



Efficacy of “HLE”—a multidrug efflux-pump inhibitor—as a disinfectant against surface bacteria

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ABSTRACT

We evaluated the efficacy of a new disinfectant product, HLE, to inhibit multiple species of planktonic and biofilm bacterial cultures. The HLE disinfectant comprised of EDTA, lactic acid and hydrogen peroxide, and our data indicated that the disinfectant had effective antimicrobial and anti-biofilm activity even at low concentrations (0.15% to 0.4% HLE, v/v). Furthermore, the HLE disinfectant destabilized biofilm structures eradicated them due to the synergistic effect of EDTA and both antimicrobials (lactic acid and hydrogen peroxide), as revealed by confocal laser scanning microscopy. Additionally, sub-inhibitory concentrations of HLE disinfectant, with EDTA as an efflux pump inhibitor, inhibited the expression of multidrug EfrAB, NorE and MexCD efflux pumps in both planktonic and biofilm cultures. This could provide an alternative way to disinfect surfaces to avoid spreading multi-drug resistant strains in the food chain and the environment by decreasing efflux pump expression and consequently reducing the antibiotic selective pressure caused by systemic antibiotics and disinfectant use.

1. Introduction

Bacterial biofilms are complex structures comprising of a consortium of multiple species of microorganisms. They prevail as part of the bacterial lifestyle as they contribute a crucial role in protecting bacterial populations from environmental hardships (e.g., exposures to antimicrobials, pH change, osmotic shock, UV radiation and several stresses), and they also provide enhanced nutrient availability, removal of toxic metabolites, and facilitate the acquisition of new genetic traits (Donlan and Costerton, 2002; Kokare et al., 2009). These irreversible microbial aggregations form on most surface types, including: plastic, metal, wood, glass, medical devices, tissues, implants, food products and soil particles. Thus, they become a source of contamination in food preparations, water sources and medical settings.

From the public health perspective, biofilm control in the food industry and medical settings poses an arduous task and responsibility since their presence is associated with increased threat of drug resistance to society and pharmaceutical industries, and thus diminishing the efficacy of chemical treatments and therapy. Several types of bacteria develop their resistance to disinfectants, and other antimicrobial agents, by forming biofilms to limit the diffusion of chemicals through

the exopolysaccharide (EPS) matrix as an effective physical barrier (McDonnell and Russell, 1999). Further, biofilm antimicrobial resistance involves additional multifaceted responses including intrinsic factors, e.g.: the matrix, micro-environments, small sub-populations as persisters, and oxidative-stress responses; and the extrinsic or induced resistance factors, e.g.: increased mutation, increased horizontal gene transmission, production of antibiotic degradative enzymes, targets with lowered affinity, and over-expression of efflux pumps with broad range of substrate targets (Paraje, 2011). Therefore, the eradication and elimination of these highly resistant structures, which serve as protective niches, including for bacterial pathogens, remains a big challenge, and these intrinsic and extrinsic resistance factors work synergistically to enhance their survival. To address this growing problem, new disinfection strategies are required to prevent biofilm formation and reduce (or to avoid) the spread of biofilm-forming bacteria and their resistance genes in different ecosystems.

Current literature reports several disinfection products and their efficacies; however, the over-use of disinfectants containing some biocides such as quaternary ammonium compounds may increase resistance traits (e.g. Buffet-Bataillon et al., 2012; Hegstad et al., 2010). As the biocides diffuse into the biofilm matrix, they generate an

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antimicrobial gradient that promotes differential gene expression and triggering of different antimicrobial-induced factors through the biofilm (Costerton et al., 2003; Macfarlane and Dillon, 2007).

Here, we developed a new disinfection product, HLE, that contains natural substances (hydrogen peroxide, lactic acid and EDTA) and avoids quaternary ammonium compounds or toxic detergents; rather, we strategically aimed to utilize compounds that synergistically eradicate preformed biofilms and inhibit further biofilm establishment on different surfaces. Furthermore, we analysed the effect of HLE on the expression of efflux pump genes, which is a means of spreading antimicrobial resistance in the environment.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Staphylococcus aureus CECT 4468, *Listeria monocytogenes* CECT 4032, *Enterococcus faecalis* S-47, *Bacillus cereus* CECT 5148, *Escherichia coli* CCUG 47553 and *Salmonella* Enteritidis UJ3449 were used in this study on the basis of their pathogenic character, their ability to form biofilms, and their resistance to antimicrobials. Strains were cultured in Tryptone Soya Broth (TSB) (Fluka, Madrid, Spain) at 37 °C for 24 h. Cultures were maintained in 20% glycerol at – 20 °C and – 80 °C for short- and long-term storage, respectively.

2.2. Effect of HLE antimicrobial product on planktonic cell growth

To determine the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC) of HLE (3–6% H₂O₂, 2.2–4.4% lactic acid and 12.5–25 mM EDTA in water), we used the broth micro-dilution method. Overnight bacterial cultures, grown in TSB broth at 37 °C for 24 h, were diluted 1/10 (v/v) in fresh TSB broth and 20 µl were added to each well of 96-well microtiter plates. 180 µl of TSB broth supplemented with HLE at different concentrations (0.25–50%, v/v) were then added to the wells and incubated at 37 °C under aerobic conditions for 24 h. Bacterial growth was evaluated by the presence of turbidity. From wells that lacked turbidity, cells were subjected to viable count determination (CFU/ml; colony-forming units) by plating 10 µl-samples on Tryptone Soya Agar (TSA) and incubated at 37 °C for 24 h. MIC was defined as the lowest concentration of HLE that inhibited visible growth, and MBC was defined as the lowest concentration of HLE that killed bacteria (> 99% removal). Each experiment was done in triplicate.

2.3. Determining the effect of HLE on biofilm development

The anti-adhesion properties of HLE to different bacterial strains (*S. aureus* CECT 4468, *L. monocytogenes* CECT 4032, *E. faecalis* S-47, *B. cereus* CECT 5148, *E. coli* CCUG 47553 and *S. Enteritidis* UJ3449) and a cocktail (mixture) of all strains were tested in microtiter plates. Overnight bacterial cultures, grown in TSB broth at 37 °C for 24 h, were diluted 1/10 (v/v) in fresh TSB broth, and 20 µl were added to each well of the microtiter plate. The wells were then added with 180 µl of TSB broth supplemented with HLE at sub-MIC concentrations (ranging from ½-level of MIC for each strain to its full MIC). Controls without HLE consisted solely of 180 µl of TSB broth. Plates were incubated at 37 °C under aerobic conditions for 24 h, and the wells were then washed with 200 µl of phosphate buffered saline (PBS). The anti-adhesion activity of HLE was determined by staining the washed wells with 100 µl of 1% (w/v) crystal violet and allowing them to incubate at room temperature for 15 min. Then, 200 µl PBS were added to the wells, and the absorbance at 590 nm was determined using a microplate reader (iMark Microplate Absorbance Reader, Bio-Rad instrument). The percentage of inhibition of biofilm formation was determined using the following formula as described by Zmantar et al. (2017):

$$\frac{OD_{590} \text{ growth control} - OD_{590} \text{ sample}}{OD_{590} \text{ growth control}} \times 100\%$$

2.4. Antimicrobial effect of HLE on preformed biofilms

Inoculum of 1% of each bacteria and the cocktail of all strains in TSB was used for the preparation of biofilms, which were grown in 96-well microtiter plates for 24 h at 37 °C. After incubation, the culture broth containing non-adhered bacteria was removed and the wells were then washed with sterile PBS. The biofilms were treated with HLE (100%) for different time periods (5, 10, 15, 20 and 30 min) at room temperature. After treatments, HLE was removed and the wells were incubated with 200 µl of D/E Neutralizing broth (Difco, Barcelona) for 5 min at room temperature and then they were washed with 200 µl of PBS. Biofilms were resuspended in 200 µl PBS and serially diluted (with PBS) before plating on TSA. The plates were incubated at 37 °C for 24 h for the determination of CFU/ml.

2.5. Microscopic evaluation of HLE effects on biofilms

Imaging of HLE-treated biofilm was done by using LIVE/DEAD BacLight™ (Thermo Fisher Scientific, Waltham, MA, USA) and a confocal laser scanning microscope (LEICA TCS-SP5, Mannheim, Germany) equipped with the Plan-Apochromat 63 × /1.4 objective. After biofilm cultivation on a microtiter plate (200 µl) as described above, some wells were not treated with HLE (Control) and the other ones were subjected to HLE treatment for 5 and 10 min at room temperature (HLE-treated samples), washed with sterile PBS and resuspended in 50 µl PBS. Then, 20 µl of the suspensions (Control and HLE-treated) were amended with 0.5 µl of LIVE/DEAD stain, subsequently spotted on a glass slide, and imaged using a confocal laser scanning microscope. Alternatively, preformed biofilms on sterile glass slides were subjected (or not, as controls) to the effect of HLE for 5 and 10 min durations at room temperature as described above; however, staining was done directly on the slides and then imaged using a confocal laser scanning microscope.

2.6. PCR amplification to detect efflux-pump genes

Total DNA extractions were done using ZymoBIOMICS DNA Miniprep Kit (Zymo Research, California, USA) according to the manufacturer's instructions. DNA quantification and quality assessment were carried out by using a NanoDrop 2000 spectrophotometer (Thermo Scientific). PCR amplification of well-known structural genes of efflux pumps (*EfrAB*, *AcrA*, *NorA*, *NorE*, *MefA*, *QacC*, *YvcC*, *EvgA*, *MexAB*, *MexCD*, *MexXY*) was done as described elsewhere (Oh et al., 2004; Lee et al., 2003; Nishino and Yamaguchi, 2002; Patel et al., 2010; Smith and Hunter, 2008; Steinfels et al., 2004; Sutcliffe et al., 1996; Swick et al., 2011).

2.7. Effect of sub-inhibitory HLE concentrations on efflux-pump gene expression

Six bacterial strains (1%) were each dosed (or not, as a control) with ½-level of MIC of HLE in TSB broth (2 ml) and then incubated for 18 h at 37 °C in either sterile tubes (for planktonic cell growth) or in 24-well microtiter plate for biofilm formation. RNA extraction was done using Direct-zol™ RNA Miniprep (Zymo Research, California, USA) according to the manufacturer's instructions. RNA quantification and quality assessment were carried out by using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNAs were adjusted to a concentration of 500 ng/ml and frozen at – 80 °C until required for analysis.

The expression of *efrA* and *efrB* genes (coding for *EfrAB*), and *norE* (coding for *NorE*) gene by both the planktonic cells and biofilms (controls and treated samples with ½-MIC and ¼-MIC of HLE) was determined by quantitative, real-time PCR (qRT-PCR) using

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