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.

Keywords:

Bacterial biofilm; Pathogens; Disinfectant; Antimicrobials; Efflux pumps.

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., 2008). 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

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% H_2O_2, 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 37ºC 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 37ºC 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 37ºC 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 37ºC 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 37ºC 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):

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

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 37ºC 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 63x/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 37ºC 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 ºC 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 ¾-MIC of HLE) was determined by quantitative, real-time PCR (qRT-PCR) using SensiFAST^{TM} SYBR & Fluorescein One-Step Kit (BIOLINE). Phenylalanyl-tRNA synthase alpha-subunit (*pheS*) gene was used as a housekeeping gene, and a no-template control (NTC) was used as negative control. Primers and annealing temperatures used in this study are described in Table 1. Quantitative PCRs (qPCRs) were performed in triplicate on a CFX96 TouchTM Real-Time PCR Detection System from BioRad using 2 Power SYBR green chemistry. PCR-grade water served as a negative control.

2.8. Statistical analysis

All analyses were performed in triplicate. Statistical analyses were conducted using Excel 2007 (Microsoft Corporation, Redmond, Washington, US) program to determine means and standard deviations. Statistical evaluation of the effect of HLE on biofilm development assays were conducted by analysis of variance (*ANOVA*) using Statgraphics Centurion XVI software (Statpoint Technologie, Warrenton, Virginia, US). The same software was used to perform Shapiro–Wilk and the Levene tests to check data normality and to perform two-sided Tukey's multiple contrast to determine the pair-wise differences between strains, where level of significance was set at *P*-value of < 0.05.

3. Results

3.1. Antimicrobial activity of HLE on planktonic cells

Table 2 shows the MICs and MBCs of HLE for the different bacterial strains used in this study and also the cocktail (mixture) of six bacterial strains. Planktonic cells had great susceptibilities, exhibiting lower MICs ranging from 0.15% to 0.4% HLE (v/v), with *L. monocytogenes* CECT 4032 and *E. faecalis* S-47 being the most susceptible strains. *S. aureus* CECT 4468 was the least susceptible (Table 1). The MBC values very similarly ranged from 0.2% to 0.5% HLE (v/v). As such, the HLE disinfectant effectively inhibited and killed Gram-positive and Gram-negative bacteria; furthermore, the cocktail comprising of the six bacteria was also inhibited by a low concentration of HLE (0.5%) (Table 2).

3.2. HLE inhibition of biofilm formation

Strong inhibition of biofilm development was achieved using the MIC of HLE for each bacterial strain/cocktail, and the results showed 80-91% inhibition of developing biofilm was achieved for individual strains and also the cocktail (Table 3). Furthermore, the use of ½-level of MIC of HLE provided 33-50% inhibition of biofilm development (Table 3). These results indicate that HLE impacted bacterial adherence to polystyrene depending on the bacterial strain (Table 3).

3.3. Evaluation of antimicrobial effect of HLE on biofilms

Preformed biofilms (24 h) of each bacterial strain were exposed to HLE for several contact times (5, 10, 15, 20 and 30 min) in microtiter plates. The results demonstrated that HLE had bactericidal effects against all bacterial strains at all contact times (5 - 30 min.; Fig. 1). Furthermore, confocal microscopy revealed that HLE decreased the viability of many cells (stained in green) in treated biofilms (5 and 10 min; Fig. 2B-D) versus untreated controls (Fig. 2A); as such, only dead cells (stained in red) were visualized (Fig. 2C and D).

Multi-species biofilms—formed on glass slides, treated with HLE, and stained with LIVE/DEAD BacLight^{TM}—exhibited strong susceptibility to the bactericidal effect of HLE. Figure 3 shows that the HLE-treated biofilms exhibited larger channels amongst the bacterial aggregates, and no viable cells were observed after 10 min exposure to HLE, when compared with the control (Fig. 3).

3.4. Effect of HLE on efflux-pump gene expression

PCR amplification of different genes related to efflux pumps revealed only the presence of *efrA* and *efrB* in all bacteria; however, *norE* and *mexD* genes were detected in some strains (*norE* gene was detected in all bacteria except *L. monocytogenes* CECT 4032 and *B. cereus* CECT 5148; *mexD* gene was detected in all bacteria except *S. aureus* CECT 4468). Thus, to check whether a low concentration of HLE had any inhibitory effect, mRNA levels from *efrA*, *efrB, norE* and *mexD* genes were quantified from both planktonic and biofilm cells. The results showed differential expression of *efrA*, *efrB, norE* and *mexD* genes depending on the physiological state of the bacteria (planktonic or biofilm). In general, the expression of efflux-pump genes in biofilms was remarkably lower than those in related planktonic cells, which were 50x higher when not treated with HLE (data not shown). Furthermore, sub-inhibitory concentrations (½-level of MIC and ¾-level of MIC) of HLE inhibited the expression *efrA*, *efrB, norE* and *mexD* genes in planktonic cells, with the differences in mRNA levels being statistically significant (Fig. 4A). Similarly, biofilms formed in the presence of sub-inhibitory concentrations (½-level of MIC and ¾-level of MIC) of HLE had decreased expression of all multidrug efflux pump genes, versus the controls without HLE exposure (Fig. 4B).

In all cases, the down-regulation of EfrAB, NorE and MexCD efflux-pump gene expression caused by HLE was more remarkable in planktonic cells than biofilms (Fig. 4). However, the HLE impacted gene expression in all cells.

4. Discussion

A biofilm's increased tolerance to antimicrobial agents (e.g., biocides and antibiotics) represents a serious problem in the food industry and medical settings, which leads to substantial economic and health concerns related to contamination and infections (Bridier et al., 2011; Davies, 2003; Stewart, 2015). Biofilm formation occurs within both the natural and medical environments, and their tolerance to antimicrobials has been largely documented by *in vitro* and *in vivo* studies (Davis et al., 2008; Sabir et al., 2017). Food processing plants provide conditions for biofilm proliferation (e.g., abundant source of nutrients), and nowadays there are no strategies that inactivate or completely remove biofilms without undesirable effects (Simões et al., 2010). Furthermore, multi-species biofilms complicate effective eradication due to synergistic interactions among microbes and their enhanced tolerance/virulence. Thus, the eradication or control of biofilm formation remains an important issue, especially when biofilms are pathogenic.

As such, we investigated HLE disinfectant on growing and mature biofilms comprised of Gram-positive (spore-forming and non spore-forming bacteria) or Gram-negative bacteria, and a combination of both. Firstly, low concentrations of HLE demonstrated to be inhibitory (MIC of 0.15% to 0.4% HLE, v/v) and bactericidal (MBC of 0.2% to 0.5% HLE, v/v) to planktonic cells. Preformed biofilms, however, required greater HLE concentrations and contact times (data not shown). Key factors contributing to biofilms' increased resistance versus planktonic cell state include the physiological heterogeneity of cells (e.g., those that are growing, stress-adapted, dormant, inactive), differences in gene expression, low diffusion of antimicrobials through the matrix, or the direct interaction of exopolysaccharides with antimicrobials. As reviewed by Mah and O´Toole (2001), cells within a biofilm can become 10–1000 times more resistant to antimicrobial agents; so, susceptibility differences between cells in plankton versus biofilm states were not surprising, but in this experiment they were (by comparison to aforementioned range in literature) relatively minimal. Thus, we demonstrated the effective eradication of preformed biofilms of *Staphylococcus aureus* CECT 4468, *Listeria monocytogenes* CECT 4032, *Enterococcus faecalis* S-47, *Bacillus cereus* CECT 5148, *Escherichia coli* CCUG 47553 or *Salmonella* Enteritidis UJ3449 and also the cocktail (a mixture of the six strains) with HLE treatment after 5 min at room temperature. Additionally, the HLE disinfectant inhibited biofilm formation at lower concentrations: 33-50% inhibition at ½-level MIC or 80-91% at full MIC (0.15%

to 0.4% HLE, v/v). As such, surface biofilm formation could be prevented by the application of HLE disinfectant at low concentration.

The composition of the HLE disinfectant in this study consisted of 3-6% hydrogen peroxide, 2.2-4.4% lactic acid and 12.5-25 mM EDTA, and the contact time for complete eradication of individual and multispecies biofilms was 5 min at room temperature (Submitted Patent OEPM P201731462).

Lactic acid and hydrogen peroxide are amongst the antimicrobial substances commonly produced by lactic acid bacteria (LAB), in addition to bacteriocins, other organic acids and diacetyl (Salminen, 1995). Lactic acid, as the unique or the predominant fermentation product in homo-fermentative and hetero-fermentative LAB (respectively), plays a crucial role in food preservation with concentrations up to 8% in fermentation process (Urbonaviciene et al., 2015), and the antimicrobial properties of lactic acid have been widely reported to inhibit bacterial growth by disrupting cytoplasmic membranes, leading to the loss of proton motive force and leakage of intracellular ions (e.g., Alakomi et al., 2000; Ricke, 2003). Similarly, Wang et al. (2015) indicated that 0.5% lactic acid sufficiently inhibits the growth of planktonic *Salmonella* Enteritidis, *E. coli* and *L. monocytogenes*; further, they observed the release of intracellular proteins and suggested that lactic acid caused physiological and morphological changes in bacterial cells. Furthermore, from the safety point of view, lactic acid is not carcinogenic and does not pose any chronic risk to human health or the environment (Boomsma et al., 2015).

Regarding hydrogen peroxide, it denatures many enzymes and protein thiol groups; it also causes the peroxidation of membranes, which leads to increased permeability and loss of cell integrity (Denyer and Stewart, 1998). Its mode of action depends on the target microorganism (Araújo et al., 2011); the biocide may act on the cytoplasm of bacteria, on the core of a bacterial spore, the ribosome of fungi, and the thiol groups of a virus. However, several studies have reported the high resistance of biofilms (Khakimova et al., 2013; Leung et al., 2012) due to catalase-producing bacteria that may exist in the matrix and thus neutralize the hydrogen peroxide. Interestingly, *S. aureus*, *L. monocytogenes*, *E. coli* and *B. cereus*, used in this study, represent catalase-producing bacteria; however, the synergistic effect of all antimicrobials in HLE still led to effective eradication of their biofilms. On the other hand, several studies have shown that hydrogen peroxide, or any other biocide alone, is ineffective in eliminating biofilms

completely (Lin et al., 2011; Smith and Hunter, 2008), suggesting combination of approaches is often required for effective biofilm control.

EDTA is a known as metal-chelating agent, which destabilizes bacterial cell walls and biofilms by sequestering divalent cations (i.e., calcium, magnesium, zinc, and iron) required for growth and maintenance of cell structure, thus impacting several other cellular processes. For those reasons, its incorporation into several antimicrobial formulations with alcohol, antibiotics, organic acids, quaternary ammonium compounds, iodine and surfactants often improved their efficacy against biofilms (Kite et al., 2005; Lambert et al., 2004). In this study, the inclusion of EDTA as an antimicrobial and anti-biofilm agent (Finnegan and Percival, 2015) has shown a synergistic effect with the other compounds in the HLE (i.e., lactic acid and hydrogen peroxide), since individual components of HLE showed less antimicrobial effect on multi-species biofilms, i.e., less than 50% biofilm eradication when compared with HLE after 5-min exposure (data not shown). Thus, the synergistic effect of all HLE components contributed to the effective elimination of mono- and multispecies biofilms after 5-min exposure at room temperature, inhibition of biofilm (80-91%) development at 0.15% to 0.4% HLE v/v, and also complete eradication of pre-formed biofilms.

Regarding the effect of HLE on biofilm structure, confocal laser scanning microscopy revealed that treated biofilms had a greater porous structure with larger channels among the cell clusters versus the control. This suggests that the HLE disinfectant produces a biofilm structure with greater clusters of dead bacteria.

Furthermore, it has been reported that EDTA has an inhibitory effect on multidrug efflux pumps (i.e., as an efflux pump inhibitor, "EPI") in *E. coli*, *Pseudomonas aeruginosa* and *Enterococcus* sp. by decreasing their gene expression, and thus allowing other antimicrobial agents to accumulate in the bacterial cells and resulting in greater impairment on cellular functions (Chaudhary and Payasi, 2012; Chaudhary et al., 2012; Lavilla Lerma et al., 2014). In this study, we explored the effect of sub-inhibitory concentrations (i.e., ½- and ¾-levels of MIC) of HLE (containing EDTA as an EPI) on the expression of genes encoding for EfrAB, NorE and MexCD multidrug efflux pumps. Our data revealed that sub-inhibitory concentrations of HLE decreased the expression of EfrAB, NorE and MexCD MDR efflux pumps. This study represents the first report describing the role of EDTA as an EPI on NorE and MexCD pumps; in this manner, it has been shown that EDTA acts as a multi-drug-resistance (MDR), efflux-pump inhibitor for: NorE—a member of the major facilitator superfamily (MFS);

EfrAB–an ATP-binding cassette (ABC) pump; and MexCD—which belongs to RND-family drug efflux pumps. It was reported by Kvist et al. (2008) that other EPIs such as NMP, PAßN and Thioridazine blocks the activity of several efflux pumps (AcrAB, AcrEF, MexAB, MexCD, MexEF, NorA) and also biofilm formation in many bacteria. Further, it was reported by Lavilla Lerma et al. (2014) that EfrAB, a multidrug efflux pump, was generally implicated in the resistance of different antibiotics and biocides in enterococci isolated from fermented foods, and 3-mM EDTA effectively down-regulated its gene expression and reduced the MICs of almost all antibiotics tested against the bacteria. As such, the EDTA present in HLE can diminish efflux-pump gene expressions in multiple cultures and synergistically enhance the antibacterial effects of lactic acid and hydrogen peroxide. As such, EfrAB, NorE and MexCD efflux pumps could be attractive targets for inhibition, not only in the food industry, but also in medical setting.

Bacterial biofilms and planktonic cells exhibit differences in growth and metabolic rates, and also the regulation of many genes including those encoding for efflux pumps as strategy for waste management. In this sense, Zhang and Mah (2008) and Kvist et al. (2008) showed that efflux pumps were highly active in bacterial biofilms, as they exude toxic metabolites that accumulate when cells grow in close proximity; unfortunately this contributes to their antimicrobial resistance. As such, EDTA inhibiting efflux pumps and impacting biofilm formation could have lasting effects on the bacteria and help prevent resistance development. Although disinfection may be accomplished with diluted HLE (½-level of MIC) and the subsequent growth inhibition is not completely achieved, the spread of multidrug resistant strains could be avoided by consequently increasing the susceptibility of microbes to other antimicrobials. In this study, HLE effectively eradicates multi-species biofilms; at sub-inhibitory concentrations, it impacts biofilm formation and efflux pump expression, thus decreasing the risk of antibiotic-selective pressures caused by the sole use of systemic antibiotics or disinfectants.

5. Conclusions

The present study demonstrated that HLE disinfectant effectively inhibited planktonic cells and biofilm establishment with low concentrations of HLE. Furthermore, HLE disinfectant eradicated preformed biofilms and destabilized their structure. Further, sub-inhibitory concentrations of the disinfectant inhibited the

expression of multidrug EfrAB, NorE and MexCD efflux pumps, which could represent a good alternative to avoid the spread of these multidrug resistant bacteria in the food chain and also the environment, and consequently minimizing the selective pressures by the use of systemic antibiotics and disinfectants.

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Figure legend

Figure 1. Antibacterial activity of HLE (100%) on mono- and multi-species biofilms (the cocktail of six bacteria: *Staphylococcus aureus* CECT 4468, *Listeria monocytogenes* CECT 4032, *Enterococcus faecalis* S-47, *Bacillus cereus* CECT 5148, *Escherichia coli* CCUG 47553 and *Salmonella* Enteritidis UJ3449) as determined by viable count (Log_{10} CFU/ml) after 0 min (Control), 5 min (T5), 10 min (T10), 20 min (T20) and 30 min (T30) exposure at room temperature.

Figure 2. Confocal laser scanning microscopy images of multi-species biofilms after treatment with HLE disinfectant, suspension and staining with the BacLight Live/Dead viability kit (Invitrogen). The treated biofilms with HLE (100% v/v) disinfectant during 0 min (A, Control), 2 min (B), 5 min (C) and 10 min (D) HLE exposure at room temperature were resuspended in PBS and stained. All images were obtained using confocal microscope (x63 objective) and digital zoom of 2.5x (A and D), 1.5x (C) and 1x (B).

Figure 3. Confocal laser scanning microscopy images of multi-species biofilms (grown on glass slides) after treatment with HLE disinfectant and staining with the BacLight Live/Dead viability kit (Invitrogen). The biofilms were treated with HLE (100% v/v) disinfectant during 0 min (A, Control), 5 min (B) and 10 min (C) HLE exposure at room temperature. All images were obtained using confocal microscope (x63 objective; 3x digital zoom) (A, B and C). The arrows indicate the channels among the bacterial clumps.

Figure 4. Effect of sub-inhibitory HLE concentrations (½-level of MIC and ¾-level of MIC) on the expression of EfrAB, NorE and MexCD efflux-pump genes in planktonic cells (A) and biofilms (B).