Hurdle technology based on the use of microencapsulated pepsin, trypsin and carvacrol to eradicate Pseudomonas aeruginosa and Enterococcus faecalis biofilms

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ABSTRACT
The biofilm lifestyle plays a major role in the resistance and virulence of Pseudomonas aeruginosa and Enterococcus faecalis. In this study, two microencapsulated proteases (pepsin ME-PEP and trypsin ME-TRYP) were evaluated for their biofilm dispersal activity and their synergistic effect with microencapsulated carvacrol (ME-CARV). Spray-drying was used to protect enzymes and essential oil and enhance their activities. Cell count analysis proved the synergistic activity of enzymes and carvacrol treatment as biofilms were further reduced after combined treatment in comparison to ME-CARV or enzymes alone. Furthermore, results showed that sequential treatment in the order ME-TRYP - ME-PEP - ME-CARV resulted in more efficient biofilm removal with a maximum reduction of 5 log CFU mL\(^{-1}\) for P. aeruginosa and 4 log CFU mL\(^{-1}\) for E. faecalis. This study proposes that the combination of microencapsulated proteases with ME-CARV could be useful for the effective control of P. aeruginosa and E. faecalis biofilms.

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Introduction
The operating environments in the food and medical sectors allow bacteria to adhere to surfaces, resulting in the potential development of resistant pathogenic bacterial biofilms. These pathogenic structures are involved in several foodborne diseases and health-care associated infections (Hall-Stoodley et al. 2004; Alav et al. 2018). Pseudomonas aeruginosa has become an important model organism in the study of bacterial biofilm formation. This bacterium is an opportunistic pathogen for humans that can induce life-threatening infections in patients who have compromised immune systems (Moradali et al. 2017). In addition, the implication of P. aeruginosa in food spoilage has been reported (Raposo et al. 2016). Enterococcus faecalis is another opportunistic biofilm-forming pathogenic bacterium. It can survive under arduous conditions, including high concentrations of salt and a wide range of temperatures (10°C to 45°C) (Arias and Murray 2012). E. faecalis is widely spread in nature and the gastrointestinal tracts of humans, animals and insects. It is a good indicator of faecal contamination of water and food. In addition, this bacterium can cause health-care associated infections (Tornero et al. 2014; Shridhar and Dhanashree 2019).

Bacterial cells in biofilms secrete extracellular polymeric substances (EPS), consisting mainly of polysaccharides, proteins, DNA, and lipids. They form a viscous film that encloses the bacterial cells (Flemming and Wingender 2010; Simøes et al. 2010). The EPS matrix is structurally and functionally complex, which plays a major role in biofilm formation, survival, and development. It provides not only a barrier of protection against external stresses, but also a nutrient and enzyme source, and an intercellular connection (Pinto et al. 2020). Furthermore, the EPS matrix ensures the high antimicrobial resistance of biofilms (Pinto et al. 2020). Consequently, a new strategy for the efficient inactivation of bacterial cells in biofilms is needed. Approaches using enzymes that degrade the EPS matrix to remove biofilms have
already been investigated (Kim et al. 2013). Many studies have highlighted the destructive and destabilizing power of protease-based enzymes when used against several bacterial biofilms (Fagerlund et al. 2016; Mohamed et al. 2018; Lim et al. 2019; Jee et al. 2020; Baidamshina et al. 2021).

However, due to the complexity of biofilms, the single use of enzymes is often insufficient for eradicating an entire biofilm (Yuan et al. 2021). In addition, treatment with enzymes may result in the contamination of other areas with dispersed bacteria, thus allowing the redevelopment of neoformed biofilms. Therefore, it is essential to use a combination of enzymes and other active agents (Hurdle technology) to attack different targets within biofilms (EPS matrix and biofilm cells) (Borges et al. 2020). The combination of enzymes and biobased antimicrobials would provide a promising approach to control biofilms. The enzymes destroy and destabilize the EPS matrix of the biofilm and thus the bacterial cells can be more effectively killed by the antimicrobials. It has been reported that the combined use of EPS-degrading enzymes and biocides is a promising approach for potential applications in biofilm control (Wang et al. 2016; Rodriguez-López et al. 2017; Zhou et al. 2018; Lim et al. 2019; Baidamshina et al. 2021). Currently, biobased antimicrobial substances have attracted attention due to their high efficacy, safety, and nontoxic effects (Nazzaro et al. 2013). Carvacrol, a monoterpenoid phenol, is a major component of Origanum vulgare essential oil (EO) and can be found in several other EOs such as Thymus vulgaris and Satureja bachtiarica (Trevisan et al. 2018). Several studies have demonstrated its anti biofilm activity on various surfaces (Amaral et al. 2015; Trevisan et al. 2018; Walczak et al. 2021). However, essential oils are generally volatile and highly vulnerable to photolysis and oxidation. The low water solubility of these active compounds can also minimize their antimicrobial efficacy (Gharsallaoui et al. 2007; Shan et al. 2007; Liu et al. 2018). In addition, the activity of enzymes is highly influenced by environmental factors, such as temperature and pH. Another disadvantage of using enzymes is their self-degradation, which causes their instability (Cordeiro and Werner 2011).

Encapsulating these active molecules could be a good strategy for overcoming these limitations. Microencapsulation is a good tool to increase stability and reduce water immiscibility of EOs and maintain high enzymatic activity and stability during long storage time (Tikhonov et al. 2021; Mechmechani et al. 2022). This technique allows the controlled release of these two active compounds and reduces their physicochemical interactions with the biofilm matrix components, often associated with a decrease in their biological activity.

In the present work, novel microcapsules of proteolytic enzymes, pepsin and trypsin, and carvacrol using spray-drying were engineered. The physicochemical properties and microscopic morphology of the developed microcapsules were also characterized. In addition, the single and combined effect of enzyme dispersive activity and antimicrobial activity of carvacrol were investigated against P. aeruginosa and E. faecalis biofilms.

Materials and methods

Bacterial strains and reagents

The target microorganisms used in this study were Enterococcus faecalis (isolated from French cheese) and Pseudomonas aeruginosa (CIP 103467). The strains were stored at −80°C in tryptic soy broth (TSB; Biokar Diagnostics, France) supplemented with 40% (v/v) glycerol. Pectin was purchased from Cargill (Bauppe, France). Maltodextrins DE 19 (dextrose equivalent value of 19) were obtained from Roquette-freres SA (Lestrem, France). Pepsin was obtained from MP Biomedicals (Strasbourg, France, CAS number: 9001-75-6, EC number 232-629-3). Trypsin was obtained from Sigma-Aldrich (St Quentin Fallavier, France, CAS number: 9002-07-7, EC number:232-650-8). Carvacrol (98% purity), analytical grade imidazole (C2H4N2), acetic acid, hydrochloric acid (HCl), sodium hydroxide (NaOH), and ethanol were obtained from Sigma-Aldrich (St Quentin Fallavier, France). Glycine HCL buffer (100 mM, pH 3) was used to prepare the pepsin solution, potassium phosphate buffer (PPB; 100 mM, pH 7.6) was used to prepare the trypsin solution, and distilled water was used in the preparation of the all-other solutions.

Microencapsulation of enzymes and carvacrol

To prepare carvacrol emulsions, carvacrol and sodium caseinate were dissolved in water then mixed with maltodextrin powders and homogenized using Ultra Turrax PT 4000 homogenizer (Polytron, Kinematica, Switzerland) for 5 min at 18,000 rpm to obtain spray-drying feed emulsions. To prepare enzyme solutions, pepsin or trypsin was mixed with pectin and maltodextrin powders in imidazole-acetate buffer (5 mmol L−1). All solutions were prepared under stirring at room temperature until complete hydration. Then,
the pH of each solution was adjusted to 7 with HCl (0.1 or 1.0 mol L\(^{-1}\)) or NaOH (0.1 or 1.0 mol L\(^{-1}\)). The concentrations of the prepared solutions were as follows: carvacrol: 1% (w/w), sodium caseinate: 0.5% (w/w) and maltodextrins: 20% (w/w) for carvacrol microencapsulation and pepsin: 0.2% (w/w), trypsin: 0.2% (w/w), pectin: 0.2% (w/w), and maltodextrins: 20% (w/w) for enzyme microencapsulation. Solutions were stored at room temperature and pH was readjusted to 7 before use.

Carvacrol emulsions and enzyme solutions were stirred for 30 min and then spray-dried using a lab-scale device equipped with a 0.5 nm nozzle atomizer (Mini Spray-Dryer Buchi B-290, Switzerland) (Wang et al. 2022). The operating conditions of the drying process were as follows: inlet air temperature 180 ± 2°C, outlet air temperature 80 ± 5°C, feed flow rate 0.5 L h\(^{-1}\) and air pressure 3.2 bar. After spray-drying, the amount of carvacrol in dried microcapsules was calculated to be 4.65% and that of enzymes to be 10%. Microcapsules without enzymes and carvacrol were also prepared and used as negative controls. The resulting powders were gathered separately and stored at 4°C until use (Mechmechani et al. 2022).

Zeta potential analysis

The electric charge known as zeta potential (\(\zeta\)-Potential) of carvacrol emulsion droplets at pH 7 was measured in a zetameter (Zetasizer Nano ZS90, Malvern Instruments, Malvern, UK). If required, the samples were suspended in water or imidazole-acetate buffer of the corresponding pH and slightly agitated before analysis. The measurements were performed in triplicate at 25°C, and the average \(\zeta\)-Potential values were expressed in millivolts (mV).

Scanning electron microscopy observation of particles

To analyze the inner and outer structures of the prepared microcapsules, a scanning electron microscope (SEM; model JSM-7800FLV, JEOL, Japan) was used with a scanning voltage of 3 kV. The experiment was performed according to Khelissa et al. (2021a) with some modifications. The outer structures of the microcapsules containing carvacrol and enzymes were studied by depositing a simple layer of each dry microcapsule on a sample holder with a double-sided adhesive (Agar Scientific Oxford). For the study of the inner structure of the microcapsules, the powder of carvacrol and enzymes microcapsules was crushed by moving a razor blade perpendicularly through a layer of microcapsules in a glass Petri dish. Before SEM analysis, samples were critical point dried and coated with a thin layer of carbon using a precision etch coating system (PECS; Gatan model 682) to obtain electrical conductivity.

Biofilm formation assay

Bacteria were pre-cultured before use by inoculating 100 µL of the stored frozen strains in 5 mL of TSB medium and incubating for 24 h at 37°C. Subsequently, 100 µL of the pre-culture was utilized to inoculate 50 mL of TSB medium and incubated for 16 h at 37°C with agitation at 160 rpm to prepare the cultures. Cells were pelleted by centrifugation (5,000 g, 5 min, 20°C), then harvested cells were washed twice with 20 mL of PPB (100 mM, pH 7), resuspended in PPB and finally dispersed by sonication (37 kHz, 5 min, 20°C) (Elmasonic S60H, Elm@). Stainless steel (SS) slides (304 L, Equinox, France) of 41 mm diameter and 1 mm thickness were used for biofilm development. Before use, slides were immersed in 95% ethanol (Fluka, Sigma-Aldrich, France) overnight, then rinsed with distilled water. After rinsing, the slides were soaked in 1% DDM ECO detergent (ANIOS, France) for 15 min at 20°C. Then, the slides were vigorously washed with distilled water five times for 1 min, and again three times with ultrapure water (Milli-Q® Academic, Millipore, France) to completely remove the detergent traces. Finally, the SS slides were air-dried before being autoclaved at 121°C for 20 min (Abdallah et al. 2015).

The sterile slides were placed in a sterile static biofilm system, called the NEC biofilm system, as previously described by Abdallah et al. (2015). The formation of biofilms was initiated by depositing 3 mL of bacterial suspension (10⁶ CFU mL⁻¹) of P. aeruginosa and E. faecalis on the SS slides in each reactor and incubated at 20°C for 1 h, under static conditions, to allow the adhesion of bacterial cells. Then the 3 mL were discarded and the slides were washed twice with PPB to remove loosely adhered cells. The slides were subsequently covered with 5 mL of TSB medium and the closed systems were incubated for 24 h at 37°C. After the biofilm formation, the old TSB medium was removed and the biofilm-covered slides were rinsed twice with PPB to discard planktonic cells.
Combination of microencapsulated carvacrol and enzymes for the treatment of biofilms

The effect of a single and combined antimicrobial and enzyme treatment against 24-hour-old *P. aeruginosa* and *E. faecalis* biofilms was evaluated by the quantification of biofilm cells. For enzymes treatment, rinsed biofilms were treated with 3 mL of PPB containing 1 mg mL⁻¹ of ME-TRYP or glycine HCL buffer containing 1 mg mL⁻¹ of ME-PEP, individually or sequentially, and incubated for 1 h at 37°C for each treatment. For ME-CARV treatment, the slides were placed in 3 mL of ME-CARV solution at a final concentration of 0.625 mg mL⁻¹ for *P. aeruginosa* and 0.312 mg mL⁻¹ for *E. faecalis* (corresponding to the 1/2 minimal inhibitory concentration (MIC) of ME-CARV against each bacterium) and incubated for 1 min for *P. aeruginosa* and 5 min for *E. faecalis* at 20°C (corresponding to the time kill of ME-CARV against each strain). These MIC and time-kill values were chosen based on a previous study (Mechmechani et al. 2022). For combined microencapsulated enzymes and carvacrol treatment, biofilms were treated with enzymes, individually or sequentially, followed by treatment with ME-CARV and incubated for the required time of each strain. Biofilms treated with glycine HCL and PPB without enzyme served as the control for enzymes, and biofilms treated with tryptone salt broth (TS) served as the control for ME-CARV. The antimicrobial action of ME-CARV was stopped by immersing the slides in 5 mL of neutralizing solution (Toté et al. 2010). Afterwards, the slides were rinsed once with PPB and vortexed for 30 s in 20 mL TS broth using a sterile 100 mL pot, followed by sonication for 5 min (37 kHz, vortexed for 30 s in 20 mL TS broth using a sterile

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Epifluorescence microscopy analysis

To analyze the antimicrobial activity of ME-CARV, rinsed slides were treated with ME-CARV solution at a final concentration of 0.625 mg mL⁻¹ for *P. aeruginosa* and 0.312 mg mL⁻¹ for *E. faecalis* and incubated for 1 min for *P. aeruginosa* and 5 min for *E. faecalis* at 20°C. Then, the slides were placed in new Petri dishes and the action of the biocide was blocked by applying 3 mL of neutralizing solution on the surface of the slides (Toté et al. 2010). Control biofilms were treated with tryptone salt broth. The treated biofilms were stained with the LIVE/DEAD BacLight kit (Invitrogen Molecular Probes, USA) for 15 min in the dark according to the manufacturer’s instructions. In addition, to analyze the dispersive activity of enzymes, rinsed slides were treated with ME-PEP and ME-TRYP solutions, individually or sequentially, at a final concentration of 1 mg mL⁻¹ and incubated at 37°C for 1 h, for each treatment. Control biofilms were treated with tryptone salt broth. Control biofilms were treated with tryptone salt broth. Control biofilms were treated with tryptone salt broth.

Statistical analysis

Each experiment was repeated at least three times. Statistical significance was determined by GraphPad Prism 9.0 software using one-way ANOVA (Tukey’s method). Values of *p* < 0.05 were considered statistically significant.

Results

Zeta potential analysis

During carvacrol microcapsules preparation, the emulsions were stabilized with sodium caseinate at pH 7. This emulsifier is a milk protein with an isoelectric point around 4.5 and a negative charge above this value due to the ionization of carboxyl groups (COOH → COO⁻). Thus, the average ζ-potential measured for the carvacrol emulsion was −25.65 mV. In general, absolute ζ-potential values of 30 mV can be considered as high to achieve good stability because higher absolute values indicate higher repulsive forces between dispersed droplets and therefore lower collision and aggregation frequencies that increase particle stability (Luna et al. 2022, Yammine et al. 2022). The ζ-potential value of the carvacrol emulsions was high, which can impede possible aggregation between the particles and, therefore, result in good emulsion stability.

Morphology and structure of spray-dried microcapsules

Figure 1 shows the scanning electron microscopy micrographs of microcapsules obtained by spray-
drying carvacrol and enzyme solutions. The observation of the outer structure of microcapsules showed that all microcapsules consisted of spherical and well-separated particles with a non-uniform diameter (Figure 1Aa–Ca). In addition, Figure 1Ab–Cb show that all microcapsules had blunt shapes and generally embossed surfaces, with the presence of a few small shrunken particles with rough surfaces. The observation of the inner structure shows that microcapsules had a central void (Figure 1Ac–Cc). Moreover, Figure 1Ad shows that carvacrol microcapsules had a thick, rough and hollow wall matrix with an obvious encapsulated core material retained inside. However, Figure 1Bd and Cd show that the microcapsules of enzymes (ME-PEP, ME-TRYP) had a thick, smooth and homogeneous wall matrix.

**Antimicrobial assessment of ME-CARV**

The MIC values of ME-CARV against *P. aeruginosa* and *E. faecalis* were determined and were 1.25 mg mL\(^{-1}\) and 0.625 mg mL\(^{-1}\), respectively (Mechmechani et al. 2022). Therefore, experiments have been conducted to decrease the concentration of carvacrol at a minimum level but still active against *P. aeruginosa* and *E. faecalis* biofilms. The results demonstrated that the use of \(\frac{1}{2}\) MIC against each bacterium caused a significant reduction of biofilms. Hence, the antimicrobial activity of ME-CARV using \(\frac{1}{2}\) MIC for each bacterium was investigated by direct analysis using epifluorescence microscopy. *P. aeruginosa* and *E. faecalis* biofilms were stained with SYTO9 and propidium iodide (PI). For both bacteria, the results showed that the control treated with TS presented a biofilm composed of viable cells predominantly stained by SYTO9 (green bacteria) with some dead bacteria stained by PI (red bacteria). Microcapsules without carvacrol showed no antimicrobial activity (biofilms mainly stained by SYTO9). However, when *P. aeruginosa* and *E. faecalis* biofilms were exposed to ME-CARV, the results showed a significant decrease in SYTO9-stained cells (viable cells) with a significant increase in PI-stained cells (dead cells) (Figure 2).

**Determination of enzyme dispersive activity**

To study the dispersive activity of the two microencapsulated enzymes, after the individual or sequential treatment, the biofilms were stained by AO and
observed by epifluorescence microscopy. In this experiment, we did not need to study the viability of biofilms since the enzymes are not antimicrobial agents. AO staining allows visualization of the biofilm shape to investigate the dispersive activity of enzymes, regardless of the viability of biofilm cells. Thus, the total amount of living and dead cells in biofilms was assessed by AO (Stiefel et al. 2016). Figure 3 shows that the control biofilm of *P. aeruginosa* and *E. faecalis* exhibited thick biomass. Microcapsules without enzymes showed no dispersive activity against biofilms. However, after treatment with ME-PEP (1 mg mL\(^{-1}\)) or ME-TRYP (1 mg mL\(^{-1}\)) alone, the results show a significant reduction in both biofilm mass compared to the control (Figure 3). This reduction was more significant after the treatment of biofilms with enzymes in sequential order (ME-PEP followed by ME-TRYP or ME-TRYP followed by ME-PEP) which caused a thin layer of biofilm to remain for both bacteria (Figure 3).
**Single and combined treatment of microencapsulated enzymes and carvacrol**

**Pseudomonas aeruginosa case**

The antibiofilm effect of ME-CARV (0.625 mg mL\(^{-1}\)), ME-PEP (1 mg mL\(^{-1}\)) and ME-TRYP (1 mg mL\(^{-1}\)) against *P. aeruginosa* biofilm grown on SS coupons for 24 h at 37°C was assessed in terms of culturable counts. *P. aeruginosa* biofilm exhibited a bacterial cell population of approximately 7 log CFU mL\(^{-1}\) (Figure 4A). The microcapsules without enzymes and carvacrol did not show any antibiofilm activity against *P. aeruginosa* strain biofilm. When enzymes were added to the biofilm, none of the ME-PEP or ME-TRYP treatments alone reduced the number of culturable cells significantly (\(p > 0.05\)). However, treatment with ME-CARV caused a significant reduction of 2.2 log CFU mL\(^{-1}\) (\(p < 0.05\)) (Figure 4A). Using the combined treatment of ME-PEP or ME-TRYP followed by ME-CARV, leads to a reduction in viable cells which was more significant and reached 4.2 log CFU mL\(^{-1}\) and 3.8 log CFU mL\(^{-1}\) (\(p < 0.05\)), respectively (Figure 4A). In addition, the use of sequential treatment of both enzymes followed by ME-CARV showed a notable and more important reduction in culturable cell numbers. Specifically, there was a substantial synergistic inactivation of 5 log CFU mL\(^{-1}\) (\(p < 0.05\)) after treatment in the order ME-TRYP, ME-PEP, and ME-CARV (Figure 4A). Interestingly, a different sequential enzyme treatment in the order ME-PEP, ME-TRYP, followed by ME-CARV was also efficient, but at a lower extent — 4.5 log CFU mL\(^{-1}\) (\(p < 0.05\)). However, no significant reduction in biofilm cells was observed after the two different treatments without ME-CARV (\(p < 0.05\)) (Figure 4A).

**Enterococcus faecalis case**

The antibiofilm effect of ME-CARV (0.312 mg mL\(^{-1}\)), ME-PEP (1 mg mL\(^{-1}\)) and ME-TRYP (1 mg mL\(^{-1}\)) against *E. faecalis* biofilm grown on SS coupons for 24 h at 37°C was also studied in culturable counts. The biofilm of *the E. faecalis* strain presented a bacterial cell population of 7 log CFU mL\(^{-1}\) (Figure 4B). The microcapsules without enzymes and carvacrol did not show any antibiofilm against *E. faecalis* strain biofilm. After biofilm treatment with microencapsulated enzymes, individually or sequentially, there was no significant reduction in viable cells. In contrast, after treatment with ME-CARV, the biofilm was reduced by 1.8 log CFU mL\(^{-1}\) (\(p < 0.05\)) (Figure 4B). The combined treatment using ME-PEP followed by ME-CARV showed an improved biofilm reduction by 2.8 log CFU mL\(^{-1}\) (Figure 4B). Moreover, the combined treatment with ME-TRYP first followed by ME-CARV was also effective in improving ME-CARV activity, but lower than that of ME-PEP with ME-CARV treatment, with a maximum reduction of 2.3 log CFU mL\(^{-1}\) (\(p < 0.05\)) (Figure 4B). Nevertheless, sequential treatment of the two enzymes, in the order of ME-PEP - ME-TRYP or ME-TRYP - ME-PEP, followed by ME-CARV treatment caused a significant and similar reduction in biofilm cells of 4 log CFU mL\(^{-1}\) (\(p < 0.05\)) approximatively (Figure 4B).

**Discussion**

The EPS matrix is the protective barrier of biofilms against external agents, such as conventional detergents or disinfectants (Al Kassaa et al. 2019; Karygianni et al. 2020). Several enzymes can disrupt the biofilm matrix by breaking down its structure (Meireles et al. 2016). Dispersion of the biofilm matrix leads to depletion of the biofilm viscoelastic properties, and weakening of its cohesion, which improves antimicrobial efficacy (Lim et al. 2019; Baidamshina et al. 2021). Thus, combining enzymes with biocides will improve the accessibility of biocides to the biofilm cells embedded in the EPS matrix, which will reduce the dose of disinfectants required and decrease environmental pollution (Rodríguez-López et al. 2017). In this study, the antibiofilm effect of combined treatment using microencapsulated enzymes (pepsin and trypsin) with carvacrol, a natural antimicrobial, was investigated against *E. faecalis* and *P. aeruginosa* biofilms. Microencapsulation is a
powerful tool used to separate active compounds from their environment by trapping them in microcapsules (Khelissa et al. 2021a, Khelissa et al. 2021b). This procedure protects the active compounds from degradation and allows their passage through the biofilm matrix to be released at a specific location and time (Gharsallaoui et al. 2007; Khelissa et al. 2021b). The microencapsulation of enzymes and carvacrol is a promising new technology to enhance their stability and improve their biological activity. Sodium caseinate was chosen for the microencapsulation of carvacrol as an emulsifier as it is an abundant and inexpensive protein of animal origin with very good emulsifying properties and resistance to high temperatures. On the other hand, pectin was used for enzyme microencapsulation to increase the resistance of the wall material by reinforcing its structure (Gharsallaoui et al. 2007; Fathi et al. 2021). Maltodextrin was selected as the wall material because it is a biodegradable and safe plant-derived polysaccharide. In addition, maltodextrin is non-toxic and easily obtained by starch hydrolysis, with relatively low viscosity at high temperatures and good solubility in water (Gharsallaoui et al. 2007; Aguiar et al. 2016).

The SEM observation was used to study the outer and inner structures of carvacrol and enzymes spray-dried microcapsules. The results showed that all microcapsules had a spherical shape and bumpy surfaces of different sizes, with no appearance of fissures or cracks, demonstrating the high structural integrity and impermeability of the microcapsules, which enhances the protection and retention of the active materials (Carneiro et al. 2013). In addition, some carvacrol and enzyme microcapsules have an irregular surface that may be caused by shrinkage during the drying and cooling steps (Botrel et al. 2012; Hijo et al. 2015). These indentation and roughness of the surface were greater in the small particles than in the large particles, indicating that wall solidification occurred before microcapsule expansion (Rosenberg and Young 1993). The internal morphology analysis showed that all microcapsules have a central void which could be the result of air expansion by the spray drying process in the drops (Teixeira et al. 2004). In addition, the results show that the carvacrol microcapsules were hollow, showing that the volatile active compound adhered to the surface as small droplets embedded in the matrix of the wall materials. However, the enzyme microcapsules had no holes and the enzymes were mixed with the matrix of microcapsules and are not apparent in the SEM micrographs. The obtained results are in agreement with the observations of other studies that showed encapsulated active compounds with almost similar properties using different wall materials (Sheu and Rosenberg 2008; Botrel et al. 2012; Rocha et al. 2012; Carneiro et al. 2013; Fernandes et al. 2013; Khelissa et al. 2021a; 2021b).

In this study, the efficacy of microencapsulated proteolytic enzymes, pepsin and trypsin, and microencapsulated carvacrol was assessed against P. aeruginosa and E. faecalis biofilms formed for 24 h on SS coupons. SS is a material widely used in the food and medical sectors due to its high corrosion resistance, mechanical strength and exceptional physicochemical properties (Yammine et al. 2022). It is characterized as an abiotic surface to which bacterial cells, including P. aeruginosa and E. faecalis, can adhere in a relatively short time (Barnes et al. 1999; Castro et al. 2018). NEC biofilm system was used for biofilm development. This system is a closed bioreactor that guarantees high repeatability and facilitative handling conditions for biofilm deposition (Abdallah et al. 2015).

Pepsin and trypsin are mammalian digestive enzymes, widely available and have the potential to be produced on an industrial scale for food applications. Previous studies have already demonstrated that biofilms from Staphylococcus lentus, Staphylococcus cohnii, Staphylococcus saprophyticus and P. aeruginosa can be degraded by trypsin (Banar et al. 2016; Fagerlund et al. 2016). Another study demonstrated the potential of pepsin to destabilize multi-species biofilms (Marcato-Romain et al. 2012). Matrix-associated proteins are reported to be important components of P. aeruginosa and E. faecalis biofilms, contributing to pathogenesis and biofilm stability (Matsukawa and Greenberg 2004; Tendolkar et al. 2004; Borlee et al. 2010; Zhang et al. 2015; Taglialegna et al. 2020). P. aeruginosa matrix proteome is composed of secreted proteins, periplasmic proteins, cytoplasmic membrane proteins, cytoplasmic proteins, and predominantly outer membrane proteins that are typically found in the outer membrane vesicles (OMVs) (Toyofuku et al. 2012). Similarly, Schooling and Beveridge (2006) have demonstrated that OMVs are a major constituent of the biofilm matrix of P. aeruginosa and play an important role in biofilm formation and resistance. In addition, Taglialegna et al. (2020) reported that enterococcal surface protein (Esp), a biofilm-associated protein (Bap) known as Bap-orthologous protein, is widely produced by E. faecalis and contributes significantly to matrix building and biofilm formation. The enzyme microencapsulation can protect and stabilize...
them and thus retain a high catalytic activity (Chaize et al. 2004; Tetter and Hilvert 2017; Zdarta et al. 2018). These microencapsulated enzymes can be used as an efficient tool to eradicate biofilm adhered to abiotic surfaces (Orgaz et al. 2007; Elchinger et al. 2015; Baidamshina et al. 2021). To directly visualize the dispersive effect of ME-PEP and ME-TRYP, biofilms were stained with AO after treatment and observed by epifluorescence microscopy. The results showed that ME-PEP and ME-TRYP disperse significantly the biofilm of both bacteria and this dispersion is more effective after treatment with both enzymes sequentially. These results strongly suggest that these proteolytic enzymes induced the degradation of proteins present in the biofilm matrix of *P. aeruginosa* and *E. faecalis* and could be an effective tool to reduce pre-formed biofilms. However, Banar et al. (2016) demonstrated that the trypsin enzyme had a weaker effect on *P. aeruginosa* biofilms as proteins are one of the sub-components of the *P. aeruginosa* biofilm (Banar et al. 2016).

Although the epifluorescence microscopy results showed the dispersed activity of the enzymes, the results of cell count showed that treatment with enzymes, individually or sequentially, did not significantly affect the biofilm cell counts. These results are not due to the weak effect of the enzymes but because proteolytic enzymes are not antimicrobial agents, so they will destabilize biofilms without killing cells.

Essential oil components, such as carvacrol, have become increasingly used as an alternative natural biocide due to their potential antimicrobial activity against a wide variety of microbial pathogens in suspension and biofilm states (Jafri et al. 2019). These alternative biocides provide the required treatment by overcoming the disadvantages of conventional antimicrobials used (Wińskiego et al. 2019). The microencapsulation of antimicrobial essential oils can improve their bioactivity and efficiency in removing biofilm from surfaces (Dohare et al. 2014; Duncan et al. 2015; Cui et al. 2016), as well as reduce their volatility and cytotoxicity, and enhance their stability and solubility in water (Bilia et al. 2014). To analyze the antimicrobial activity of ME-CARV, biofilms of *P. aeruginosa* and *E. faecalis* were stained using the Live Dead stains and then observed by epifluorescence microscopy. The results show that after treatment of both bacterial biofilms with ME-CARV, the number of dead cells increased significantly compared to the control biofilms. These results are consistent with the biofilm culturable counts, which showed a significant reduction in the number of viable cells after ME-CARV treatment for both bacteria. These findings proved the high antimicrobial potential of ME-CARV to kill the embedded biofilm cells. Several previous studies have also demonstrated the strong antimicrobial activity of encapsulated essential oils against several types of biofilms due to their improved solubility and enhanced interaction with the biofilm matrix (Chifriju et al. 2012; Bilcu et al. 2014; Duncan et al. 2015; Jamil et al. 2016; Khelissa et al. 2021).

The use of EPS-degrading enzymes combined with biocides has been widely investigated for potential applications in biofilm control (Wang et al. 2016; Rodríguez-López et al. 2017; Zhou et al. 2018; Lim et al. 2019; Baidamshina et al. 2021). These enzymes destabilize the biofilm matrix so that the matrix-embedded cells of the biofilm will be increasingly attacked by antimicrobial agents, thereby increasing the effectiveness of the biocides. The results showed that the combined treatments using ME-PEP or ME-TRYP followed by ME-CARV further reduced substantially the number of culturable cells in both biofilms compared to ME-CARV treatment alone. Furthermore, the results also demonstrated that sequential treatment using the two microencapsulated enzymes followed by ME-CARV was significantly more effective than single enzymes in biofilm control. Similarly, Cui et al. (2016) showed that *Escherichia coli* biofilm was greatly reduced by combined treatment using microencapsulated protease and essential oil (Cui et al. 2016). Lim et al. (2019) have also demonstrated that the sequential treatment with multiple enzymes followed by an antimicrobial was more effective against biofilm developed on SS surfaces than the single enzyme treatment (Lim et al. 2019). Hence, the results obtained suggest that the degradation of the EPS matrix would be a suitable strategy to improve the effectiveness of essential oils for removing *P. aeruginosa* and *E. faecalis* biofilms. Furthermore, the results showed that the combined treatment of microencapsulated enzymes followed by ME-CARV significantly reduced the biofilm of *P. aeruginosa* more than that of *E. faecalis*, compared to the treatment with ME-CARV alone, which may be due to the diversity and protein enrichment of the *P. aeruginosa* biofilm matrix (Tseng et al. 2018).

Based on the results obtained, the current study shows that the synergistic effect of ME-CARV with ME-PEP and ME-TRYP may be an effective method to disrupt *P. aeruginosa* and *E. faecalis* biofilms. Although several EOs have been investigated for their antibiofilm properties, the present study is the first reporting the synergistic combination of microencapsulated
proteolytic enzymes and a microencapsulated EO for increasing the efficacy of biofilm disruption (Supplementary materials Tables S1 and S2). Hence, such a biofilm control strategy has several limitations that could be improved by further investigations. For example, further experiments should be conducted using different types of biofilms, enzymes, and antimicrobials at different concentrations. In addition, more research can be performed by growing the biofilm under dynamic conditions for a longer period to represent better the biofilms expected on contaminated industrial surfaces. Investigations on the co-encapsulation of enzymes and antimicrobials in the same type of microcapsules would also be required to allow simultaneous activity when used for biofilm control.

Conclusions

The objective of this study was to analyze the combined effect of microencapsulated enzymes and antimicrobials, on the removal of biofilms by a dual action, matrix destabilization and bacterial killing. The selected enzymes and carvacrol were successfully microencapsulated in different types of microcapsules, using the spray-drying method, and their antibiofilm action against biofilms of P. aeruginosa and E. faecalis was demonstrated. The results suggested that the biofilm of both bacteria can be significantly reduced by ME-CARV treatment. Additionally, treatment with either ME-PEP or ME-TRYP followed by ME-CARV can synergistically inactivate cells from pre-formed biofilms. The results also suggest that sequential treatment of the two microencapsulated proteolytic enzymes followed by ME-CARV would be more appropriate for the deep removal of cells and EPS matrix from biofilms. Therefore, the combined treatment using EPS-degrading enzymes with natural disinfectants can be used as an effective alternative strategy for controlling biofilms of P. aeruginosa and E. faecalis in medical and food-associated environments.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The data used to support the findings of this research are included in the article.

References

Abdallah M, Khelissa O, Ibrahim A, Benoliel C, Heliot L, Dhulster P, Chihib N-E. 2015. Impact of growth temperature and surface type on the resistance of Pseudomonas aeruginosa and Staphylococcus aureus biofilms to disinfectants. Int J Food Microbiol. 214:38–47. doi:10.1016/j.ijfoodmicro.2015.07.022

Aguiar J, Estevinho BN, Santos L. 2016. Microencapsulation of natural antioxidants for food application – The specific case of coffee antioxidants – A review. Trends Food Sci Technol. 58:21–39. doi:10.1016/j.tifs.2016.10.012

Al Kassaa I, Mechemchani S, Zaylaa M, Ismail MB, El Omari K, Dabboussi F, Hamze M. 2019. Characterization of lactobacilli strains isolated from baby's feces for their potential immunobiological application. Iran J Microbiol. 11: 379–388.

Alav I, Sutton JM, Rahman KM. 2018. Role of bacterial efflux pumps in biofilm formation. J Antimicrob Chemother. 73:2003–2020. doi:10.1093/jac/dky042

Amaral VCS, Santos PR, Silva A d, Santos AR, dos Machinski M, Mikcha JMG. 2015. Effect of carvacrol and thymol on Salmonella spp. biofilms on polypropylene. Int J Food Sci Technol. 50:2639–2643. doi:10.1111/ijfs.12934

Arias CA, Murray BE. 2012. The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol. 10: 266–278. doi:10.1038/nrmicro2761

Baidamshina DR, Koroleva VA, Olshannikova SS, Trizna E, Bogachev MI, Artyukhov VG, Holyavka MG, Kayumov AR. 2021. Biochemical properties and anti-biofilm activity of chitosan-immobilized papain. Mar Drugs. 19:197. doi:10.3390/md19040197

Banar M, Emaneini M, Satarzadeh M, Abdellahi N, Beigverdi R, Leeuwen W v, Jabalameli F. 2016. Evaluation of mannosidase and trypsin enzymes effects on biofilm production of Pseudomonas aeruginosa isolated from burn wound infections. PLoS One. 11: e0164622. doi:10.1371/journal.pone.0164622

Barnes L-M, Lo MF, Adams MR, Chamberlain AH. 1999. Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. Appl Environ Microbiol. 65: 4543–4548. doi:10.1128/AEM.65.10.4543-4548.1999

Bilcu M, Grumezescu AM, Oprea AE, Popescu RC, Mogosanu GD, Hristu R, Stanciu GA, Mihaiescu DF, Lazar V, Bezirtzoglou E, et al. 2014. Efficiency of vanilla, patchouli and ylang ylang essential oils stabilized by iron oxide@c14 nanostructures against bacterial adherence and biofilms formed by Staphylococcus aureus and...
Klebsiella pneumoniae Clinical Strains. Molecules. 19: 17943–17956. doi:10.3390/organisms191117943

Bilia AR, Guccione C, Isacchi B, Righeschi C, Firenzuoli F, Bergonzi MC. 2014. Essential oils loaded in nanosystems: a developing strategy for a successful therapeutic approach. Evid-Based Complement Altern Med ECAM. 2014:1–14. doi:10.1155/2014/651593

Borges A, Meireles A, Mergulhão F, Melo L, Simões M. 2020. Biofilm control with enzymes. In Simoes M, Borges Anabel, Chaves Simoes I, editors. Recent trends in biofilm science and technology. London: Academic Press; p. 249 – 271. doi:10.1007/978-0-12-819497-3_4.0011-8

Borlee BR, Goldman AD, Murakami K, Samudrala R, Botrel DA, Borges SV, Botrel DA, Silva EK, Costa JMG, da, Queiroz F. 2013. Microencapsulation of rosemary essential oil: characterization of particles. Dry Technol. 31:1245–1254. doi:10.1080/07373937.2013.785432

Flemming H-C, Wingender J. 2010. The biofilm matrix. Nat Rev Microbiol. 8:623–633. doi:10.1038/nrmicro2415

Gharsallaoui A, Roudaut G, Chambin O, Voilley A, Saurel R. 2007. Applications of spray-drying in microencapsulation of food ingredients: an overview. Food Res Int. 40: 1107–1121. doi:10.1016/j.foodres.2007.07.004

Hajati H, Ansari FA, Ahmad I. 2019. Chapter 9 - Prospects of essential oils in controlling pathogenic biofilm. In: Ahmad Khan MS, Ahmad I, Chattopadhyay D, editors. New look to phytomedicine. London: Academic Press; p. 203–236. doi:10.1016/B978-0-12-814619-4.00009-4

Jamil B, Abbasi R, Abbasi S, Imran M, Khan SU, Ihsan A, Javed S, Bokhari H, Imran M. 2016. Encapsulation of cardamom essential oil in chitosan nano-composites: in-vitro efficacy on antibiotic-resistant bacterial pathogens and cytotoxicity studies. Front Microbiol. 7:1580. doi:10.3389/fmicb.2016.01580

Jee S-C, Kim M, Sung J-S, Kadam AA. 2020. Efficient biofilms eradication by enzymatic-cocktail of pancreatic protease type-i and bacterial ß-amylase. Polymers. 12:3032. doi:10.3390/polym12123032

Karygianni L, Ren Z, Koo H, Thurnheer T. 2020. Biofilm matrixome: extracellular components in structured microbial communities. Trends Microbiol. 28:668–681. doi:10.1016/j.tim.2020.03.016

Khelissa S, El Fannassi Y, Mechmechani S, Alhuthali S, El Amrani MA, Gharsallaoui A, Barras A, Chihib N-E. 2021c. Water-soluble ruthenium (ii) complex derived from optically pure limonene and its microencapsulation are efficient tools against bacterial food pathogen biofilms: escherichia coli, Staphylococcus aureus, Enterococcus.
faecalis, and Listeria monocytogenes. Front Microbiol. 12: 711326. 10.3389/fmicb.2021.711326.

Khellisa S, Gharsallaoui A, Fadel A, Barras A, Jama C, Jbilou F, Chibih N-E. 2021b. Microencapsulation of benzalkonium chloride enhanced its antibacterial and anti-biofilm activities against Listeria monocytogenes and Escherichia coli. J Appl Microbiol. 131:1136–1146. doi:10.1111/jam.15010

Khellisa S, Gharsallaoui A, Wang J, Dumas E, Barras A, Jama C, Jbilou F, Loukili N, Chibih N-E. 2021a. Anti-biofilm activity of dodocycltrimethylammonium chloride microcapsules against Salmonella enterica serovar Enteritidis and Staphylococcus aureus. Biofouling. 37: 49–60. doi:10.1080/08927014.2021.1873958

Kim LH, Kim S-J, Kim C-M, Shin MS, Kook S, Kim IS. 2013. Effects of enzymatic treatment on the reduction of extracellular polymeric substances (EPS) from biofouled membranes. Desalination Water Treat. 51:6355–6361. doi:10.1080/19443994.2013.780996

Lim ES, Koo O, Kim M, Kim J-S. 2019. Bio-enzymes for inhibition and elimination of Escherichia coli O157: h7 biofilm and their synergistic effect with sodium hypochlorite. Sci Rep. 9:9920. doi:10.1038/s41598-019-46363-w

Liu C, Guo D-A, Liu L. 2018. Quality transitivity and traceability system of herbal medicine products based on quality markers. Phytomedicine. 44:247–257. doi:10.1016/j.phymed.2018.03.006

Luna M, Beltran O, Encinas-Basurto DA, Ballesteros-Monrreal MG, Topete A, Hassan N, López-Mata MA, Reyes-Márquez V, Valdez MA, Juarez J. 2022. High antibacterial performance of hydrophobic chitosan-based nanoparticles loaded with Carvacrol. Colloids Surf B Biointerfaces. 209:112191. doi:10.1016/j.colsurfb.2021.112191

Marcato-Romain CE, Pechaud Y, Paul E, Girbal-Neuhauser E, Dossat-Létisse V. 2012. Removal of microbial multispecies biofilms from the paper industry by enzymatic treatments. Biofouling. 28:305–314. doi:10.1080/08927014.2012.673122

Matsukawa M, Greenberg EP. 2004. Putative exopolysaccharide synthesis genes influence Pseudomonas aeruginosa biofilm development. J Bacteriol. 186:4449–4456. doi:10.1128/JB.186.14.4449-4456.2004

Mechmechani S, Gharsallaoui A, Fadel A, Omari KE, Khellissa S, Hamze M, Chibih N-E. 2022. Microencapsulation of carvacrol as an efficient tool to fight Pseudomonas aeruginosa and Enterococcus faecalis biofilms. PLoS One. 17:e0270200. doi:10.1371/journal.pone.0270200

Mechmechani S, Khellissa S, Gharsallaoui A, Omari KE, Hamze M, Chibih N-E. 2022. Hurdle technology using encapsulated enzymes and essential oils to fight bacterial biofilms. Appl Microbiol Biotechnol. 106:2311–2335. doi:10.1007/s00253-022-11875-5

Meireles A, Borges A, Giacouris E, Simões M. 2016. The current knowledge on the application of anti-biofilm enzymes in the food industry. Food Res Int. 86:140–146. doi:10.1016/j.foodres.2016.06.006

Mohamed SH, Mohamed MSM, Khalil MS, Azmy M, Mabrouk MI. 2018. Combination of essential oil and ciprofloxacin to inhibit/eradicate biofilms in multidrug-resistant Klebsiella pneumoniae. J Appl Microbiol. 125: 84–95. doi:10.1111/jam.13755

Moradali MF, Ghods S, Rehm BHA. 2017. Pseudomonas aeruginosa lifestyle: a paradigm for adaptation, survival, and persistence. Front Cell Infect Microbiol. 7:39. 10.3389/fcimb.2017.00039.

Nazzaro F, Fratianne F, De Martino L, Coppola R, De Feo V. 2013. Effect of essential oils on pathogenic bacteria. Pharmaceuticals (Basel). 6:1451–1474. doi:10.3390/ph6121451

Orgaz B, Neufeld RJ, Sanjose C. 2007. Single-step biofilm removal with delayed release encapsulated pronase mixed with soluble enzymes. Enzyme Microb Technol. 40: 1045–1051. doi:10.1016/j.enzmictec.2006.08.003

Pinto RM, Soares FA, Reis S, Nunes C, Van Dijck P. 2020. Innovative strategies toward the disassembly of the eps matrix in bacterial biofilms. Front Microbiol. 11:952. doi:10.3389/fmicb.2020.00952

Raposo A, Pérez E, Faria C d, Ferrús MA, Carrascosa C. 2016. Food spoilage by Pseudomonas spp.—An overview. In: Singh OV, editor. Foodborne pathogens and antibacterial resistance. Hoboken, NJ: Wiley; p. 41–71. doi:10.1002/9781119139188.ch3

Rocha GA, Fávaro-Trindade CS, Grosso CRF. 2012. Microencapsulation of lycopene by spray drying: characterization, stability and application of microcapsule. Food Bioprod Process. 90:37–42. doi:10.1016/j.fbp.2011.01.001

Rodríguez-López P, Puga CH, Orgaz B, Cabo ML. 2017. Quantifying the combined effects of pronase and benzalkonium chloride in removing late-stage Listeria monocytogenes–Escherichia coli dual-species biofilms. Biofouling. 33:690–702. doi:10.1080/08927014.2017.1356290

Rosenberg M, Young S. 1993. Whey proteins as microencapsulating agents. Microencapsulation of anhydrous milkfat - structure evaluation. Food Struct. 12:952. https://digitalcommons.usu.edu/foodmicrostructure/vol12/iss1/4.

Schooling SR, Beveridge TJ. 2006. Membrane vesicles: an overlooked component of the matrices of biofilms. J Bacteriol. 188:5945–5957. doi:10.1128/JB.00257-06

Shan B, Cai Y-Z, Brooks JD, Corke H. 2007. The in vitro antibacterial activity of dietary spice and medicinal herb extracts. Int J Food Microbiol. 117:112–119. doi:10.1016/j.ijfoodmicro.2007.03.003

Sheu T-Y, Rosenberg M. 2008. Microstructure of microcapsules consisting of whey proteins and carbohydrates. J Food Science. 63:491–494. doi:10.1111/j.1365-2621.1998.tb15770.x

Shridhar S, Dhanashree B. 2019. Antibiotic susceptibility pattern and biofilm formation in clinical isolates of Enterococcus spp. Interdiscip Perspect Infect Dis. 2019:2019–2335. doi:10.1128/JB.00257-06

Simões M, Simões M, Simoes L, Vieira M. 2010. A review of current and emergent biofilm control strategies. Lebensm-Wiss Technol. 43:573–595. doi:10.1016/j.lwt.2009.09.008

Stiefel P, Rosenberg U, Schneider J, Maurerhofer S, Maniura-Weber K, Ren Q. 2016. Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. Appl Microbiol
Biotechnol. 100:4135–4145. doi:10.1007/s00253-016-7396-9

Taglialegna A, Matilla-Cuenca L, Dorado-Morales P, Navarro S, Ventura S, Garnett JA, Lasa I, Valle J. 2020. The biofilm-associated surface protein Esp of Enterococcus faecalis forms amyloid-like fibers. NPJ Biofilms Microbiomes. 6:1–12. doi:10.1038/s41522-020-0125-2

Teixeira MI, Andrade LR, Farina M, Rocha-Leão MHM. 2004. Characterization of short chain fatty acid microcapsules produced by spray drying. Mater Sci Eng C. 24: 653–658. doi:10.1016/j.msec.2004.08.008

Tendolkar PM, Baghdayan AS, Gilmore MS, Shankar N. 2004. Enterococcal surface protein, Esp, enhances biofilm formation by Enterococcus faecalis. Infect Immun. 72: 6032–6039. doi:10.1128/IAI.72.10.6032-6039.2004

Tetter S, Hilvert D. 2017. Enzyme encapsulation by a ferritin cage. Angew Chem Int Ed Engl. 56:14933–14936. doi:10.1002/anie.201708530

Tikhonov SL, Tikhonova NV, Kudryashov LS, Kudryashova OA, Moskovenko NV, Tretjakova IN. 2021. Efficiency of microencapsulation of proteolytic enzymes. Catalysts. 11:4906–3142. doi:10.3390/catal11111270

Torneros E, Senneville E, Euba G, Petersdorf S, Rodriguez-Pardo D, Lakatos B, Ferrari MC, Pilares M, Bahamonde A, Trebse R, et al. 2014. Characteristics of prosthetic joint infections due to Enterococcus spp. and predictors of failure: a multi-national study. Clin Microbiol Infect. 20:1219–1224. doi:10.1111/1469-0691.12721

Totó K, Horemans T, Berghé DV, Maes L, Cos P. 2010. Inhibitory effect of biocides on the viable masses and matrices of Staphylococcus aureus and Pseudomonas aeruginosa biofilms. Appl Environ Microbiol. 76:3135–3142. doi:10.1128/AEM.02095-09

Toyofuku M, Roschitzki B, Riedel K, Eberl L. 2012. Identification of proteins associated with the Pseudomonas aeruginosa biofilm extracellular matrix. J Proteome Res. 11:4906–4915. doi:10.1021/pr3003959

Trevisan DAC, Silva A d, Negri M, Abreu Filho B d, Junior M, Patussi M, Campanerut-Sá EV, Mikcha PAZ, Trevisan JMG, Silva DAC, et al. 2018. Antibacterial and antibiofilm activity of carvacrol against Salmonella enterica serotype Typhimurium. Braz J Pharm Sci. 54:e17229. doi:10.1590/s2175-97902018000117229

Tseng BS, Reichhardt C, Merrihew GE, Araujo-Hernandez SA, Harrison JJ, MacCoss MJ, Parsek MR. 2018. A biofilm matrix-associated protease inhibitor protects Pseudomonas aeruginosa from proteolytic attack. mBio. 9:e00543-18. doi:10.1128/mBio.00543-18

Walczak M, Michalska-Sionkowska M, Olkiewicz D, Tarnaw ska P, Warzyńska O. 2021. Potential of carvacrol and thymol in reducing biofilm formation on technical surfaces. Molecules. 26:2723. doi:10.3390/molecules26092723

Wang J, Sun J, Sun P, Yang K, Dumas E, Gharsallaoui A. 2022. Formation of lysosome-caseinate heteroprotein complexes for encapsulation of lysozyme by spray-drying: effect of mass ratio and temperature. Int J Biol Macromol. 215:312–320. doi:10.1016/j.ijbiomac.2022.06.123

Wang H, Wang X, Xing T, Wu N, Xu X, Zhou G. 2016. Removal of Salmonella biofilm formed under meat processing environment by surfactant in combination with bio-enzyme. LWT - Food Sci Technol. 66:298–304. doi:10.1016/j.lwt.2015.10.049

Wieńska K, Mącżka W, Łyczko J, Grabarczyk M, Czubaszek A, Szumny A. 2019. Essential oils as antimicrobial agents—myth or real alternative? Molecules. 24:2130. doi:10.3390/molecules24112130

Yasmine J, Gharsallaoui A, Fadel A, Mechemachi S, Karam L, Ismail A, Chihib N-E. 2022. Enhanced antimicrobial, antibiofilm and ectotoxic activities of nanoencapsulated carvacrol and thymol as compared to their free counterparts. Food Control. 143:109317. doi:10.1016/j.foodcont.2022.109317

Yuan L, Sadiq FA, Wang N, Yang Z, He G. 2021. Recent advances in understanding the control of disinfectant-resistant biofilms by hurdle technology in the food industry. Crit Rev Food Sci Nutr. 61:3876–3891. doi:10.1080/10408398.2020.1809345

Zdarta J, Meyer AS, Jesionowski T, Pinelo M. 2018. A general overview of support materials for enzyme immobilization: characteristics, properties, practical utility. Catalysts. 8:92. doi:10.3390/catal8020092

Zhang W, Sun J, Ding W, Lin J, Tian R, Lu L, Liu X, Shen X, Qian P-Y. 2015. Extracellular matrix-associated proteins form an integral and dynamic system during Pseudomonas aeruginosa biofilm development. Front Cell Infect Microbiol. 5:40. 10.3389/fcimb.2015.00040.

Zhou J, Bi S, Chen H, Chen T, Yang R, Li M, Fu Y, Jia A-Q. 2017. Anti-biofilm and antivirulence activities of metabolites from Plectosphaerella cucumerina against Pseudomonas aeruginosa. Front Microbiol. 8:769. doi: 10.3389/fmicb.2017.00769

Zhou J-W, Luo H-Z, Jiang H, Jian T-K, Chen Z-Q, Jia A-Q. 2018. Hordenine: a novel quorum sensing inhibitor and antibiofilm agent against Pseudomonas aeruginosa. J Agric Food Chem. 66:1620–1628. doi:10.1021/acs.jafc.7b05035