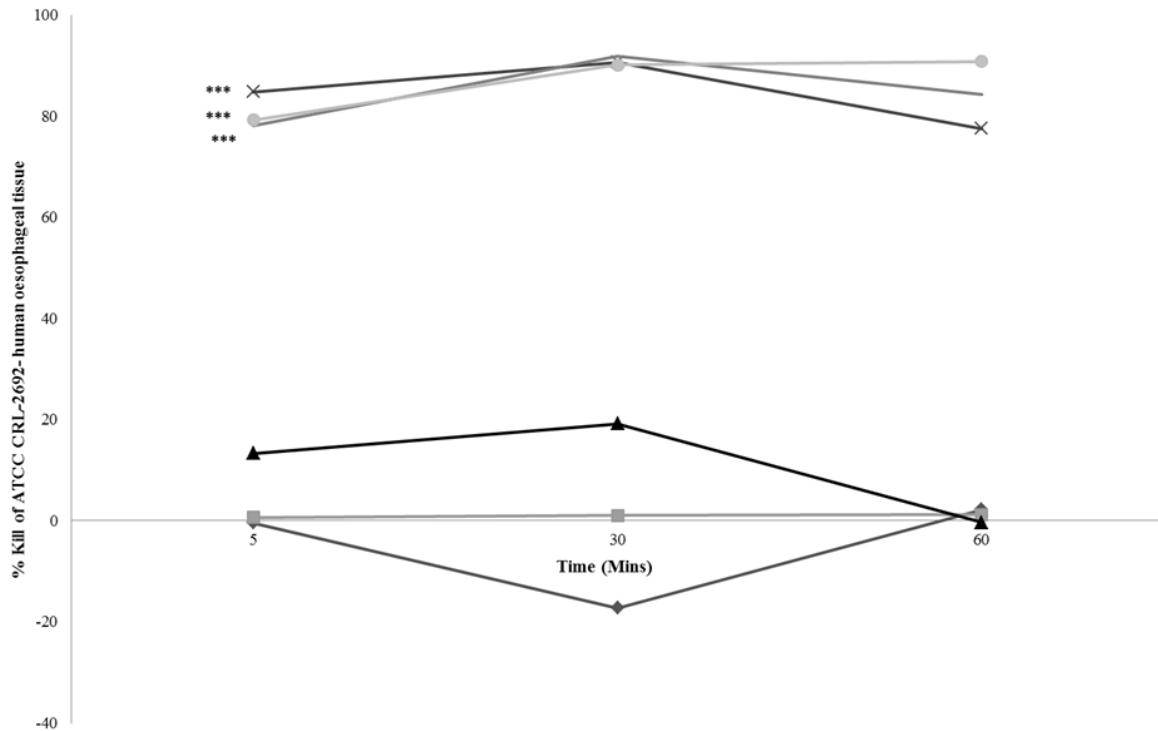


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509 **Fig. 11. The percentage kill of CCL 1 [NCTC clone 929]- murine fibroblasts subcutaneous**  
 510 **connective tissue monolayer cells after 5, 30 and 60 minutes exposure to varying concentrations**  
 511 **of ML:8 emulsion and Chlorhexidine digluconate (CHX). Results are obtained via the use of an**  
 512 **alamarBlue® assay (10 hour development time). Key:**

513 **◆ 0.25% v/v ML:8      ■ 0.125% v/v ML:8      ▲ 0.0625% v/v ML:8**  
**× 0.25% v/v CHX      — 0.125% v/v CHX      ● 0.0625% v/v CHX**

514 **ns: no significant difference (P>0.05), \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference**  
 515 **between cytotoxicity of ML:8 Emulsion and Chorhexidine digluconate at same time point and**  
 516 **concentration.**



517  
 518 **Fig. 12. The percentage kill of ATCC CRL-2692- human oesophageal tissue monolayer cells**  
 519 **after 5, 30 and 60 minutes exposure to varying concentrations of ML:8 emulsion and**  
 520 **Chlorhexidine digluconate (CHX). Results are obtained via the use of an alamarBlue® assay (10**  
 521 **hour development time). Key:**

522 **◆ 0.25% v/v ML:8      ■ 0.125% v/v ML:8      ▲ 0.0625% v/v ML:8**  
 523 **✕ 0.25% v/v CHX      — 0.125% v/v CHX      ● 0.0625% v/v CHX**

524 **ns: no significant difference (P>0.05), \*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, significant difference**  
 525 **between cytotoxicity of ML:8 Emulsion and Chlorhexidine digluconate at same time point and**  
 526 **concentration.**

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