

## ORIGINAL ARTICLE

# Antimicrobial activity of alexidine alone and associated with N-acetylcysteine against *Enterococcus faecalis* biofilm

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The purpose of this study was to assess the efficacy of alexidine (ALX), alone and combined with N-acetylcysteine (NAC), in eradicating two *Enterococcus faecalis* strain biofilms. The biofilms of *E. faecalis* ATCC 29212 and the clinical isolate *E. faecalis* D1 were grown in the MBEC-high-throughput device for 24 h and were exposed to five twofold dilutions of ALX (2%–0.007 8%) alone and combined with 100 mg·mL<sup>-1</sup> NAC, for 1 and 5 min. Eradication was defined as 100% kill of biofilm bacteria. The Student's *t*-test was used to compare the efficacy of the associations of the two irrigants. After 1-min contact time, ALX eradicated the biofilms at all concentrations except for 0.007 8% and 0.015 6%–0.007 8% with *E. faecalis* ATCC 29212 and *E. faecalis* D1, respectively. Similar results for eradication and concentration were obtained when it was combined with 100 mg·mL<sup>-1</sup> NAC. After 5 min of contact time, ALX alone and combined with NAC eradicated all enterococci biofilms. ALX showed antimicrobial properties against the two *E. faecalis* strain biofilms tested at very low concentrations, and its combined use with NAC was not seen to enhance its activity.

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## INTRODUCTION

Bacteria and their by-products play an essential role in the establishment and development of pulpal and periapical alterations.<sup>1</sup> Once bacteria gain access to the complex root canal system, they organize in communities known as biofilms.<sup>2</sup> Although the bacterial biofilm associated with periradicular infections is mostly constituted of obligatory anaerobic organisms, some facultative anaerobes like *Enterococcus faecalis* are involved in persistent diseases.<sup>3–5</sup>

This type of infection cannot be eliminated by host defense mechanisms, and needs to be treated by chemical–mechanical procedures.<sup>6</sup> Irrigation by chemical solutions should be aimed to eliminate both the smear layer—an organic and inorganic layer formed during instrumentation—and the biofilm. As the latter is a more difficult structure to remove compared with bacteria in a planktonic state, the irrigating solutions must exert antimicrobial activity as well as be capable of removing the exopolymeric substance (EPS) to guarantee disruption of the biofilm.<sup>7</sup>

Chlorhexidine (CHX) is a widely used antimicrobial endodontic irrigant. It is a bisbiguanide with proven antimicrobial activity and substantivity in contaminated root canals (for a review, see ref. 8). Another irrigant in this group is alexidine (ALX), which differs chemically from CHX because of the presence of two hydrophobic ethylhexyl groups in its structure; it provides for faster bactericidal activity

and bacterial permeabilization.<sup>9</sup> Also, 1% ALX can be as effective against *E. faecalis* infection as 2% CHX in bovine dentin blocks,<sup>10</sup> and it has been demonstrated that its interactions with sodium hypochlorite (NaOCl) do not produce precipitates.<sup>11</sup>

N-acetylcysteine (NAC) is a potent thiol-containing antioxidant and a mucolytic agent widely used in medical treatment of chronic bronchitis and acetaminophen overdose.<sup>12</sup> Its principal mechanism of action consists of reducing the production of extracellular polysaccharide, thereby disrupting mature biofilms and reducing adhesion of bacteria on surfaces.<sup>13–16</sup> However, its antimicrobial activity against biofilms appears to depend on the bacterial strain.<sup>13,15,17</sup> It is very effective against certain Gram-negative bacilli, such as *Klebsiella pneumoniae*,<sup>18</sup> and some Gram-positive cocci.<sup>19</sup> Recently, NAC was found to be effective against *E. faecalis* biofilm.<sup>20</sup> As a mucolytic agent, its combination with other antimicrobial substances could result in enhanced efficacy against biofilms.<sup>17</sup> Thus, the aim of this study was to evaluate the efficacy of ALX alone and associated with NAC, in eradicating two *E. faecalis* strain biofilms after 1 and 5 min of exposure time.

## MATERIALS AND METHODS

### Biofilm cultivation

Single biofilms of *E. faecalis* strain ATCC 29212 and *E. faecalis* strain D1<sup>21</sup> isolated from a failed endodontic treatment were grown in the

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MBEC-high-throughput (MBEC-HTP) device (Innovotech, Edmonton, Alberta, Canada), previously described by Ceri et al.<sup>22</sup> and adapted for *E. faecalis*.<sup>23–24</sup> The MBEC-HTP has two parts. The top half is a lid with 96 polystyrene pegs, and the bottom half is a fluted trough that guides inoculated growth medium across the pegs when the device is placed on a rocker. We inoculated the trough with approximately  $1 \times 10^7$  colony forming units (CFUs) per mL of each *E. faecalis* strain suspended in 22 mL of brain heart infusion (BHI; Scharlau Chemie S.A., Barcelona, Spain). The device was then placed on a rocking table (model Swing Sw 8 10000-00015; OVAN, Badalona, Spain) and incubated at 37 °C for 24 h under aerobic conditions at five rocks per minute.

Biofilms forming on the lid of the MBEC-HTP device were rinsed once with 0.9% saline solution for 2 min to remove loosely adherent planktonic bacteria. To verify biofilm formation, four pegs were removed from each device, placed in 200 µL 0.9% saline and sonicated on a water-table sonicator (model 5510E-MT; Branson, Danbury, CT, USA) for 10 min.<sup>23</sup> The disrupted biofilms were diluted serially from  $10^{-1}$  to  $10^{-5}$  and plated for viable cell counting. This growth control was used to evaluate the initial number of bacteria formed in the biofilm in each assay.

#### Root canal irrigants

An initial solution of 2% ALX in 30% ethanol (pH 2.63) was prepared. Nine twofold dilutions were made of this solution in sterile distilled water up to a concentration of 0.007 8% ALX in 0.117% ethanol. The action of ethanol on the biofilms at the concentrations used in the ALX solutions was previously determined, showing that ethanol achieved a reduction of  $\leq 0.5$  logarithmic units at all the concentrations tested, which is considered trivial. To prepare the NAC combined with ALX, we first established the biofilm eradicating ability of NAC at pH 11 with NaOH (Merck, Darmstadt, Germany) and at pH 2.63 without NaOH, both for 1 min. The latter was selected because it demonstrated some antimicrobial activity. NAC (Acofarma, Terrassa, Barcelona, Spain) was dissolved in ethanol at 20% to obtain a final concentration of 100 mg·mL<sup>-1</sup>. All the dilutions were carried out using sterile distilled water and were stored at room temperature until use, for no more than 60 min. To prepare the combinations, dilutions of ALX were mixed 1:1 with NAC 100 mg·mL<sup>-1</sup>. The contact times of the irrigants on biofilms were 1 and 5 min.

#### Susceptibility testing of *E. faecalis* biofilms

The susceptibility tests were done in a microtiter plate known as the challenge plate (Nunclon Delta Surface; Nunc, Roskilde, Denmark). The dilutions of the irrigating solutions were placed along the length of the challenge plate, allowing the first and the last wells of each row to serve as the sterility and growth controls, respectively. The peg lid was submerged in the challenge plate (for 1 and 5 min). After exposure, the biofilms were rinsed twice by placing the lid on two additional microtiter plates containing 180 mL of 0.9% saline solution for 2 min to remove loosely adherent planktonic bacteria, and they were then placed in a microtiter recovery plate with 180 mL of BHI per well and sonicated 10 min. The disrupted biofilms were diluted serially from  $10^{-1}$  to  $10^{-5}$  in 0.9% saline and 10 µL aliquots were plated for viable cell counting. Ten replicates per irrigant concentration, time and enterococci biofilm were performed. The effect of each test agent on the biofilm was determined by calculating the percentage of viable bacteria kill as follows:

$$[1 - (\text{Mean CFU}_{\text{irrigant}} / \text{Mean CFU}_{\text{initial bacterial number}})] \times 100\%$$

The term eradication was used to denote the death of 100% of the bacterial population. When the percentage varied from 100%, the Student's *t*-test was used, previously subjecting data to the Anscombe transformation.<sup>25</sup>

#### RESULTS

The NAC at pH 11 had no antimicrobial effect and at pH 2.63 showed limited antimicrobial effect, with a mean of reduction of 1.14 of logarithmic units. The results of the antimicrobial activity of ALX alone and associated with NAC against both *E. faecalis* strain biofilms are shown in Table 1. Globally, the two strains tested showed very similar sensitivity to ALX alone and combined with NAC. After 1-min contact time, ALX eradicated the biofilms at all concentrations except for 0.007 8% for *E. faecalis* ATCC 29212 and 0.015 6%–0.007 8% for *E. faecalis* D1. When combined with NAC, similar results were obtained regarding eradication/concentration, the exception being ALX at 0.015 6% against *E. faecalis* ATCC 29212; when used alone, it achieved eradication but not when combined. After 5-min contact time, ALX alone and combined with NAC eradicated all enterococci biofilms.

**Table 1 Percentages of reduction of *Enterococcus faecalis* biofilms by alexidine alone and combined with NAC after 1 min of exposure: mean (SD)**

ALX concentrations/%	<i>E. faecalis</i> ATCC 29212		<i>E. faecalis</i> D1	
	Alone	Combined with NAC	Alone	Combined with NAC
2	E	E	E	E
1	E	E	E	E
0.5	E	E	E	E
0.25	E	E	E	E
0.125	E	E	E	E
0.062 5	E	E	E	E
0.031	E	E	E	E
0.015 6	E	99.75 (0.18) <sup>a,1</sup>	99.92 (0.13) <sup>a,2</sup>	99.70 (0.39) <sup>a,1,2</sup>
0.007 8	99.46 (0.57) <sup>1</sup>	97.22 (2.00) <sup>b,2</sup>	99.50 (0.48) <sup>b,1</sup>	99.23 (0.50) <sup>b,1</sup>

ALX, alexidine; E: eradication, 100% bacterial kill; NAC, N-acetylcysteine, 100 mg·mL<sup>-1</sup>.

Read vertically, the same letter shows differences not statistically significant.

Read horizontally, the same number shows differences not statistically significant.

*E. faecalis* ATCC 29212: ALX 0.007 8% vs. ALX 0.007 8%+NAC, *P*<0.001.

*E. faecalis* D1: ALX 0.015 6% vs. ALX 0.015 6%+NAC, *P*=0.104; ALX 0.007 8% vs. ALX 0.007 8%+NAC, *P*=0.163.

## DISCUSSION

This study aimed to evaluate the antimicrobial activity of ALX alone and combined with NAC against two *E. faecalis* strain biofilms. The presence of EPS is one mechanism that may explain why bacteria in the form of biofilm are less sensitive to antimicrobial agents than planktonic forms.<sup>7</sup> EPS acts as a barrier limiting the penetration by the agent.<sup>26</sup> In this sense, the association of an antimicrobial substance—ALX—with an agent of proven mucolytic activity or a potential EPS disruptor such as NAC should enhance antimicrobial efficacy against biofilms.

To carry out these objectives, the MBEC-HTP device<sup>20</sup> was used. It permits the formation of 96 statistically equivalent biofilms on polystyrene surfaces and moreover is easy to use, quick and gives reproducible results.<sup>22,24</sup> In this device, two single *E. faecalis* strain biofilms were grown for 24 h, an ideal time period for obtaining adequate biofilm density.<sup>23</sup> The strains studied were *E. faecalis* ATCC 29212, a strain of reference used in antimicrobial susceptibility studies and a strain isolated from a failed endodontic treatment, *E. faecalis* D1.<sup>21</sup>

Alexidine has been used as an antiseptic in mouthwashes<sup>27</sup> and as a disinfectant in contact lens solutions,<sup>28</sup> although in the latter, it has been associated with a *Fusarium* keratitis outbreak.<sup>29–30</sup> In this study, all ALX dilutions eradicated the biofilms after 5-min contact time, whereas at 1 min, the concentrations that achieved 100% kill of *E. faecalis* ATCC 29212 and *E. faecalis* D1 biofilms were 0.015 6% and 0.031%, respectively. These results evidence that ALX effectiveness is both time- and concentration-dependent, as is CHX,<sup>24</sup> and support the use of irrigants at an optimal time of exposure to ensure the killing of microbial biofilms. The activity of ALX would be comparable to that of NaOCl, which eradicated biofilms of *E. faecalis* grown in the MBEC-HTP device at a concentration of 0.006 25% after 1 min of exposure.<sup>23</sup> However, in view of the fact that ALX is a cationic molecule similar to CHX, the results obtained with ALX are noteworthy, far superior to the results obtained with CHX in a previous study using the same methodology.<sup>24</sup>

Such diverse efficacy may be attributed to structural differences,<sup>9</sup> conditioning dissimilar mechanisms of action. Both solutions disrupt the integrity of the bacterial cytoplasmic membrane, causing leakage of the intracellular contents. However, ALX has greater affinity for lipoteichoic acids than CHX, which might lead to faster permeabilization of the bacterial membrane.<sup>9</sup> Consequently, the bactericidal action would be more rapid,<sup>31</sup> and particularly evident in an exposure period not only of 5 min but of as little as just 1 min.

NAC was selected in light of its mucolytic activity. Although its antimicrobial activity against planktonic bacteria has been demonstrated, its effect on biofilms appears to depend on the microorganism in question.<sup>13–17,19</sup> In fact, Aslam and Darouiche<sup>19</sup> demonstrated an increase of non-viable cells in NAC-treated *Staphylococcus aureus* biofilms, in contrast with a minimal to null anti-biofilm activity against *E. faecalis* biofilms. We found that the antibiofilm activity of NAC is pH-dependent and very scarce with one of exposure, though exposure times of 1 week have served to demonstrate its activity against *E. faecalis*.<sup>20</sup>

Our results indicate that the combination of NAC+ALX does not improve the results of ALX alone. Eradication was obtained with 0.015 6% and 0.031% ALX (for each of the strains), and when combined with NAC it was achieved at 0.031% after 1 min of contact time. We may surmise that NAC did not contribute to the antibiofilm activity. This result could be attributed to the main mechanism of action of NAC. Its ability to disrupt the EPS in biofilms may condition its efficacy in detaching bacteria adhered

to a surface,<sup>16</sup> which would favor a higher bacterial biofilm recovery. As a result, a greater antimicrobial concentration to kill them would be needed.

Overall, no differences were obtained in the sensitivity of the two *E. faecalis* (reference and clinical isolate) strains to the irrigating solutions tested, despite the fact that strain D1 may have undergone environmental stress in a treated root canal, favoring the expression of virulence factors.<sup>32</sup> The similar behavior of the two strains, likewise observed in a previous study using the same methodology,<sup>21</sup> might be due to the fact that they are strains of the same species.

Within the limitations of this *in vitro* study, ALX showed antimicrobial properties against the two *E. faecalis* strain biofilms tested at very low concentrations, and its combined use with NAC was not seen to enhance its activity. Taking into account that sensitivity is species-dependent,<sup>19</sup> future research using other species and multispecies biofilms grown in the complex root canal system and under strictly controlled clinical conditions should be carried out to support these findings.

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