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Hurdle technology using microencapsulated proteolytic enzymes and microencapsulated carvacrol to fight pathogenic bacterial biofilms

La technologie des barrières utilisant des enzymes à pouvoir déstructurant et du carvacrol microencapsulés pour lutter contre les biofilms de bactéries pathogènes

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ABSTRACT

Hurdle technology using microencapsulated proteolytic enzymes and microencapsulated carvacrol to fight pathogenic bacterial biofilms

Abstract

The ambient operating environments in the food and medical sectors allow bacteria to adhere and develop on the substrates, resulting in the growth of resistant pathogenic bacterial biofilms. These pathogenic structures are involved in several foodborne diseases and health-care associated infections. Consequently, to combat this public health burden, several strategies have recently been proposed which include chemical and mechanical removal. This work presents the different factors that influence bacterial adhesion and biofilm formation on abiotic surfaces, as well as biofilm resistance to disinfectants. The different strategies for biofilm prevention and eradication are described. Microencapsulation using spray-drying method for the formulation of anti-biofilm active components as a tool to ensure their stability and improve their biological activities are also presented. In this context, a study was conducted using carvacrol, a natural antimicrobial agent, to control biofilms of *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Indeed, these two bacteria are responsible for several infections worldwide due to their persistence on abiotic surfaces in hospitals and food processing industries. Furthermore, in order to enhance the antimicrobial activity of carvacrol and reduce its volatility and low solubility in water, feed emulsions were prepared with sodium caseinate and maltodextrins and then spray-dried to obtain dry carvacrol microcapsules. The results showed that carvacrol had a strong antimicrobial activity against both bacterial biofilms. Furthermore, our findings revealed that microencapsulation by spray-drying significantly increased the antimicrobial activity of carvacrol while reducing the amounts used. Indeed, microencapsulated carvacrol was able to reduce biofilm below the detection limit for *Pseudomonas aeruginosa* and 5.5 log CFU mL^{-1} for *Enterococcus faecalis* after 15 min of treatment. However, the complete removal of biofilms from abiotic surfaces in medical and food sectors has proven difficult with the single use of disinfection strategy due to the high protection of the biofilm cells by the extracellular polymeric matrix. This matrix provides an initial protective barrier for the biofilm cells, and makes biofilms highly resistant to antimicrobial agents. The effectiveness of hurdle technology in removing biofilms using different strategies is discussed in this work. One of the hurdle technology approaches is the use of matrix-degrading enzymes that can disperse bacteria embedded in biofilms for more efficient disinfection when combined with biocide agents. Indeed, two proteolytic enzymes, pepsin and trypsin, targeting matrix proteins, have been studied for their potential to degrade biofilms of *Pseudomonas aeruginosa* and *Enterococcus faecalis* and their synergistic effect when combined with carvacrol. The direct analysis using epifluorescence microscopy allowed visualization of the dispersive activity of proteases and the lethal activity of carvacrol against the two bacterial biofilms. In addition, the combined pepsin or trypsin treatment with carvacrol showed more significant reduction of both biofilms compared to carvacrol treatment alone. Moreover, this reduction was more substantial after sequential treatment of both enzymes followed by carvacrol. However, the enzyme activity is highly influenced by environmental factors and is only optimal under restricted conditions. Another disadvantage of using enzymes is self-degradation, leading to instability. Indeed, protease microcapsules containing pepsin or trypsin complexed with pectin and maltodextrin have been prepared. The combined use of these miocrocapsules with microencapsulated carvacrol was also investigated in this study against *Pseudomonas aeruginosa* and *Enterococcus faecalis* biofilms. The results showed that enzyme microcapsules were also able to enhance the antimicrobial properties of encapsulated carvacrol with a retained and even improved activity compared to free enzymes and carvacrol. The physicochemical properties and the microscopic morphology of the realized capsules allowed to a better understanding of the mechanism of action of these microcapsules.

Keywords: Biofilms; antimicrobial activity; dispersive activity; microencapsulation; carvacrol; pepsin; trypsin; *Pseudomonas aeruginosa*; *Enterococcus faecalis*.

RESUME

La technologie des barrières utilisant des enzymes à pouvoir déstructurant et du carvacrol microencapsulés pour lutter contre les biofilms de bactéries pathogènes

Résumé

L'environnement opératoire dans les secteurs alimentaire et médical permet aux bactéries de se fixer et de se développer sur les surfaces, ce qui entraîne la formation de biofilms bactériens pathogènes et résistants. Ces structures pathogènes sont responsables de nombreuses maladies d'origine alimentaire et d'infections associées aux soins. Par conséquent, pour lutter contre ce problème de santé publique, plusieurs stratégies ont récemment été proposées, notamment l'élimination chimique et/ou mécanique. Ce travail présente dans une première partie bibliographique les différents facteurs qui influencent l'adhésion bactérienne et la formation de biofilms sur des surfaces abiotiques, ainsi que la résistance des biofilms aux désinfectants. La microencapsulation par la méthode de séchage par atomisation pour la formulation de composants actifs anti-biofilm en vue d'assurer leur stabilité et améliorer leurs activités biologiques est également présentée. Dans ce contexte, l'étude menée a pour objectif d'utiliser le carvacrol, un agent antimicrobien naturel, pour contrôler les biofilms de *Pseudomonas aeruginosa* et *Enterococcus faecalis*. En effet, ces deux bactéries sont responsables de nombreuses infections dans le monde en raison de leur persistance sur des surfaces abiotiques dans les hôpitaux et les industries agroalimentaires. Par ailleurs, afin de renforcer l'activité antimicrobienne du carvacrol et de réduire sa volatilité et sa faible solubilité dans l'eau, des émulsions ont été préparées avec du caséinate de sodium et des maltodextrines, puis séchées par atomisation pour obtenir des microcapsules de carvacrol sèches. Les résultats ont montré que le carvacrol exerce une forte activité antimicrobienne contre les deux biofilms bactériens. De plus, nos résultats ont révélé que la microencapsulation par séchage par atomisation améliore d'une manière significative l'activité antimicrobienne du carvacrol tout en réduisant les quantités utilisées. En effet, le carvacrol microencapsulé a été capable de réduire le biofilm en dessous de la limite de détection pour *Pseudomonas aeruginosa* et de 5.5 log CFU mL^{-1} pour *Enterococcus faecalis* après 15 min de traitement. L'efficacité de la technologie hurdle pour éliminer les biofilms en utilisant différentes stratégies est discutée dans ce travail. Une des approches de la technologie hurdle est l'utilisation d'enzymes qui peuvent dégrader la matrice et disperser les bactéries intégrées dans les biofilms pour une désinfection plus efficace lorsqu'elles sont combinées avec des agents biocides. En effet, deux enzymes protéolytiques, la pepsine et la trypsine, ciblant les protéines de la matrice, ont été étudiées pour leur potentiel de dégradation des biofilms de *Pseudomonas aeruginosa* et *Enterococcus faecalis* et leur effet synergique lorsqu'elles sont combinées au carvacrol. L'analyse directe par microscopie à épifluorescence a permis de visualiser l'activité dispersive des protéases et l'activité létale du carvacrol contre les deux biofilms bactériens. En outre, le traitement combiné avec la pepsine ou la trypsine et le carvacrol a entraîné une réduction plus significative des deux biofilms par rapport au traitement avec le carvacrol seul. De plus, cette réduction était plus importante après un traitement séquentiel avec les deux enzymes suivi d'un traitement avec du carvacrol. Cependant, l'activité enzymatique est fortement influencée par les facteurs environnementaux et n'est optimale que dans des conditions restreintes. Un autre inconvénient de l'utilisation des enzymes est l'auto-dégradation, qui entraîne leur instabilité. En effet, des microcapsules de protéase contenant de la pepsine ou de la trypsine en présence de la pectine et de maltodextrines ont été préparées. L'utilisation combinée de ces miocrocapsules avec du carvacrol microencapsulé a également été étudiée dans cette étude contre les biofilms de *Pseudomonas aeruginosa* et *Enterococcus faecalis*. Les résultats ont montré que les microcapsules d'enzymes étaient capables de renforcer l'activité antimicrobienne du carvacrol microencapsulé avec un maintien et même une amélioration d'activité biologique par rapport aux enzymes et au carvacrol libres. Les propriétés physicochimiques et la morphologie microscopique des capsules obtenues ont permis de mieux comprendre le mécanisme d'action de ces microcapsules.

Mots clés: Biofilms; activité antimicrobienne; activité dispersive; microencapsulation; carvacrol; pepsine; trypsine; *Pseudomonas aeruginosa*; *Enterococcus faecalis*.

CONTENTS

Contents

ABBREVIATIONS

Abbreviations List

Symbol	Description
EPS	Extracellular polymeric substances
EO	Essential oil
eDNA	Extracellular DNA
CDC	Centers for Disease Control
CFU	Colony-Forming-Unit
CIP	Collection de l'Institut Pasteur
WHO	World Health Organization
QS	Quorum sensing
PSL	Polysaccharide synthesis locus
PEL	Pellicle polysaccharides
PIA	Staphylococcal polysaccharide intercellular adhesin
Esp	Enterococcal surface protein
F-CARV	Free carvacrol
ME-CARV	Miroencapsulated carvacrol
ME-PEP	Microencapsulated pepsin
ME-TRYP	Microencapsulated Trypsin
GRAS	Generally recognized as safe
PPB	Potassium phosphate buffer
DMSO	Dimethyl sulfoxide
ζ-potential	Zeta potential
SEM	Scanning electron microscopy
TS	Tryptone Salt broth
TSB	Tryptic soy broth
TSA	Tryptic Soy Agar
MHB	Müller-Hinton Broth
MHA	Mueller Hinton agar
MOPS	Morpholinopropane sulfonic buffer
SS	Stainless steel

Symbol	Description
MDC	Minimum dispersive concentration
MIC	Minimal inhibitory concentration
OD	Optical density
PI	Propidium iodide
AO	Acridine orange
min	Minute
s	Seconde
mM	millimolar
USA	United states of America
U.S.	United states
UV	Ultraviolet
FDA	Food and Drug Administration

General Introduction

GENERAL INTRODUCTION

The persistence of pathogens in the health care and food sectors constitutes a critical public health issue. The contamination of abiotic surfaces with pathogenic bacteria results in serious human infections worldwide, such as health-care associated infections (HCAIs) and foodborne ones. These infections have a substantial impact on increasing patient morbidity, mortality and healthcare costs (Scharff 2012; Haque et al. 2018). In both natural and artificial ecosystems, bacteria tend to live tethered to surfaces. These sessile cells can develop a complex structure called biofilm (Flemming and Wuertz 2019). Actually, the biofilm formation is a complicated process characterized by a succession of steps. The first step is the adhesion of bacteria to a support surface, then the proliferation of the adhered cells, leading to the accumulation of multi-layered cell clusters simultaneously with the matrix production (Carniello et al. 2018). The final stage of the biofilm life cycle is the dispersal of bacterial cells and their subsequent colonization of new substrates. This stage plays a crucial role in the spread of bacteria and the dissemination of infections (Kaplan 2010). Biofilms are known by their extreme resistance to antimicrobials and other stressors, which makes them very difficult to remove (Khelissa et al. 2019). In addition, several studies have shown that health-care and foodborne infections are largely caused by biofilms formed on equipment in the food and medical sectors. Hence, the control of biofilms, in the context of these areas, seems to be urgent to overcome these issues.

In order to combat this public health threat, researchers have applied several strategies, including chemical and mechanical removal. Nevertheless, complete eradication of biofilms through the single use of these methods appeared to be difficult to achieve due to the high protection of the biofilm cells by the matrix (Tan et al. 2018). This matrix is composed of extracellular polymeric substances (EPS) (Fulaz et al. 2019) and, in the most cases, represents 90% of the biofilm dry mass, with microorganisms making up the remaining (Flemming and Wingender 2010). Even though the chemical and physical content of EPS differs between species, it is mostly composed of polysaccharides, proteins, and extracellular DNA (eDNA); playing an important role in adhesion, aggregation, cohesion, structural integrity and protective barrier of biofilm (Fulaz et al. 2019). Hence, the combined use of two or more biofilm control methods (Hurdle technology) is a potentially effective strategy for removing biofilms from abiotic surfaces, as they attack biofilms

in different ways (Khan et al. 2017b). In this way, combining enzymes with antimicrobials will provide a promising method to control biofilms such that the enzymes destroy the biofilm matrix, by binding and breaking down the EPS components, so that the matrix-embedded cells are effectively removed by the antimicrobials. Pre-treatment of biofilms with enzymes can replace biocides or significantly reduce their concentration, since the enzymatic effect on the EPS matrix promotes access of biocides to the cells (Meireles et al. 2016).

Currently, the bio-based antimicrobial substances have attracted public attention due to their high efficacy, safety and non-toxicity. Essential oils, which are aromatic oily liquids derived from plant materials are known as natural and safe biocides. Several studies have demonstrated their anti-biofilm activity by eliminating and preventing biofilm formation (Oh et al. 2017; Engel et al. 2017; Mohamed et al. 2018). However, the application of essential oils is limited due to their low stability, high volatility and poor water solubility (Liu et al. 2018). In addition, enzyme activity is highly affected by environmental factors and is only optimal under limited conditions. Moreover, enzymes can autodegrade, resulting in instability (Hijo et al. 2015). A new strategy, such as encapsulation of these molecules in different material supports, is therefore necessary to overcome these problems. Microencapsulation of EO is an effective strategy to improve their stability and reduce their immiscibility in water (Mechmechani et al. 2022). Additionally, the microencapsulation of the enzymes allows to maintain their stability and high activity during a long storage period (Tikhonov et al. 2021). Moreover, this technique allows to control the release of these two active conpounds and reduces their physico-chemical interactions with the biofilm matrix components. These interactions are often associated with a decrease in the effectiveness of anti-biofilm molecules (Cui et al. 2016; Engel et al. 2017; Mechmechani et al. 2022).

Thus, this dissertation is organized into three chapters:

Chapter I, (Article I), summarizes the different factors that influence bacterial adhesion and biofilm formation on abiotic surfaces, as well as the resistance of biofilms to disinfectants. In addition, the problems related to biofilms in the food and medical sectors, and the different strategies to prevent and eradicate biofilms will be highlighted. As well, the efficiency of hurdle technology to control biofilms using different bio-sourced molecules will be also discussed.

Finally, the place of microencapsulation as a tool for the formulation of bio-based biocides for biofilm control will be presented.

Chapter II, (Article II), presents the efficacy of free and encapsulated carvacrol, a monoterpenoid phenol, to fight *Pseudomonas aeruginosa* and *Enterococcus faecalis* biofilms. For this purpose, feed emulsions were prepared using sodium caseinate as emulsifier and maltodextrins as drying matrix and then spray-dried to obtain dry carvacrol microcapsules. The physicochemical properties and microscopic morphology of the realized capsules were characterized. The minimal inhibitory concentration and the mode of action of free and encapsulated carvacrol were investigated against planktonic cells of *Pseudomonas aeruginosa* and *Enterococcus faecalis*. In addition, the anti-biofilm activity of free and encapsulated carvacrol was evaluated against both bacterial biofilms grown on stainless steel surfaces. The main objective was to improve the efficacy of this bio-based antimicrobial compound against pathogenic bacterial biofilms through its spray-dryied encapsulation, while reducing the amount used.

Chapter III is devised into two parts. The first one, (Article III), reports experimental results on the efficacy of two proteolytic enzymes, pepsin and trypsin, to degrade *Pseudomonas aeruginosa* and *Enterococcus faecalis* biofilms by targeting proteins-associated matrix. Moreover, these enzymes were combined with carvacrol to study their synergistic effect on biofilms removal. This study aims to demonstrate the potential effects of proteolytic enzymes to degrade barrier properties by possible interacting with proteins-associated matrix of *Pseudomonas aeruginosa* and *Enterococcus faecalis*, thus facilitating carvacrol penetration and reducing cell survivability. The minimal dispersive concentrations of proteolytic enzymes against bacterial biofilms grown on polysterene surfaces were evaluated. In addition, the dispersive activity of enzymes and the antimicrobial activity of carvacrol, as well as the synergistic effect of these two active components against bacterial biofilms grown on stainless steel surfaces, were demonstrated using direct microscopic analysis and indirect cell counting. On the other hand, the second part, (Article IV), presents the effect of encapsulated pepsin and trypsin, and carvacrol to fight *Pseudomonas aeruginosa* and *Enterococccus faecalis* biofilms. Microencapsulation using spray-drying method was performed in order to ensure the stability and long-term activity of these active compounds, as

well as to limit their interactions with the biofilm matrix components. Two types of microcapsules were prepared containing pepsin or trypsin complexed with pectin and maltodextrin. The previously prepared encapsulated carvacrol was also used in this study. The characterization of these capsules was studied. Moreover, the single and combined effect of the dispersive activity of the enzymes and the antimicrobial activity of carvacrol, were also studied against both bacterial biofilms.

Finally, this thesis is concluded with a general conclusion and the perspectives of the present study.

Objectives

In order to fight against foodborne and healthcare associated infections, our study aimed to investigate the effect of hurdle technology using proteolytic enzymes (pepsin and trypsin) and essential oil (carvacrol) in biofilm control. In addition, in order to improve the antibiofilm activity of enzymes and carvacrol, microencapsulation of these active agents was performed using spray drying. Thus, the main goals of our study are:

1) to study the antibiofilm activity of carvacrol against *P. aeruginosa* and *E. faecalis* biofilms and to improve the efficacy of this antimicrobial agent through its spray-dried formulation, while reducing the amount used.

2) to investigate the effect of free and microencapsulated carvacrol on cell membrane permeability of *P. aeruginosa* and *E. faecalis*.

3) to investigate the potential efficacy of pepsin and trypsin to degrade *P. aeruginosa* and *E. faecalis* biofilms and their synergistic effect when combined with free carvacrol, and to evaluate the effect of microencapsulation on their antibiofilm activity.

Chapter I
Article I-Literature Review

Hurdle technology using encapsulated enzymes and essential oils to fight bacterial biofilms

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Abstract

Biofilm formation on abiotic surfaces has become a major public health concern because of the serious problems they can cause in various fields. Biofilm cells are extremely resistant to stressful conditions, because of their complex structure impedes antimicrobial penetration to deep-seated cells. The increased resistance of biofilm to currently applied control strategies underscores the urgent need for new alternative and/or supplemental eradication approaches. The combination of two or more methods, known as Hurdle technology, offers an excellent option for the highly effective control of biofilms. In this perspective, the use of functional enzymes combined with biosourced antimicrobial such as essential oil (EO) is a promising alternative anti-biofilm approach. However, these natural antibiofilm agents can be damaged by severe environmental conditions and lose their activity. The microencapsulation of enzymes and EOs is a promise new technology for enhancing their stability and improving their biological activity. This review article highlights the problems related to biofilm in various fields, and the use of encapsulated enzymes with essential oils as antibiofilm agents.

Key points

- *Problems associated with biofilms in the food and medical sectors and their subsequent risks on health and food quality.*
- *Hurdle technology using enzymes and essential oils is a promising strategy for an efficient biofilms control*
- *The microencapsulation of enzymes and essential oils ensures their stability and improves their biological activities.*

Keywords Biofilm; Enzymes; Essential oils; Microencapsulation.

Introduction

Pathogenic bacterial contaminations of abiotic surfaces in food and medical sectors represent a serious public health problem, as they can lead to severe human infections worldwide (Abdallah et al. 2014; Khelissa et al. 2017). Foodborne infections generally occur after consumption of food and drink contaminated with pathogens. These contaminations can occur during any step of food processing, through food handlers and contaminated food contact surfaces and equipment (Verraes et al. 2013). According to the annual report of the Centers for Disease Control and Prevention (CDC), 841 foodborne disease outbreaks were reported in the United States in 2017, resulting in 14,481 illnesses, 827 hospitalizations, 20 deaths, and 14 food product recalls (CDC 2019). The World Health Organization (WHO) reported that an estimated 600 million people - nearly one in ten people worldwide - become ill from consuming contaminated food and 420,000 die each year of which 30% occur among children under 5 years old. In addition, 110 billion US$ are lost annually in medical expenses and productivity due to unsafe food in low- and middle-income countries (WHO 2020). In 2019, 1,783 foodborne illnesses were reported in France, affecting 15,641 people, of which 609 (4%) were hospitalized (hospitalization or emergency room visit) and 12 (0.08%) died (SPF 2021). Healthcare-associated infections (HCAIs) are the most common adverse event in the healthcare field worldwide. These infections can occur in all types of healthcare settings through healthcare personnel's hands and contaminated devices and surfaces (catheters, surgical instruments, endoscopes, respiratory systems, needles, etc.) (Weber et al. 2013; Ssekitoleko et al. 2020). According to the U.S. Centers for Disease Control and Prevention, almost

1.7 million hospitalized patients contract HCAIs each year while treated for other health problems, and more than 98,000 patients (one in 17) die from them (Klevens et al. 2007). In 2017, the National Point Prevalence Survey (PPS) on HCAI in France showed that one in twenty patients hospitalized in a healthcare facility were infected. The four main sites of HCAI, accounting for 71.5% of documented infectious sites - urinary tract infections (28.5%), surgical site infections (15.9%), pneumonia (15.6%) and bloodstream infections (11.4%) – were identical in 2012 and 2017 (SPF 2019).

In natural and artificial environments, bacteria tend to live attached to the abiotic surfaces and to develop a complex structure called biofilm. It has been found that approximately 40-80% of the bacterial cells on earth are able to form biofilms (Flemming and Wuertz 2019). Biofilms, unlike planktonic cells, are a self-protected grown cluster of bacteria. They are defined as a structured microbial community, adhering to a surface, to interfaces and to each other, and embedded in a self-produced polymer matrix that offers a highly protective environment against biocide attack (Donlan and Costerton 2002; Karygianni et al. 2020). The formation of biofilms poses serious problems in many fields due to the potential increased resistance to chemical biocides, antibiotics and UV radiation; increased secretion of secondary metabolic products and high gene exchange rates (de Carvalho 2017; Xu et al. 2017; Gebreyohannes et al. 2019; Rodrigues and Černáková 2020). Several studies have shown that industrial ecosystems are favourable areas for bacterial growth and biofilm formation (Coughlan et al. 2016). In hospitals environment, biofilms have been found to survive and persist on many medical device surfaces and on the tissue of patients, resulting in several persistent infections (Dongari-Bagtzoglou 2008; Percival et al. 2015). Thus, the control of biofilms remains the most important task for many industries to reduce the microbiological risk associated with its persistence in these areas. Several strategies have recently been proposed to combat biofilms, which include chemical removal such as detergents, biocides and surfactants; and mechanical removal such as thawing, freezing, sonication and scraping (de Carvalho 2007; Zea et al. 2020). However, complete removal of biofilm by the single use of these methods has been shown to be difficult to achieve due to the high protection of biofilm cells by EPS that act as an initial protective barrier to the biofilm cells, and make biofilm 10-1,000 times more resistant to antimicrobial agents than the planktonic cells (Singh et al. 2017; Tan et al. 2018). In addition,

although the sanitation is one of the most widely used and essential techniques to control biofilm in the industries, it is important to note that the application of these sanitizers for many decades could be a major cause of the emergence of antibiotic resistance in bacteria and their spread to pathogens, which has led to the search for new natural antimicrobial agents to overcome these issues (Bayoumi et al. 2012; al Kassaa et al. 2021).

The combined use of two or more hurdle methods to control biofilm (Hurdle technology) is a potentially effective strategy for an efficient biofilm cells removal from abiotic surfaces, as they would attack microorganisms in different ways (Khan et al. 2017). The synergistic effect of reducing bacterial contamination from abiotic surfaces using Hurdle technology has been successfully demonstrated in numerous studies (Lequette et al. 2010; Pechaud et al. 2012; Ban and Kang 2016; Lim et al. 2017; Jung et al. 2018; Lim et al. 2019; Hussain et al. 2019). In this way, the combination of enzymes with bio-based antimicrobials will be a promising method for controlling biofilm in such a way that the enzymes would destabilize and destroy the biofilm matrix, so that bacteria protected by the matrix would be eliminated more effectively by the antimicrobials. It's now established that enzymes control and eliminate biofilms owing to their ability to degrade major components of the biofilm matrix, promote cell lysis, induce biofilm disruption, and disrupt cell-to-cell signals that govern biofilm maintenance and formation (Mohamed et al. 2018). Essential oils (EOs), which are aromatic oily liquids derived from plant materials are known as natural and safe bio-based biocides for synthetic drugs and antiseptics, and have been widely tested in vitro against a broad range of pathogenic bacteria (Oulkheir et al. 2017). In addition to their antibacterial activity, numerous studies have demonstrated their anti-biofilm activity by removing and preventing biofilm formation (Vázquez-Sánchez et al. 2015; Oh et al. 2017; Engel et al. 2017; Mohamed et al. 2018). The use of EOs and their application have to face many challenges such as their volatility, stability issues and their poor water solubility which may decrease their activity. It is therefore necessary to find out a new strategy, such as encapsulating these molecules in different material supports, to effectively improve their activity. Microencapsulation of EOs is a good tool to increase their stability, decrease their water immiscibility, control their release and limit the physicochemical interactions between these molecules and the biofilm matrix components. These interactions are often associated with a

decrease in the efficacy of the antibacterial molecules (Cui et al. 2016a; Cui et al. 2016c; El Asbahani et al. 2015; Engel et al. 2017).

In this regard, this review will focus on the various factors that influence the adhesion of bacteria and the formation of biofilms on abiotic surfaces, as well as the biofilms resistance towards disinfectants. Moreover, the problems associated with biofilms in food and medical sectors, and the different strategies for biofilm prevention and eradication will be highlighted, in addition to the effectiveness of hurdle technology in removing biofilms using different bio-based molecules. Finally, the place of the microencapsulation as a tool for the formulation of bio-based biocides to fight biofilm will be discussed.

What is a bacterial biofilm and how is it formed?

Biofilms are generally defined as microbial populations that are irreversibly associated (cannot be removed by mild rinsing) with a surface and wrapped in a self-produced EPS matrix (Donlan 2002; Hall-Stoodley and Stoodley 2009). Microorganisms structured within biofilm differ from their planktonic counterparts by the transcribed genes. Bacteria are able to form biofilms when the growth conditions are suitable on different abiotic surfaces encountered in healthcare, food industries, industrial or potable water systems, and natural aquatic systems (Abdallah et al. 2014).

Biofilm formation steps

The process of biofilm formation is complex and several factors may be involved. Bacterial cell adhesion and biofilm formation are significantly linked to the substratum properties such as hydrophobicity, electric charge and rugosity. In addition, cell surface and the presence of pili, flagella, glycocalyx, or fimbriae are important for cell adhesion and biofilm formation (Donlan 2001; Roy et al. 2017; Hage et al. 2021). A bacterial biofilm can be structured in four common stages (Fig. 1). In the first stage, the bacterial cells bind to a biotic or abiotic surface (1), then the cells cluster multiply and form microcolonies (2) followed by the formation mature biofilm (3). The last stage is the detachment and dispersion of bacterial cells in the surrounding environment (4) (Abdallah et al. 2014; Khelissa et al. 2017).

The initial stage is governed by the reversible interactions mediated by the non-specific Lifshitz-van der Waals, Lewis acid-base and electrostatic forces (Kaplan 2010), and requires the presence of specific adhesins located on the host (e.g. fimbriae, flagella) (1) (Rosan and Lamont 2000; Abdallah et al. 2014). In the second step, the adherent bacteria synthesize exopolysaccharide proteins and other components of the polymer matrix that maintain the bacterial cells together in a mass and tightly fixe the bacterial mass to the surface and contribute to the irreversible adhesion (2). In the third stage, the biofilm becomes mature and able to express different genes and contributes to the antimicrobial resistance of the biofilm (3) (Kaplan 2010; Mah and O'Toole 2001; Chakraborty and Kumar 2019). The final biofilm formation step is the dislocation of cells from the biofilm and their dispersion in the environment (4). Cell detachment can be triggered by a variety of factors such as mechanical disturbances, polymer matrix enzymatic degradation, surfactant production and exopolysaccharide release (Kaplan 2010). These cells have the ability to adhere to new surfaces, re-form a biofilm and may contribute to biological dispersion, bacterial survival and disease transmission that are known as biofilm lifecycle. As at other stages of biofilm development, bacteria respond to multiple environmental signals (e. g. nutrient concentrations), signal transduction pathways, effectors and bacterial cell density, a phenomenon better known as quorum sensing (QS) (Karatan and Watnick 2009; Liu et al. 2019). QS is the regulation of gene expression in response of cell density by the liberation of chemical signal molecules called autoinducers (acylated homoserine lactones as autoinducers of Gram-negative bacteria and oligo-peptides as autoinducers of Gram-positive bacteria), that allows the differentiation of bacterial biofilm. When these molecules attain a minimal threshold stimulatory concentration, the activation or repression of new genes occurs (Zhao et al. 2020). Thus, QS allows bacteria to display many responses that benefit the population, such as enhanced accessibility to nutrients and more favourable environment, and promotes action against competing bacteria and environmental stresses (Zhao et al. 2020).

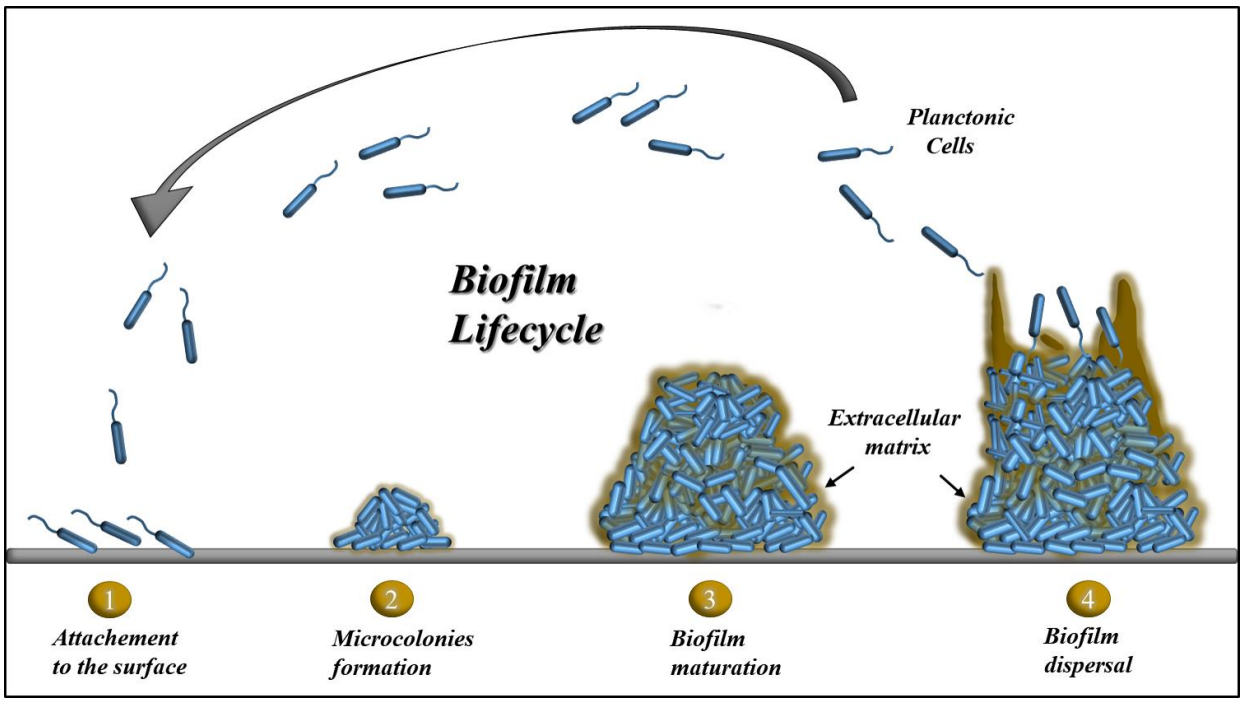


Fig. 1 Different stages of biofilm formation

Extracellular Polymeric Substance, composition and functions

A biofilm is composed of attached microbial cells encased within a matrix of EPS. EPS was initially designated as "extracellular polysaccharides", but it has been renamed as "extracellular polymeric substance " because it can also contain many others substances (Flemming and Wingender 2010). EPS may represent 50% to 90% of the total organic carbon of biofilms (Evans 2014), The composition of the matrix varies according to the bacterial species, strains and growing conditions, it is highly hydrated (can contain up to 97% water) and is mainly composed of proteins, polysaccharides and extracellular DNA (eDNA) (Fulaz et al. 2019). Biofilm matrix can also contain surfactants, lipids, glycolipids, extracellular enzymes and cations (Karatan and Watnick 2009; Flemming and Wingender 2010; Karygianni et al. 2020). Most of the today knowledge has been generated by using model organisms forming biofilms, in particular *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Streptococcus mutans* and *Vibrio cholerae* (Fig. 2). Several advanced reviews have detailed the functional role

and composition of EPS in various matrices formed by these organisms as biofilms of a single species (Zarnowski et al. 2014; Peterson et al. 2015; Hobley et al. 2015; Flemming et al. 2016; Dragoš and Kovács 2017; Bowen et al. 2018).

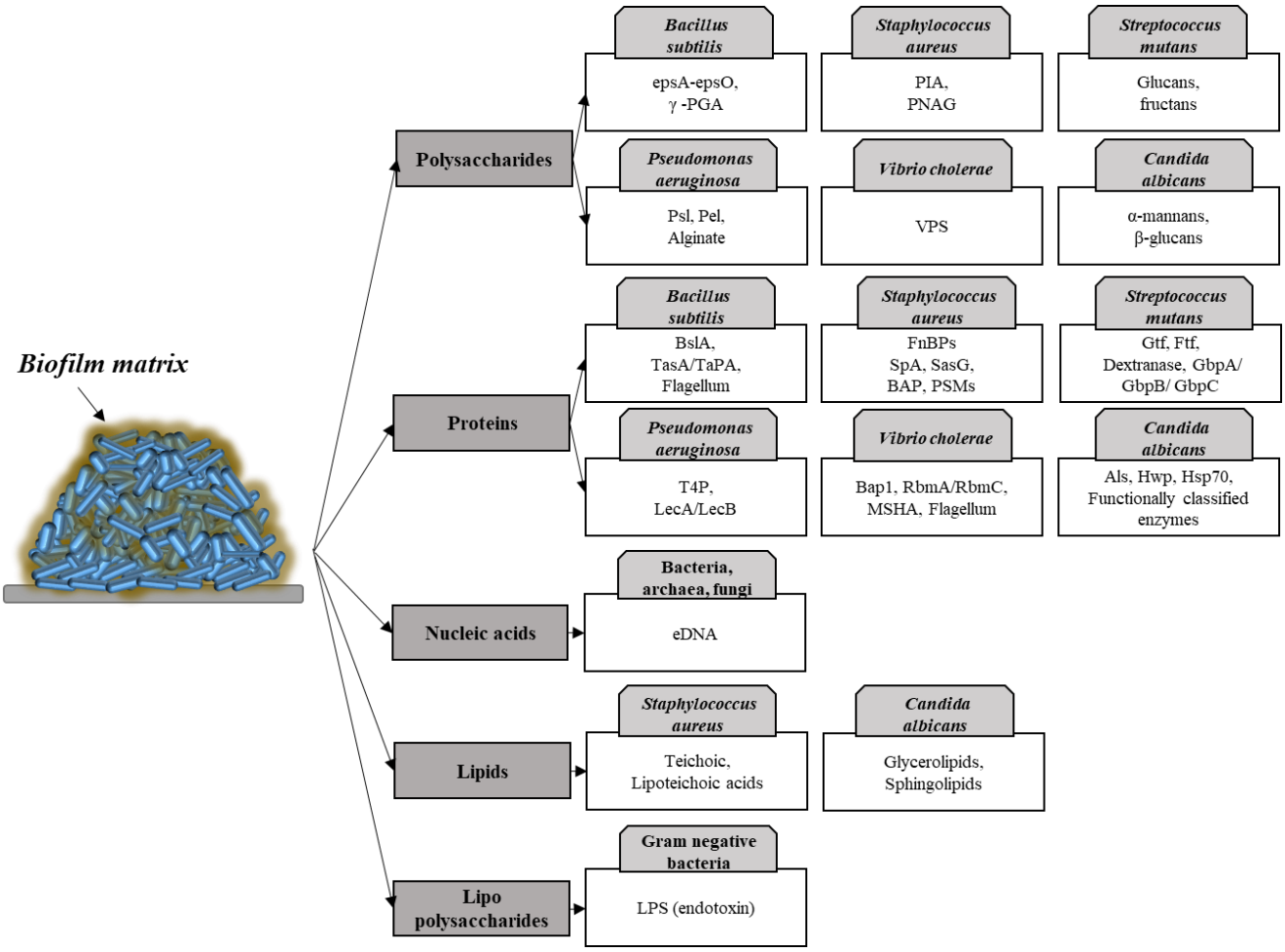


Fig. 2 Composition of extracellular polymeric substances (EPS) in the biofilms of some model organisms, epsA-epsO operon-encoded exopolysaccharide, poly-γ-glutamate (γ-PGA), Polysaccharide intercellular adhesin (PIA), poly-β(1-6)-N-acetylglucosamine (PNAG), Vibrio cholerae Vibrio polysaccharide (VPS), Biofilm surface layer protein (BslA), Translocation-dependent antimicrobial spore component (TasA)/TasA anchoring and assembly protein (TapA), Fibronectin-binding proteins (FnBPs), Staphylococcal Protein A (SpA), *Staphylococcus aureus* surface protein G (SasG), Biofilm associated protein (BAP) Extracellular, Phenol-soluble modulins (PSMs), Glucosyltransferases (Gtf), fructosyltransferases (Ftf), Glucan binding proteins (GbpA, GbpB, GbpC), Type IV pilins (T4P), Lectins (LecA/LecB), Biofilm-associated protein (Bap1), Rugosity and biofilm modulators (RbmA/RbmC), Mannose-sensitive hemagglutinin (MSHA) pili, Agglutinin-like sequence protein (Als), Hyphal wall proteins (Hwp) Cell wall, Heat-shock proteins (Hsp70). Further details on EPS components, including those from other microbes (such as

Escherichia coli), are available from the following references (Mann and Wozniak 2012; Vlamakis et al. 2013; Zarnowski et al. 2014; Teschler et al. 2015; Hobley et al. 2015; Ibáñez de Aldecoa et al. 2017; Dragoš and Kovács 2017; Cochet and Peri 2017; Bowen et al. 2018; Nett and Andes 2020)

EPS, which has been termed as the "dark matter of biofilms", may provide a potential number of challenges, and play a very crucial role in adhesion, aggregation, cohesion, structural integrity and protective barrier of biofilm (Fulaz et al. 2019). The EPS matrix provides key architectural and protective support for microbial communities in the biofilm, blocking access of xenobiotic and antimicrobials to biofilm cells and providing protection against environmental stressed such as pH change, UV radiation, desiccation and osmotic shock (Al Kassaa et al. 2019; Karygianni et al. 2020).

The polysaccharides play a fundamental role in the biofilm formation, antibiotic tolerance and as a virulence factor in opportunistic pathogens. Examples of the most common polysaccharides are alginate, cellulose, polysaccharide synthesis locus (PSL), and pellicle (PEL) polysaccharides Pel, Psl, and the staphylococcal polysaccharide intercellular adhesin (PIA) (Fulaz et al. 2019; Bundalovic-Torma et al. 2020). The proteins play also an essential role in biofilm adhesion and cohesion and, in some cases, are found in higher proportions than polysaccharides (Karygianni et al. 2020). The common proteins presented in the biofilm matrix are amyloid fibers (Fulaz et al. 2019). Furthermore, previous studies have shown that eDNA plays a significant role in the structural stability, formation, and integrity of the bacterial biofilms (Devaraj et al. 2019) (Table 1).

Table 1 The role of EPS in biofilm formation

Function	Relevance for biofilm	EPS components involved	References
Adhesion	Allows the first steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term fixation of entire biofilms to the surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules	(Flemming et al. 2007)
Aggregation of bacterial cells	Enable binding between bacterial cells and biofilm cohesion that allows bridge construction between cells, the increase in cell density, the overall mechanical stability of biofilm and the determination of the biofilm architecture	Polysaccharides, proteins and DNA	(Dunne 2002)
Cohesion of biofilms	Formation of the biofilm matrix, ensuring the mechanical stability of biofilms, determining the architecture of the biofilm and allowing communication between cells	Charged and neutral polysaccharides, proteins (such as lectins and amyloids), and DNA	(Karygianni et al. 2020)
Retention of water	Retains a highly hydrated microenvironment around the biofilm which contributes to their tolerance to desiccation in water-poor environments	Hydrophilic polysaccharides and, possibly, proteins	(Flemming 2016)
Protective barrier	Defensive barrier by providing resistance to defenses during infection by host or nonspecific defenses and provides tolerability to various antimicrobial agents as well as environmental stress	Polysaccharides and proteins	(Leid 2009)
Sorption of organic and inorganic substances	Promotes the accumulation of nutrients from the environment, the sorption of xenobiotics, the formation of polysaccharide gels, the exchange of ion, the formation of minerals and the accumulation of metallic toxic ions (thus	Charged or hydrophobic polysaccharides and proteins	(Fulaz et al. 2019)

	contributing to the detoxification of the environment)		
Enzymatic activity	Allows the sequestration of dissolved and particulate substances in the environment, which provide nutrients to biofilm organisms and the degradation of the matrix EPS which permits the liberation of cells from biofilms	Proteins	(Flemming et al. 2007; Dewasthale et al. 2018)
Nutrient source	Offers a source carbon, nitrogen and phosphorus for consumption by the biofilm cells	Potentially all EPS components	(Flemming and Wingender 2010)
Exchanging of genetic material information	Promotes cross-gene transfer between biofilm cells	DNA	(Madsen et al. 2012)
Redox activity	Play an important role in extracellular electron transfer reactions	Proteins (for example, those forming nanowires and pili) and, possibly, humic substances	(Cao et al. 2011)
Over-energy storage	Storage of excess carbon	Polysaccharides	(Bester et al. 2011)
Binding of enzymes	Accumulation, retention and stabilization of enzymes by their interaction with polysaccharides	Polysaccharides and enzymes	(Lens et al. 2003)

Biofilm architecture

Although some structural properties of biofilm can generally be regarded as universal, it has been reported that each biofilm community is unique (Tolker-Nielsen and Molin 2000). The morphology of biofilm can be rough smooth and flat or filamentous, furthermore the biofilm can change on its degree of porosity, with mushroom-like macrocolonies surrounded by voids filled with water (Flemming and Wingender 2010). The concept of this diversity of structure is descriptive not only

for mixed crop biofilms (environmental biofilms) but also for pure crop biofilms common to medical devices and those associated with infectious diseases (Donlan 2002). Several parameters can explain this heterogeneity, including the surface properties (e. g. hydrophobicity, roughness, electrochemical properties), hydrodynamic forces (e. g. mass transfer, shear forces, frictional drag, form drag), the presence of nutrients or inhibitors (e.g. concentration, antimicrobial properties, mass transfer properties, reactivity) and the consortia and ecological diversity of the biofilm (e.g. cell signal, presence of morphotypes, motility, food chains, trophic structure) (Stoodley et al. 1997). In addition, the structured communities of biofilm depend highly on the quantity, characteristic and the three-dimensional structure (the dense areas, pores and channels) of the EPS (Sutherland 2001).

Factors influencing bacterial cells adhesion

The attachment of a cell to a substrate is called adhesion, and the attachment of one cell to another is called cohesion (Garrett et al. 2008). For the first steps of adhesion, the interactions between the conditional layer and the substrate strongly influence the growth of cellular communities; this layer can be composed of many organic or inorganic particles and modifies the substrates which facilitate the accessibility to bacteria. The biofilm adheres in a reversible or irreversible manner. Factors such as available energy, surface functionality, bacterial orientation, temperature and pressure conditions have a significant effect on the initial adhesion of bacterial cells. Then if the repellent forces are higher than the attraction forces, the bacteria detach from the surface, this is more apt to occur before a substrate is conditioned (Garrett et al. 2008). If the physical appendages of bacteria (flagella, fimbriae and pili) overcome the repellent physical forces of the electric double layer, a number of reversibly adsorbed cells remain immobilized and become irreversibly adsorbed (Weger et al. 1987). Some research has shown that microbial adhesion is highly dependent on the hydrophobic-hydrophilic and topography properties of interacting surfaces (Liu et al. 2004; Hage et al. 2021).

Factors of biofilm resistance

There are different factors related to the physiological and structural characteristics of a biofilm that influence its resistance to disinfectants. Biofilm can be protected against antimicrobials by the

limitation of diffusion or reaction of disinfectants, thus due to the presence of EPS and multiple layers of cells that can form a complex and dense structure, biocides have a difficult entering and achieving the inner layers, which affects their effectiveness (Bridier et al. 2011). The organic matter present in the matrix such as proteins, nucleic acids or carbohydrates can deeply interfere with the efficiency of disinfectants (Banach et al. 2015). The phenotypic adaptation of biofilm cells to non-lethal doses of disinfectants can also lead to biofilm resistance toward biocides. In addition, due to the limited penetration of antimicrobials and the low levels of exposition of the biofilm deep layers to the antimicrobial agent, the biofilm can develop adaptive responses to sublethal concentrations of the disinfectant (Bridier et al. 2011). Moreover, the physiological adaptations of biofilm cells, such as expression of specific genes according to the environmental conditions of biofilms, allow the increase of the biofilm resistance. Many studies confirmed this adaptation by the comparison of gene expression profiles, and proteomic analyses of planktonic and biofilm states in different species (Sauer 2003; Whiteley et al. 2001; Abdallah et al. 2014), such as the expression of specific genes encoding for changes in membrane composition (Wolska et al. 2016), efflux pump (Soto 2013) and enzyme production. It has been reported that in *Pseudomonas aeruginosa* biofilms, quorum sensing is involved in the expression of catalase and superoxide dismutase genes. These enzymes are involved in protection against oxidative stress (Ahmed et al. 2019). Biofilm can also develop resistance by mutations and gene transfers (plasmids, transposons or integrons) that provide cells specific characteristic such as metabolic capabilities, virulence expression and antimicrobial resistance (Bridier et al. 2011). Moreover, multi-species biofilms can protect against antimicrobials when present in complex communities, where interactions between species can lead to the formation of a large complex matrix, protection of members of bacterial communities and expanded gene pool with more efficient passive resistance, quorum sensing systems, DNA sharing, metabolic cooperation, and many other synergies (Elias and Banin 2012; Wolcott et al. 2013). For instance, *in vitro* studies conducted on polymicrobial biofilms including *Staphylococcus epidermidis* and *Candida albicans* showed an altered susceptibility of each species to antimicrobial drugs due to their mutual interactions: the EPS of *Staphylococcus epidermidis* inhibited the penetration of fluconazole, while *Candida albicans* appeared to protect *Staphylococcus epidermidis* from vancomycin (Adam et al. 2002).

Microbiological hazard associated with bacterial biofilms

Bacteria are capable of colonizing and forming biofilms on almost any type of surface, including synthetic and natural surfaces (Hall-Stoodley et al. 2004; Sweet et al. 2011). Bacterial structured biofilms improve the ability of bacteria to survive under stress and cause serious problems in many sectors such as industries, water systems, medical facilities, public health, etc (Khelissa et al. 2017; Jamal et al. 2018; Di Pippo et al. 2018; Avila-Novoa et al. 2018). The detrimental effects of biofilms on the human society are therefore multiple.

The high capacity of bacteria to adhere and form biofilms on abiotic surfaces is a main concern for industries that provide a suitable environment for their formation (Donlan 2002; Simões et al. 2010; Flemming et al. 2013). Biofilms provoke biofouling of the industrial equipment such as cooling towers and heat exchangers. The biofouling is defined operationally as the development of biofilm that exceed a defined threshold of interference (Murthy and Venkatesan 2009). This problem leads to energy loss, effective heat transfer, increased fluid friction resistance and accelerated corrosion, as well as reduced the quality of product and many process additives and chemicals (Xiong and Liu 2010).

For example, the formation of biofilm in water distribution systems leads to reduced water quality and increased health risks (Dewanti and Wong 1995; Rao et al. 1998; Barak 2006). In the paper industry, biofilms cause the breakdown of chemicals such as calcium carbonate sludge and starch that are added to pulp sludge in the wet-end processing (Barak 2006).

In addition, the presence of biofilms is widespread in food industry. They can be present on all kinds of surfaces such as plastics, glass, metal, wood and food products (Chmielewski and Frank 2003). Microbial cells adherence to food contact surfaces is a serious concern for the food service and food processing industries, as adhesion can lead to cell survival and biofilm growth, allowing cross- and post- processing contamination. This reduces the shelf life of food products, and constitute the major factor of foodborne diseases (Shi and Zhu 2009; Bridier et al. 2015). In general, abiotic surfaces in contact with product may be cleaned many times per day, whereas environmental surfaces like walls may be cleaned weekly. This provides longer time for adherent cells to grow on environmental supports. Thus, the extensive colonization of surfaces and the formation of mature biofilm may occur on these environmental surfaces. However, the most of

food product contact surfaces can hold only the adherent bacteria cells and young biofilm (Gibson et al. 1999). These adherent cells and biofilms not only pose a hygiene hazard in the food industry, but also contribute to economical costs due to technological failures, impedance of heat transfer, mechanical blockage and metal surfaces corrosion (Houdt and Michiels 2010; Téllez 2010). Thereby, the need for efficient cleaning techniques is necessary to prevent the hazardous and expensive damage that bacterial biofilms can cause (Chmielewski and Frank 2003). Although many species of bacteria are able to form biofilms in the food industry, among the major genera of foodborne bacteria that are biofilm producers are *Pseudomonas*, *Listeria*, *Enterobacter*, *Flavobacterium*, *Alcaligenes*, *Staphylococcus* and *Bacillus* (Téllez 2010). Most importantly, *Pseudomonas* contributes to the formation of polymicrobial biofilms with other foodborne pathogens providing them shelter for persistence (Bai et al. 2021).

In healthcare environments, biofilms can be found on several biomedical device surfaces (e.g., pacemakers, catheters, prosthetic heart valves, contact lenses, breast implants, and cerebrospinal fluid shunts) and on patient's tissues (dead tissues: e.g., bone sequestration and living tissues: e.g., tooth surfaces, lung tissue) (Hall-Stoodley et al. 2004; Wu et al. 2015; Alav et al. 2018). Several Gram-negative and Gram-positive bacteria can form biofilms on the biomedical devices, but the most commonly found are *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Enterococcus faecalis*. It has been reported that approximately two-thirds of infections related to medical devices are attributed to *staphylococcal* species (Hall-Stoodley et al. 2004; Shokouhfard et al. 2015; Pakharukova et al. 2018; Khatoon et al. 2018). *Pseudomonas aeruginosa* can also form biofilms on the interior surfaces in hospital water distribution systems (Loveday et al. 2014). In addition, infections caused by enterococci have become of particular concern in recent years because of their ability to develop resistance against a wide range of antimicrobial drugs used in medical practice. They are also involved in serious life-threatening infections in patients suffering from cancers or chronic diseases (Boccella et al. 2021). Furthermore, the emergence of polymicrobial infections has serious implications for patient care because of the difficulties associated with selecting the most appropriate antimicrobial therapy, particularly when multidrug-resistant pathogens are implicated (Francolini and Donelli 2010). Bacteria forming biofilms can cause several life-threatening humans' diseases and infections such as otitis media, infective

endocarditis, osteomyelitis, periodontitis, cystic fibrosis and chronic wounds (Southey-Pillig et al. 2005; Akyıldız et al. 2013; Masters et al. 2019). It has been reported that biofilm is involved for more than 65% of all microbial infections and have a high resistance to antimicrobials and host defense system components (Jamal et al. 2018; Ciofu and Tolker-Nielsen 2019). Hence, biofilms have a considerable impact on the human healthcare.

Biofilm control

The controlling of biofilm accumulation remains the most arduous task for the many industries for which it is very important that both the inactivation and removal of biofilms from surfaces have to be realized (Simões et al. 2003; Dzianach et al. 2019).

As discussed previously, bacteria structured in biofilms are more resistant, than planktonic cells, to physical and chemical methods used in cleaning and disinfection of abiotic surfaces (Martin and Feng 2009). Several methods and strategies can be used to control biofilm such as chemical treatment, mechanical removal, quorum sensing inhibition, nanotechnological method, enzymatic dispersion, biosurfactants, biosourced compounds such as essential oils derived from plants, bacteriocins, bacteriophages (Fig. 3).

It is necessary to first understand the difference between disinfectants and sanitizers used in the industry. Disinfection means irreversibly destroying or inactivating specific infectious fungi and bacteria, but not necessarily spores, on hard surfaces. However, sanitizing means reducing microorganisms to levels considered safe for humans (Allan Pfuntner 2012).

Several chemical disinfectants can be used to treat biofilms such as: NaOCl, peracetic acid, NaOH and H_2O_2. The efficiency of these disinfectants is related to their oxidation of cellular structures (Rosenberg et al. 2008; Bayoumi et al. 2012; Nam et al. 2014; Bang et al. 2014; Ban and Kang 2016; Møretrø et al. 2017; Yang et al. 2017; Alvarez-Ordóñez et al. 2019). However, previous studies have indicated that most of these disinfectants have little or no significant effect on the removal of established biofilms (Walker et al. 2007). It has been reported that disinfection with chlorine and chlorine dioxide can decrease the concentration of planktonic cells, but has no effect on biofilm biomass (Berry et al. 2006). Other studies show that treatment with sodium hypochlorite, the main commercial disinfectant, does not significantly reduce the biomass of biofilms formed by

Escherichia coli on the stainless steel surface (Lim et al. 2019). Chlorine is known as the most widespread artificial chemical disinfectant used because of its broad antimicrobial spectrum, easiness of application and cost-effectiveness. Nevertheless, it is rapidly inactivated by organic matter. Moreover, chlorine activity is pH dependent, and exhibits corrosion even to stainless steel and may combine with organic compounds to form toxic by-products (Chmielewski and Frank 2003; Guzel-Seydim et al. 2004; Houdt and Michiels 2010).

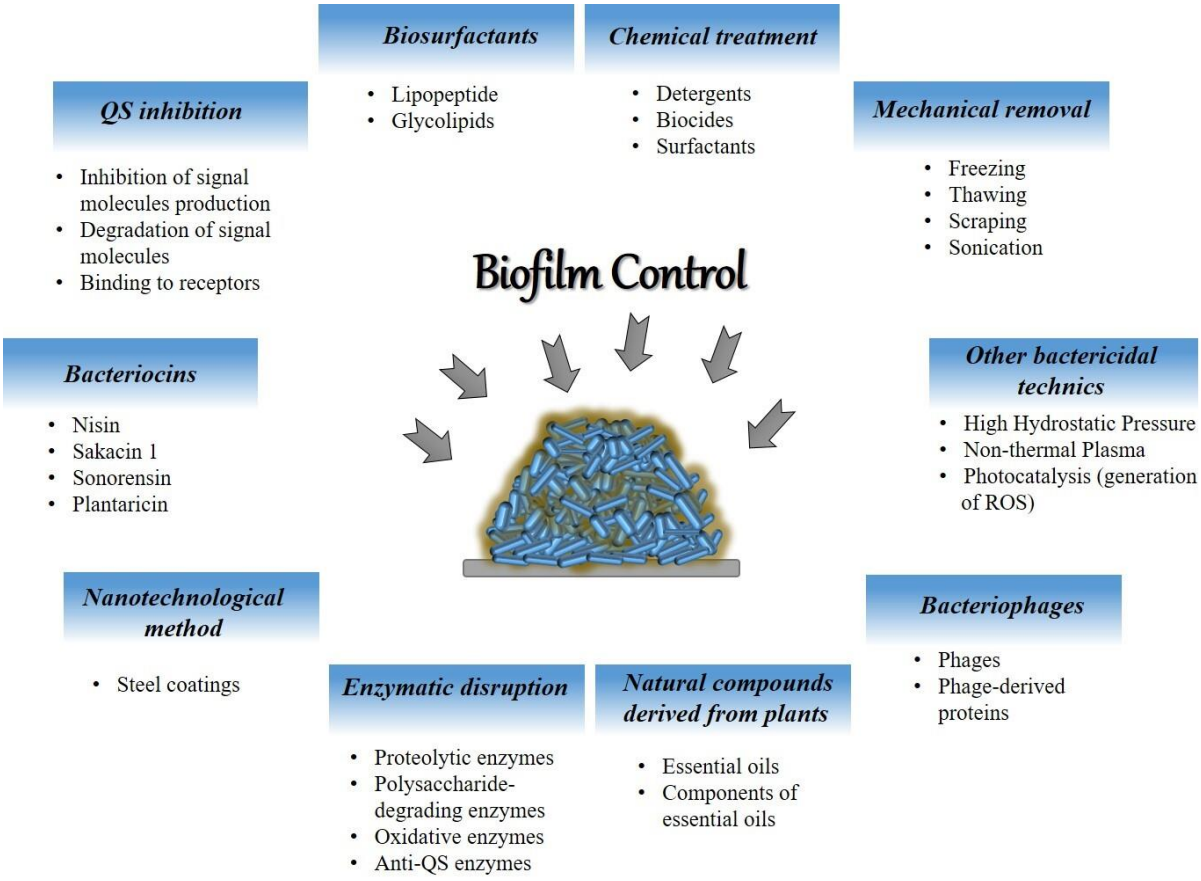


Fig. 3 Different methods for biofilm control, Quorum Sensing (QS), Reactive Oxygen Species (ROS) (de Carvalho 2007; García-Almendárez et al. 2008; Winkelströter et al. 2011; Torres et al. 2011; Bayoumi et al. 2012; Beyth et al. 2015; Silva 2015; Nobrega et al. 2015; Chopra et al. 2015; Scholtz et al. 2015; Winkelströter et al. 2015; Coughlan et al. 2016; Gutiérrez et al. 2016; Coronel-León et al. 2016; Campana et al. 2017; Nica et al. 2017; Castellano et al. 2017)

In addition, it is important to note that the use of these sanitizers for decades could be one of the main causes of the development of antibiotic resistance in bacteria and their spread to pathogens (Capita and Alonso-Calleja 2013). These issues combined with the growing consumer concerns about their own health and environmental consciousness, are leading to setup new alternative strategies to control biofilm such as the use of biosourced active molecules such as biosourced enzymes and essential oils (Knowles et al. 2005; Desai et al. 2012).

Enzymatic Disruption

The use of enzymes is an effective tool for eradicating biofilm owing to its ability to degrade the physical integrity of the EPS by binding and breaking down the components of the EPS into smaller units that can be transferred across cell membranes and then metabolized, thus destroying the multi-structural biofilm (Xavier et al. 2005; Mohamed et al. 2018). By a pre-treatment using enzymes, the biocides can be substituted or their concentration can be considerably reduced since the enzymatic effect on the EPS matrix promotes the access of the chemical biocides to the cells (Meireles et al. 2016). A wide range of enzyme applications have been described (Table 2) with the aim of reducing microbiological biofilm risk and replacing hazardous and ineffective chemical biocides, as well as providing an alternative green solution against biofilm formation due to their high biodegradability and low toxicity (Cortés et al. 2011; Srey et al. 2013). These characteristics make enzymes as a high-performance tool for controlling biofilm, thus, they are commonly used in detergents used in many industries (Torres et al. 2011; Huang et al. 2014).

Nevertheless, the enzymes effectiveness in eradicating and destroying biofilm is highly dependent on the composition of the matrix (Walker et al. 2007). Due to the heterogeneous composition of this matrix, different types of enzymes can be used to destroy biofilms. These enzymes can be applied individually or in combination with a complementary treatment (Meireles et al. 2016). There are currently four types of enzymes of potential interest in biofilm removal: polysaccharide degrading enzymes, proteolytic enzymes, anti-QS, and oxidizing enzymes that belong to three main classes: hydrolase, lyases and oxydoreductases (Boels 2011; Thallinger et al. 2013; Huang et al. 2014; Coughlan et al. 2016; Meireles et al. 2016).

Table 2 Examples of application of enzymes as anti-biofilm, their classification and targets

Types of enzyme	**Enzymes applied**	**Target Biofilm**	**Results**	**References**
Proteolytic enzymes	Savinase	*Pseudomonas fluorescens*	>75% biofilm removal	(Molobela et al. 2010)
	Endolysin (LysH5)	*Staphylococcus aureus*	1–3 log biofilm removal	(Gutiérrez et al. 2014)
	Bromelain	*Klebsiella pneumonia*	74.6% biofilm removal	(Mohamed et al. 2018)
	Savinase, Everlase Esperase Mixed protease	*Pseudomonas fluorescens*	>80% biofilm removal 74% biofilm removal 75% biofilm removal	(Molobela et al. 2010)
	Savinase	*Pseudoalteromonas*	Total biofilm removal	(Leroy et al. 2008)
	Protease P4 Protease P2 Papain P1 Papain P3	Seven types of biofilms Three types of biofilms Eight types of biofilms Nine types of biofilms	>70% biofilm removal	(Lequette et al. 2010)
	Proteinase K Trypsin	*Staphylococcus lentus* *Staphylococcus cohnii*, *Staphylococcus saprophyticus*	Strongly removing of biofilm	(Fagerlund et al. 2016)
	Dispersin B	*Staphylococcus epidermidis* *Staphylococcus aureus*	Strongly removing of biofilm	(Fagerlund et al. 2016)
	Trypsin	*Pseudomonas aeruginosa*	Strongly destroying biofilm	(Banar et al. 2016)
	Proteinase K	*Escherichia coli*	91.1–99.5% biofilm inhibition	(Lim et al. 2019)
	α-Amylase	*Staphylococcus aureus*	79% biofilm removal	(Craigen et al. 2011)
	Dispersin B	*Staphylococcus epidermidis*	40% biofilm removal	(Brindle et al. 2011)
	Fungamyl Amyloglucosidase Mixed amylases	*Pseudomonas fluorescens*	>80% biofilm removal >50% biofilm removal >70% biofilm removal	(Molobela et al. 2010)

Polysaccharide-degrading enzyme	Amylase S1 Polysaccharidase mix A	Six types of biofilms Three types of biofilms	Biofilm removal	(Lequette et al. 2010)
	α-mannosidase, β-mannosidase	*Pseudomonas aeruginosa*	Strongly destroying biofilm	(Banar et al., 2016)
	Pectin esterase	*Pseudomonas fluorescens*	Three quarters of the biofilm cells	(Orgaz et al. 2007)
	Cellulase	*Escherichia coli*	65.5–98.5% biofilm inhibition	(Lim et al. 2019)
Oxidative enzymes	DNase	*Listeria monocytogenes*	50% biofilm removal	(Nguyen and Burrows 2014)
	DNase I	Gram-negative and Gram-positive biofilm	Biofilm removal	(Tasia et al. 2020)
Anti-QS enzymes	Lactonase	*Pseudomonas aeruginosa*	>70% biofilm removal	(Kiran et al. 2011)
	acylase	*Pseudomonas aeruginosa*	60% biofilm inhibition	(Grover et al. 2016)

Essential Oils (EOs) as antibiofilm compounds

Essential oils (EOs) are volatile and aromatic liquids derived from plants. These compounds can be composed of complex mixtures and of low weight molecules, whose typical main components are dependent on the plant source (Engel et al. 2017). The biological activities of EOs and their components are largely recognized, including antimicrobial activities against bacteria, yeasts and molds (Burt 2004; Reyes-Jurado et al. 2015; Calo et al. 2015).

EOs or their purified antimicrobial components are natural alternative biocides that have recently attracted attention as potential cleaners for the following reasons: i) Many studies suggest that the chemical antimicrobial agents, currently used, trigger the development of antimicrobial resistance in the target microorganisms (Boakye et al. 2019). However, the development of bacterial resistance to EOs is limited because each EO is composed of a mixture of various active antimicrobial agents (Wińska et al. 2019). ii). The vapours emitted by EOs are highly bactericidal

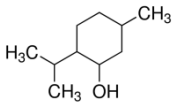
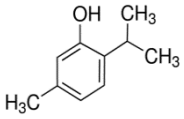
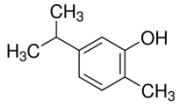
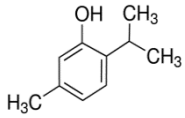
that can offer an additional advantage for the disinfection of hard-to-reach areas that need to be cleaned (López et al. 2005; López et al. 2007). iii). Due to the ongoing trend towards green technology and changing consumer attitudes, there is commercial advantage in antimicrobial agents that can be classified as "green", such as EOs of plant origin (Soni et al. 2013).

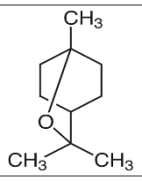
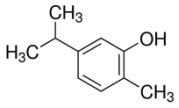
The antimicrobial activity of EOs is caused mainly by their hydrophobic characteristic, which helps them to disperse into bacterial cell membrane lipids, causing disruption of the structure and increasing its permeability. This can lead to leakage of ions and other cell molecules, and then lead to cell death (Rao et al. 2019). In general, EOs are slightly more effective against Gram-positive bacteria than Gram-negative ones (Ratledge and Wilkinson 1988; Davidson and Naidu 2000; Canillac and Mourey 2001; Cimanga et al. 2002; Delaquis et al. 2002). Gram-negative bacteria can be expected to be less sensitive to the action of EOs since they possess a lipopolysaccharide-coated outer membrane that surrounds the cell wall and limits the diffusion of hydrophobic compounds (Ratledge and Wilkinson 1988; Burt 2004). Nevertheless, not all research on antimicrobial activity of EOs has shown that Gram-positive bacteria are more susceptible (Wilkinson et al. 2003). Furthermore, the antimicrobial activity of EOs is linked to their interactions, chemical composition and the volatile molecules proportions (Dhifi et al. 2016).

EOs extracted from different plants can be used for their antibacterial effect such as: monoterpenoids (such as borneol, camphor, carvacrol, eucalyptol, limonene, pinene, thujone), sesquiterpenoids (such as caryophyllene, humulene) and flavonoids (such as cinnamaldehyde and other phenolic acids) (Campana et al. 2017). In general, EOs with a high level of phenolic compounds, such as eugenol, carvacrol, and thymol have significant antibacterial activities. These components are primarily responsible for disruption of the cytoplasmic membrane, electron flow, active transport, proton motive force and coagulation of the cell contents (Dhifi et al. 2016). Numerous studies prove the antimicrobial activity of EOs against one or more microorganisms (Sivropoulou et al. 1996; Lambert and Johnston 2001; Ooi et al. 2006; Rota et al. 2008; Xu et al. 2008). Moreover, EOs have been shown to be highly effective against the most serious foodborne pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* spp. (Braga et al. 2006). In addition, several studies (Table 3) demonstrate that essential oils have a significant

antimicrobial activity against biofilm and biofilm formation (Soni et al. 2013; Neyret et al. 2014; Amaral et al. 2015)

Table 3 Examples of application of essential oil or their components as anti-biofilm, their chemical structure and targets

Essential oil	Chemical structure	Targets Biofilm	Results	References
Menthol		*Klebsiella pneumoniae*	75.3-97.5% biofilm inhibition	(Mohamed et al. 2018)
Thymol			85.1-97.8% biofilm eradication	
Peppermint EO			69,2 to 98,2 % biofilm inhibition	
Thyme EO			80,1 to 98,0 % biofilm eradication	
Carvacrol		*Staphylococcus aureus*	1.87-2.04 log CFU/cm^2 biofilm eradication	(Engel et al. 2017)
Thymol			1.47-1.76 log CFU/cm^2 biofilm eradication	
R. officinalis EO		*Staphylococcus epidermidis*	>57% biofilm inhibition 67% biofilm eradication	(Jardak et al. 2017)
E. globulus EO		*Staphylococcus aureus*	74.74 to 89.15 % biofilm eradication Effective biofilm inhibition	(Merghni et al. 2018)

1,8-cineole			77.46 to 90.81 % biofilm eradication Effective biofilm inhibition	
M. longifolia EO		*Enterococcus faecalis,* *Escherichia coli* *Staphylococcus aureus* *Pseudomonas aeruginosa* *Klebsiella pneumonia* *Candida albicans*	Effective anti-biofilm activity	(Pazarci et al. 2019)
Carvacrol		*Salmonella typhimurium*	5.12 log CFU/cm^2 biofilm eradication	(Trevisan et al. 2018)
		Staphylococcus aureus	6 log CFU/cm^2 biofilm eradication 3 log CFU/cm^2 biofilm inhibition	(Nostro et al. 2009)
		Staphylococcus epidermidis	5 log CFU/cm^2 biofilm eradication 2 log CFU/cm^2 biofilm inhibition	

Hurdle technology as an efficient strategy to control biofilms

Due to the complexity of biofilms, a single use of a disinfectant may be insufficient to remove the entire undesirable biofilm. Hurdle technology involves the combined intelligent use of hurdles such as physical-chemical, chemical-chemical, or biological-chemical disinfection methods to achieve effective control of undesirable monomicrobial and polymicrobial biofilms by striking different targets within bacterial cells at the same time (Fig. 4) (Yuan et al. 2021). The synergistic effect of hurdle technology in reducing biofilm contamination has been proven by numerous studies (Ban and Kang 2016; Jung et al. 2018; KIM et al. 2019; Lim et al. 2019; Hussain et al. 2019; Venkatesh et al. 2009; Francolini and Donelli 2010).

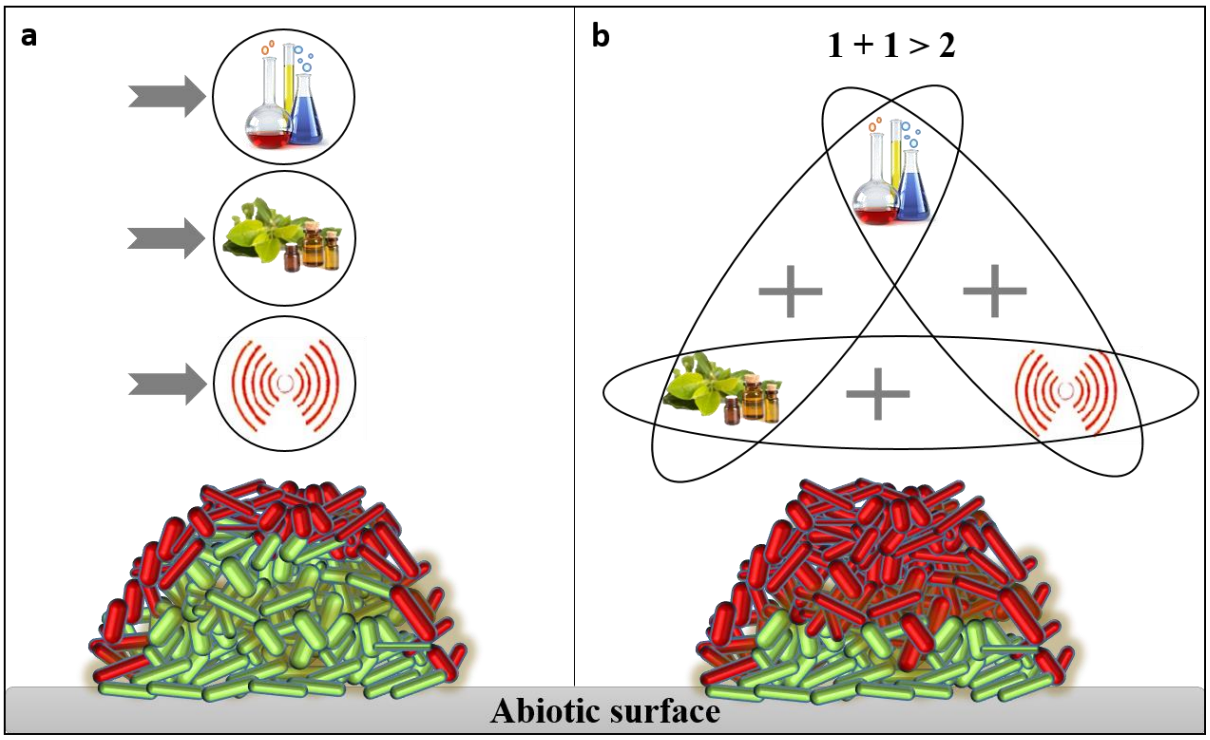


Fig. 4 Improved biofilm cell reduction by hurdle technology: (a) single disinfection strategy using a chemical, physical, or biological method (b) combined disinfection strategies using chemical-chemical, physical-chemical, or biological-chemical methods

In healthcare and food ecosystems, disinfection must be carried out economically and safely, by reducing the frequency of disinfection, and in the shortest timeframe possible, with the lowest use of chemicals, labour costs and energy, producing the least amount of waste and without damaging the equipment. Thus, Hurdle technology could be more effective in controlling biofilms compared to the single use of disinfectants. Potential solutions for combined disinfection procedures must therefore be carefully selected to achieve an effective disinfection effect.

Previous studies have shown that the combined use of physical and chemical disinfection strategies is very efficient against biofilms (Vankerckhoven et al. 2011; Kim et al. 2016; Jung et al. 2018). Indeed, the combined treatment of biofilms with ultraviolet irradiation (234 mJ/cm2) and hydrogen peroxide (5 ppm) proved to be 10 times more effective than treatment with hydrogen peroxide alone, which could lead to a more eco-friendly treatment (Vankerckhoven et al. 2011). In addition, treatment with biocide solutions that contain more than one bioactive agent was also found to be

effective in removing biofilms from industrial surfaces (Ortega Morente et al. 2013). It has been also reported that the combination of different disinfectant compounds can facilitate their diffusion into the biofilm matrix and improve their oxidative activity, resulting in high bactericidal activity even at low concentrations (Ríos-Castillo et al. 2017). Dhowlaghar et al., (2018) demonstrated that the use of a mixture of hydrogen peroxide and quaternary ammonium disinfectants, or hydrogen peroxide, octanoic acid and peracetic acid was able to completely remove *Listeria monocytogenes* biofilm from stainless steel surface, whereas treatment with a single active component in the disinfection procedure could not eliminate biofilm cells completely. In addition, the combined used of EDTA, ethanol, N-acetylcysteine and recombinant human talactoferrin with amphotericin B, fluconazole, nafcillin and vancomycin have been successfully applied as catheter lock solutions to rescue colonized catheters. It was found that these combinations were effective in inhibiting both monomicrobial and polymicrobial biofilms of *Staphylococcus epidermidis* and *Candida albicans* (Venkatesh et al. 2009).

The use of enzymes for the removal of biofilms in the industrial settings generally misses biocidal activity, making them unsuitable for bactericidal applications. To solve this problem, a combined use of enzymatic and antibacterial control approaches is desirable, as the action of the enzyme would contribute positively to the antibacterial activity of the disinfectant (Table 4). This strategy has the potential advantage of avoiding the overuse of toxic antimicrobial agents.

Many studies demonstrate that the antimicrobial agents use after enzymatic treatment can significantly inactivate microbial cells in biofilms (Table 4). EOs are biosourced compounds used as alternative natural disinfectants suitable for biofilm control. In addition, it has been reported an increased activity of these natural antimicrobials to inactivate biofilms when combined with other methods (Table 4). Thus, the use of enzymes in combination with an EO as biological hurdles seems to be a promising strategy to control biofilms. The goal of this strategy is that enzymes disrupt and destroy the biofilm matrix, so that the biosourced antimicrobials can hit the target efficiently and easily.

Table 4 Hurdle technology using enzymes and essential oils combined with another technology for biofilm control

Hurdle technology	Technique	Target biofilm	Finding	References
Enzymes + Other technology	Protease K + Soduim hypochlorite	*Escherichia coli* O157:H7	Increased sensitivity of biofilm cells (6.15 log CFU/cm^2 biofilm reduction)	(Lim et al. 2019)
	Pronase + Benzalkonium chloride	*Listeria monocytogenes*– *Escherichia coli* dual-species biofilm	Reducing dual-species biofilm cells below the detection limit Preventing secondary colonizers from further adhesion after the antimicrobial treatment	(Rodríguez-López et al. 2017)
	Cellulase + Cetyltrimethyl ammonium bromide	*Salmonella*	Completely remove biofilm (6.22 log CFU/cm^2 biofilm reduction)	(Wang et al. 2016)
	Polysaccharide depolymerase enzymes + Chlorine dioxide	*Klebsiella* spp.	Increasing of the disinfection effect (92% biofilm removal) Reducing the attachment of bacteria as well as the adhesion of EPS in the biofilm.	(Hansen et al. 2019)
	Savinase + Shear stress	*Pseudomonas aeruginosa*	Completely removing of biofilm (90% biofilm removal)	(Pechaud et al. 2012)
	Protease type XXIII, Crystalline trypsin,	*Escherichia coli*	61-96% Biofilm removal	(Oulahal-Lagsir et al. 2003)

	Cmyloglucosidase, Cysozyme, Papain + Ultrasonic waves			
Essential oils + Other technology	*Lippia sidoides*, *Thymus vulgaris*, *Pimenta pseudochariophyllus* + Peracetic acid	*Listeria monocytogenes*, *Staphylococcus aureus*	Doses required to reach a reduction greater than 4 log CFU/cm^2 in biofilms were reduced	(Vázquez-Sánchez et al. 2015; Vázquez-Sánchez et al. 2018)
	Clove oil + Cold nitrogen plasma	*Escherichia coli* O157:H7	5.48 log CFU/cm^2 reduction of biofilm was achieved by synergetic treatment	(Cui et al. 2016b)
	Thyme oil, Thymol + Cyprofloxacin	*Klebsiella pneumoniae*	Strong synergistic activities which affect the viability of biofilm cells (decrease ciprofloxacin effective dose)	(Mohamed et al. 2018)

Encapsulation as a tool to improve antibiofilm compound activities

Microencapsulation aims in protecting bioactivity of solid, liquid or gaseous materials by trapping within a surrounding matrix forming particles with a diameter of 1 to 1000 μm (Fu and Hu 2017). Microparticles can be in the form of microspheres or microcapsules. Microspheres are matrix systems in which the core is dispersed (heterogeneous microsphere) and/or dissolved in a polymer matrix (homogenous microsphere) (Silva et al. 2003), while microcapsules are particles consisting of an inner core surrounded by a material that is significantly different from that of the core. Mononuclear and polynuclear microcapsules can be classified according to the division of the core or not. However, the terms microcapsules and microspheres can be used synonymously (Singh et al. 2010).

Coating materials for microencapsulation

The coating serves as a protective film for isolating the core from inadequate exposure; the core can be released in the ideal place or at the ideal time, in various manners depending on the characteristics of the coating materials, such as physical pressure, friction, diffusion, dissolution of the wall and biodegradation (Suave et al. 2006; Qin 2016). The appropriate selection of wall material is extremely critical as it has a significant impact on the effectiveness and stability of the microcapsule. The most appropriate wall material should have the same properties: controlled release under specific conditions; non-reactive with the core; capacity to hold and stabilize the core inside the capsule; ability to protect the core from unfavourable conditions; absence of disagreeable taste in case of food application; and economic feasibility (Nazzaro et al. 2012; Gharsallaoui et al. 2012). A variety of coating materials can be used in microencapsulation such as: synthetic polymers such as non-biodegradable polymers (e.g. Poly methyl methacrylate (PMMA), acrolein, Glycidyl methacrylate Epoxy polymers) (Kreuter et al. 1983; Margel and Wiesel 1984) and biodegradable polymers (e.g. Lactides, Glycolides & their co polymers) (Wakiyama et al. 1981); and natural polymers such as proteins (e.g. albumin, gelatin, collagen) (Toshio et al. 1981) , carbohydrates (e.g. agarose, carrageenan, chitosan, starch) (Patel et al. 2011) and chemically modified carbohydrates (e.g. poly dextran, poly starch) (Jain 2000).

Control of the release of encapsulated molecules

Encapsulation should protect and isolate the core from the environment until the desired release at the appropriate time and place (Gouin 2004). Many factors affect the rate of releasing including the interactions between wall materiel and core, the volatility of core, the ratio of core to support material, the size and viscosity of particle of wall material, among others (Roberts and Taylor 2000). The release of the core is conditioned by several mechanisms involved: degradation (enzymes such as lipases and proteases that degrade lipids and proteins, respectively) (Rosen 2005), diffusion (chemical properties of core and wall material and physical properties of wall determine the releasing of core from intact wall) (Choudhury et al. 2021), use of solvent (contact with solvent that dissolve wall material) (Frascareli et al. 2012), pH (solubility of membrane wall altered by changes of pH) (Toldrá and Reig 2011), pressure (applied pressure to the capsule wall cause

releasing) (Wong et al. 2009) and temperature (expanding or collapsing of wall material in a critical temperature which name temperature-sensitive release or melting of wall material when the temperature increase which name fusion-activated release) (Park and Maga 2006). Moreover, the combination of two or more mechanisms can be used (Desai and Park 2005).

Microencapsulation methods

Many encapsulation methods such as spray drying, extrusion, coacervation are currently used for the encapsulation of the antimicrobial substances (Fig. 5). The choice of the most appropriate method depends on the capsule application, the type of core, the chemical and physical characteristics of the core and wall, the particle size required, the mechanism of release required, the scale-of-production and the cost (Suave et al. 2006).

The spray drying technique is the most common encapsulation method that has been used for decades to encapsulate mainly flavours, lipids, and pigments (Gharsallaoui et al., 2007). Several applications of spray drying in industrial field rang from encapsulation of fragrances and flavours in food industries to pigments in manufacture (Laohasongkram et al. 2011). In addition, this technique is widely used for the encapsulation of enzymes and antimicrobials substances such as EOs to ensure their activities (Schutyser et al. 2012; Dajic Stevanovic et al. 2020). However, the optimal choice of drying conditions and adapted matrix formulations is necessary to avoid serious thermal damage leading to a loss of enzymatic activity or volatility of the essential oil (Gharsallaoui et al. 2007; Schutyser et al. 2012). Spray drying is a relatively inexpensive and commercially feasible method of microencapsulation. Biomolecules used as carriers for this technique are starch, maltodextrins, chitosan and gum arabic (Dajic Stevanovic et al. 2020).

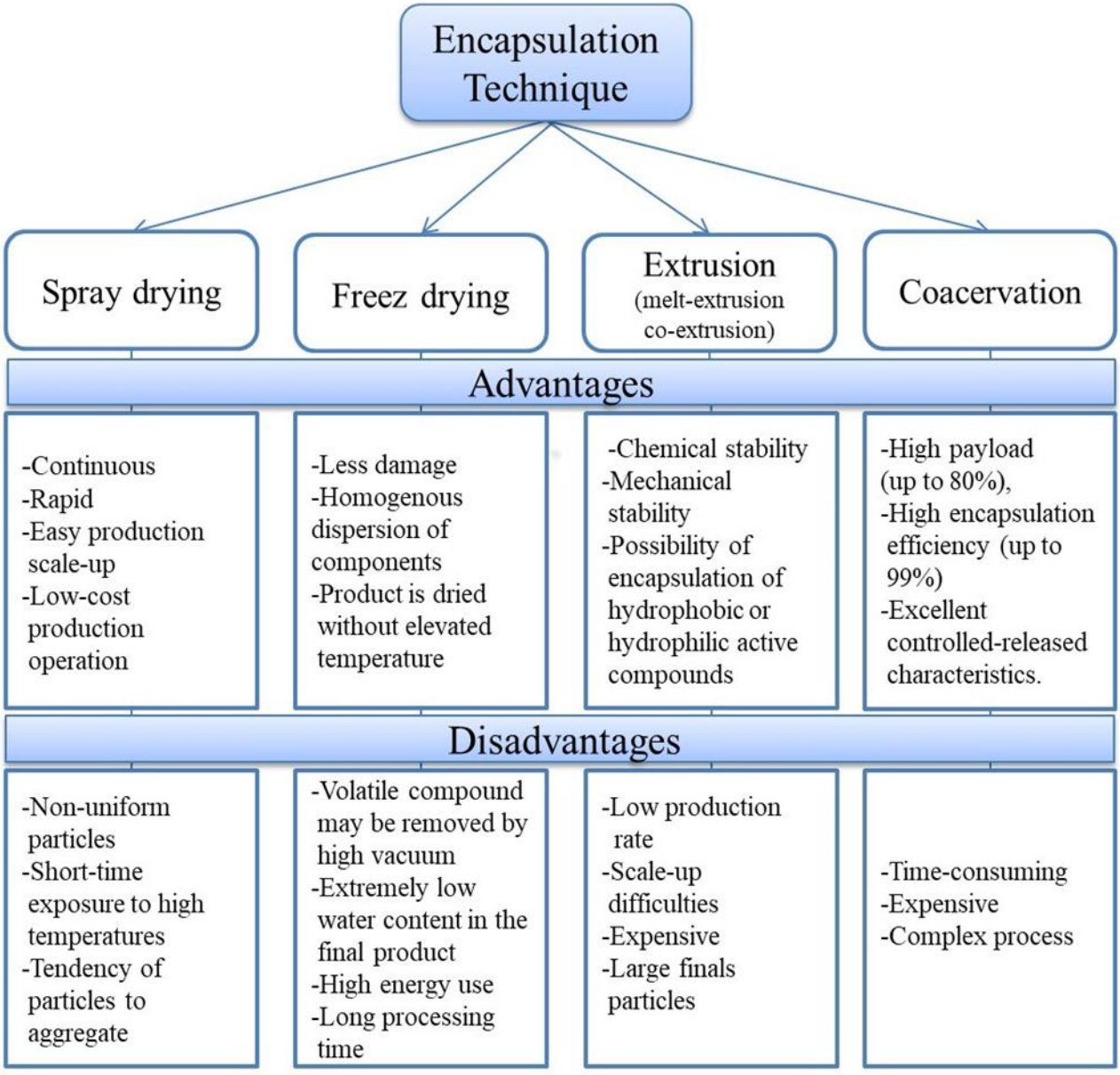


Fig. 5 Examples of different techniques for antimicrobials encapsulation with some advantages and disadvantages (Dolçà et al. 2015; Bakry et al. 2016; Wang et al. 2018; Khairnar et al. 2012; Dajic **S**tevanovic et al. 2020)

Spray drying microencapsulation involves 4 steps as shown in Fig. 6: (1) preparation of the emulsion, (2) homogenization of the emulsion, (3) atomization of the dispersion, and (4) dehydration of the atomized particles (Bakry et al. 2016). This process consists of forming an emulsion, suspension or solution containing the wall material and core, then pulverization in a drying chamber in which circulates hot air, upon contact with the hot air, the water evaporates immediately and the core is encapsulated into the wall material (Laohasongkram et al. 2011).

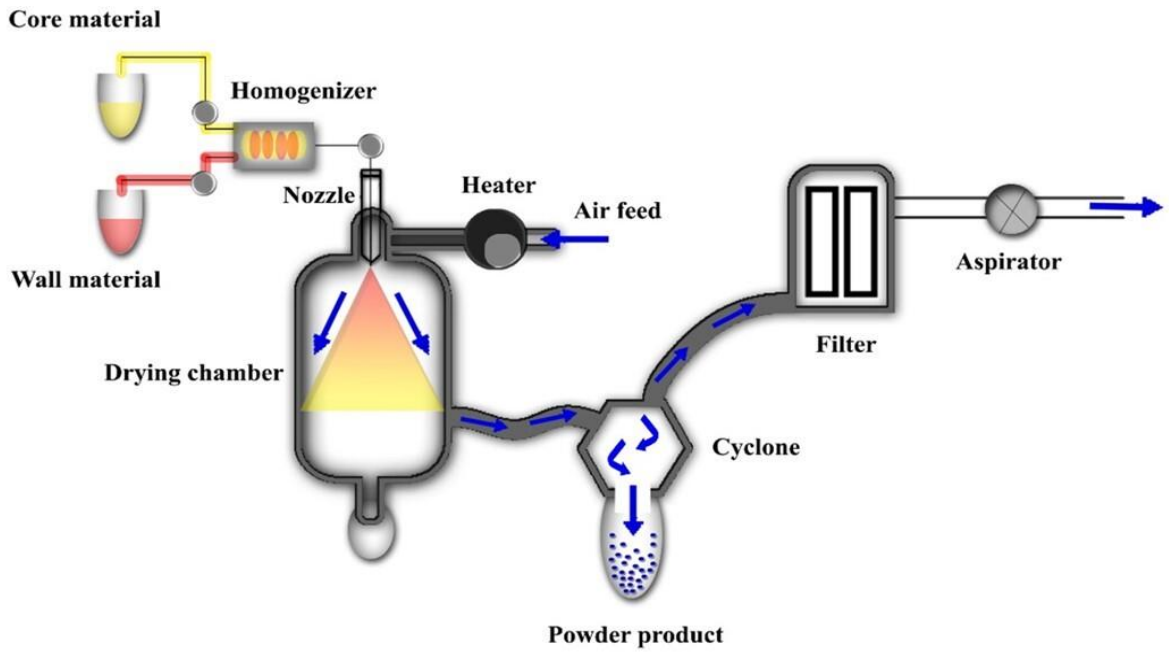


Fig. 6 Schematic representation of the spray drying microencapsulation process

Role of microencapsulation in biofilm control

Microencapsulation is widely used in the fields of medicine, food, cosmetics, pharmaceuticals, textiles, agriculture and advanced materials, which make this technique widely used in the encapsulation of active constituents: enzymes, EOs, flavours, colours, sweeteners, micro-organisms, etc (Desai and Park 2005; Fu and Hu 2017). One of the recent uses of microencapsulation is the control of biofilm on industrial equipment and materials, by the

encapsulation of antimicrobial substances to ensure their stability and long-term activity, as well as to limit their interactions with the biofilm matrix components (Khelissa et al. 2021a; Khelissa et al. 2021b).

As mentioned earlier, the use of enzymes is an important tool to remove biofilms through the enzymes ability to degrade the EPS and destroy biofilms (Xavier et al. 2005). Thus, extensive researches have been conducted to immobilize enzymes in mechanically resistant capsules, in most cases in dry microcapsules, in order to protect enzymes during storage and to control their release (Mohamad et al. 2015). Microencapsulation is a promising strategy to prevent and stabilize enzymes under severe reaction conditions from denaturation by proteolysis and dilution effects, and thus maintain high catalytic activity (Chaize et al. 2004; Tetter and Hilvert 2017; Zdarta et al. 2018). Orgaz et al. (2007) demonstrated that the combination of delayed-release encapsulated pronase with cellulase, pectin lyase or esterase leads to three to four decimal reductions in cells and detach up to 90% of the biofilm of *Pseudomonas fluorescens* after 2 h at 25 °C. These data prove that these results are more favourable than those obtained with the same application of the equivalent soluble enzyme mixtures. Tan et al. (2020) shows that the co-immobilization of deoxyribonuclease I (DNase) and cellobiose dehydrogenase (CDH) results in a bi-functional particle that targets both the microorganisms and the biofilm matrix. The assessment of the antibiofilm activities of these particles has shown a high ability to penetrate through the biofilm matrix and interfere with microbial cells, thus exhibiting a stronger activity to inhibit biofilm formation as well as to disrupt preformed biofilms.

Furthermore, the use of EOs to control biofilm has been extensively studied in recent years; a wide variety of EOs can be used as antibiofilm compounds. However, EOs are not stable and can be degraded in the presence of oxygen, light and temperature. Thus, efforts have been attempted to protect them by encapsulation in various colloidal systems such as microspheres, microcapsules, liposomes and nanoemulsions (Sherry et al. 2013). Many studies show that protecting the EOs with antimicrobial activity in a capsule can increase their bioactivity and efficiency to remove biofilm from surfaces (Dohare et al. 2014; Duncan et al. 2015; Cui et al. 2016a), as well as decreasing volatility and improving stability and water solubility (Bilia et al. 2014). For example, peppermint oil encapsulated in starch-based emulsions showed increased stability and bioavailability

characteristics and improved activity against *Staphylococcus aureus* and *Listeria monocytogenes* relative to free EOs (Liang et al. 2012). The antibacterial activity of EOs after nanoencapsulation has been shown to very often exceed the efficacy of the current antibiotic (Zaman et al. 2017). Dohare et al. (2014) reported that the encapsulation of Eucalyptus globulus oil has increased its antibiofilm activity against *Escherichia coli* biofilm from 62% to 81% compared to the soluble one, thus the use Eucalyptus globulus oil encapsulated into a nanoparticle is important for controlling biofilm associated with microbial infections and diseases. Other studies showed that the two antimicrobials, Carvacrol and eugenol, encapsulated in micellar nonionic surfactant solutions, were significantly effective against two strains of *Escherchia* coli O157:H7, reducing culturable counts by 3.5 to 4.8 log CFU/cm2 within 20 min of exposure (Pérez-Conesa et al. 2011). Furthermore, Cui et al. (2016a) evaluated the anti-biofilm effect of cinnamon oil, encapsulated in liposomes, on methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms. The results showed that the use of liposomes improves the stability of cinnamon oil, which has an effective antibacterial performance on MRSA and its biofilms, and prolongs the time of action.

Conclusion

The prevention/eradication of biofilms in the industrial and medical sectors is one of their main concerns. This field can provide an appropriate environment for the development of biofilms that threaten public health and increase economic losses. A clear understanding of the mechanisms of biofilm formation and resistance to disinfecting agents is necessary to provide an effective strategy to prevent and destroy biofilms. Biofilm resistance to disinfectants appears to be multi-factorial and involves several parameters. In addition, the side effects caused by these agents require the search for alternative natural antimicrobial agents to obtain the requested treatment and overcome the disadvantage of the conventional antimicrobial used. The biofilm matrix is the main physical barrier preventing the penetration of biocides into biofilms. Therefore, if one or more compounds capable of destroying the structural components of the matrix produced by biofilm as well as active against the microbial biofilm are found, then the "microbe city" (biofilm) would be permanently destroyed and eradicated. However, the activity and stability of the anti-biofilm agents used may be affected by several parameters. On this basis, the encapsulation of these compounds can be

useful to protect and ensure their stability and activities against matrix biofilm as well as biofilm-producing microorganisms, in order to prevent the formation and/or eradicate the establishing biofilms.

Author contributions SM and AG and KO and MH and NEC conceived the outline of this review. SM contributed the figures. All authors contributed to literature searches and the writing of the manuscript. All authors have read and approved the manuscript.

Compliance with ethical standards

- **Competing interests** The authors declare that there are no competing interests.
- **Ethical approval** Not applicable, since the work does not involve any study with human participants or animals.
- **Consent to participate** Not applicable.
- **Consent for publication** All co-authors have given their consent to publish this manuscript.

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Chapter II

Article II-Results

INTRODUCTION

Consumers have the right to expect safe and secure food products and safe healthcare services. However, foodborne and health-care associated infections (HCAIs) caused by pathogenic bacteria still exist, despite advances in the food and medical sectors (Khan et al. 2017a; Boone et al. 2021). Microbial contamination in the food and medical sectors can occur from diverse sources. In fact, the unhygienic practices of food handlers, the use of unsanitary water and unsafe equipment are the main causes of microbial contamination in the food sector. Beyond impacting food processing, contamination also affects food packaging and can provoke several diseases, such as food poisoning, botulism, and some other enteric infections by generating harmful toxic metabolites (Chatterjee and Abraham 2018). Furthermore, the HCAIs can occur during the provision of care for other diseases and even after the discharge of patients. In addition, they include occupational infections among health care personnel. The most common types of infections are catheter-related urinary tract infections, central line-related bloodstream infections, ventilator-related pneumonia, and surgical site infections (Khan et al. 2017a). The persistence of pathogenic bacteria in both of these areas leads to the formation of biofilms on food and medical equipment, thus providing a reservoir for pathogens (Bridier et al. 2011). The complex microbial biofilm community is highly tolerant to disinfectants and antibiotics and presents a persistent survival challenge. Several conventional approaches are available to combat biofilms, mechanical and/or physical removal, chemical removal, and the use of sanitizers, antimicrobials, or disinfectants to kill the biofilm embedded-cells. However, pathogens living in biofilm state are highly resistant to these approaches compared to their planktonic counterparts. Furthermore, the application of these disinfectants for several decades could be a main cause of the emergence of bacterial resistance to antibiotics and their spread to other pathogens. Therefore, new alternative strategy other than the conventional methods to control biofilms are needed to overcome these problems (Sadekuzzaman et al. 2015).

Several natural products, such as plant extracts and EOs, have been assigned antibiofilm properties and these characteristics have been widely studied. EOs are natural volatile substances derived from plants that are expected to effectively kill bacteria without promoting the development of resistance (Jafri et al. 2019). EOs have a low toxicity and degrade rapidly, making them an environmental friendly product (Jin et al. 2011). The antibacterial mode of action of EOs has been

widely reported in the literature. The common mechanisms involve disruption of the cell wall and cytoplasmic membrane, causing lysis and leakage of intracellular pool (Lopez-Romero et al. 2015). Other biological activities of essential oils have been reported, including antigenotoxic activity (Thirugnanasampandan et al. 2012).

The phenolic monoterpene carvacrol [2-Methyl-5-(1-methylethyl) phenol, isomeric with thymol] is a main component of plant essential oils of Labiatae family, including Thymus and Origanum, which have been widely used as condiments and in traditional medicine from early times (Nostro and Papalia 2012). Carvacrol has been graded as GRAS (Generally Recognized As Safe) and certified for food use (Food and Drug Administration [FDA] 2013; Suntres et al. 2015). Besides its antioxidant, anti-inflammatory, analgesic, anti-tumor, insecticidal, and anti-hepatotoxic properties, several studies have shown that carvacrol has antimicrobial activities. In addition, several other studies have assessed its antibiofilm activity on common surfaces in the food industry (Hyldgaard et al. 2012; Soni et al. 2013; Trevisan et al. 2018). The antibacterial activity of carvacrol is based primarily on the deterioration of the bacterial membrane; it leads to the dissolution of the proton-motive force and the subsequent reduction of ATP synthesis, resulting in the reduction of other energy-dependent cellular processes, including enzyme and toxin synthesis (Magi et al. 2015). Particularly, carvacrol has been extensively evaluated as an antimicrobial agent for control Gram-negative and Gram-positive pathogens in foods, including *Enterococcus faecalis, Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli O157:H7, Salmonella typhimurium, Pseudomonas fluorescens, Vibrio vulnificus* and *Vibrio cholera* (Hyldgaard et al. 2012; Langeveld et al. 2014; Magi et al. 2015).

However, as the quality and yield of EOs are highly affected by environmental conditions, an efficient extraction method is needed. Hence, an encapsulation method has been proposed to improve the stability and biological activity of EOs, decrease their low water solubility, and control their release (Engel et al. 2017; Maqsoudlou et al. 2020). The process consists of emulsifying the core materials (e.g., EOs) with the coating materials (e.g., proteins or carbohydrates), and then drying or cooling the obtained emulsion (Mechmechani et al. 2022). Encapsulation ensures strong protection against oxygen, moisture and light, thus reducing their impacts on EOs activity and shelf

life (Burhan et al. 2019; Veiga et al. 2019). The resulting products of the microencapsulation process are microcapsules ranging in size from a few micrometers to a few millimeters, in the form of capsules, particles, complexes or droplets (Weiler 1992). Currently, microencapsulation is performed using different methods including spray-drying, freeze-drying, coaxial electrospray system, emulsification, *in situ* polymerization, coacervation, supercritical fluid technology, fluidized-bed-coating, and melt-extrusion. The selection of an appropriate encapsulation technique and wall material depends on the final use of product and the processing conditions involved (Bakry et al. 2016; Tiwari et al. 2020; Tanhaei et al. 2021). Spray-drying microencapsulation is widely used in food industries due to the low cost and energy efficiency of processing, convenience of the equipment, high retention of core materials, and the ability to encapsulate thermolabile compounds with low risk of degradation, high encapsulation efficiency, and extended shelf life. This method physically transforms EOs from a hydrophobic liquid to a more convenient low-moisture powder, which helps promote the alternative use of EOs (Nguyen et al. 2021). In this context, our work aimed to study the effect of spray-drying microencapsulation on the antimicrobial activity of carvacrol against *P. aeruginosa* and *E. faecalis* biofilms adhering to stainless steel in the context of food and medical environments.

P. aeruginosa is an opportunistic and ubiquitous human pathogen that can be isolated from a variety of living sources, including humans, animals and plants. The capacity of *P. aeruginosa* to survive with minimal nutrient needs and to tolerate various physical conditions allowed this organism to persist in food and hospital environments. Despite the wide diffusion of *P. aeruginosa* in natural and artificial ecosystems, the risk of serious infections caused by this bacterium remains essentially hospital-based. *P. aeruginosa* has been isolated from a variety of medical devices such as catheters, respiratory therapy equipment, breathing apparatus and dialysis tubing (Rosenthal et al. 2012). This bacterium could be responsible for a wide range of acute and chronic life-threatening infections, particularly in patients with compromised immune systems (Moradali et al. 2017). On the other hand, the involvement of *P. aeruginosa* in food spoilage and foodborne infections is also prevalent, since it can be isolated from water and vegetables (Raposo et al. 2016). Otherwise, the Gram-positive *E. faecalis* is a commensal bacterium that has gained importance in recent decades as leading to opportunist pathogens causing health-care associated infections (Anderson et al. 2016).

E. faecalis is involved in 80-90% of all healthcare-associated Enterococcal infections including bacteremia, urinary tract infections, neonatal infections, wound infections, and meningitis (Shridhar and Dhanashree 2019). In addition, because of their high heat tolerance and ability to survive in harsh environmental conditions, *E. faecalis* can easily persist for a long periods and contaminate processed food products (Rehaiem et al. 2016; Liu et al. 2020b). The frequent incidence of this bacterium in food and cattle could allow its zoonotic transmission to humans (Anderson et al. 2016).

In this regard, the second chapter of this thesis focused on the study of the antimicrobial activity of free and encapsulated carvacrol against *P. aeruginosa* and *E. faecalis* biofilms grown on stainless steel surfaces. Spray-drying was used to encapsulate carvacrol in order to improve its stability and water solubility, and to enhance its biological activity. Indeed, feed emulsions were first prepared using sodium caseinate as emulsifier and maltodextrins as drying matrix, and then spray-dried to obtain dry carvacrol microcapsules. The mode of action of free and encapsulated carvacrol was also investigated. The main objective of this study was to improve the efficacy of this antimicrobial agent against pathogenic bacterial biofilms through its spray-dried formulation, while reducing the amount used.

Microencapsulation of carvacrol as an efficient tool to fight *Pseudomonas aeruginosa* and *Enterococcus faecalis* biofilms

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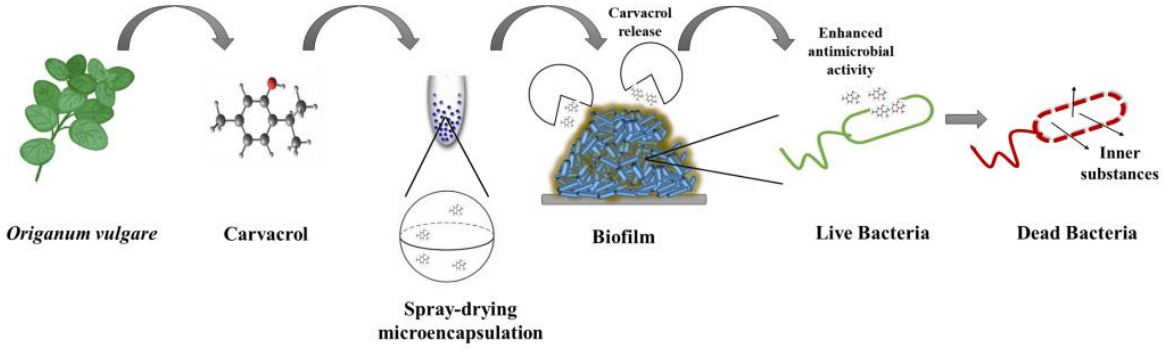
Abstract

Biofilms are involved in serious problems in medical and food sectors due to their contribution to numerous severe chronic infections and foodborne diseases. The high resistance of biofilms to antimicrobial agents makes their removal as a big challenge. In this study, spray-drying was used to develop microcapsules containing carvacrol, a natural antimicrobial agent, to enhance its activity against *P. aeruginosa* and *E. faecalis* biofilms. The physicochemical properties and microscopic morphology of the realized capsules and cells were characterized. The minimum inhibitory concentration of encapsulated carvacrol (E-CARV) (1.25 mg mL^{-1}) was 4-times lower than that of free carvacrol (F-CARV) (5 mg mL^{-1}) against *P. aeruginosa*, while it remained the same against *E. faecalis* (0.625 mg mL^{-1}). E-CARV was able to reduce biofilm below the detection limit for *P. aeruginosa* and by 5.5 log CFU ml^{-1} for *E. faecalis* after 15 min of treatment. Results also showed

that F-CARV and E-CARV destabilize the bacterial cell membrane leading to cell death. These results indicate that carvacrol exhibited a strong antimicrobial effect against both bacterial biofilms. In addition, spray-drying could be used as an effective tool to enhance the antibiofilm activity of carvacrol, while reducing the concentrations required for disinfection of abiotic surfaces.

Keywords: Antibiofilm activity, microencapsulation, carvacrol, *Pseudomonas aeruginosa*, *Enterococcus faecalis*

Graphical abstract



Introduction

Enterococcus faecalis and *Pseudomonas aeruginosa* are important opportunistic human pathogens that cause major problems in medical and food sectors. In fact, *E. faecalis* is a Gram-positive commensal bacterium that is normally associated with human as a member of the gut microflora (Selleck et al. 2019). However, *E. faecalis* is an opportunistic pathogen of considerable clinical importance, particularly as an etiologic agent of healthcare-associated infections (HCAIs) (Denstaedt et al. 2018; Boccella et al. 2021). In addition, due to their high tolerance to heat and their ability to survive in severe environmental conditions, *E. faecalis* can easily persist for long periods of time and contaminate animals' carcasses and processed products (Foulquié Moreno et

al. 2006; Rehaiem et al. 2016; Liu et al. 2020b). *P. aeruginosa* is a Gram-negative bacterium that can provoke several healthcare-associated infections, even fatal infections in immunosuppressed patients (Thi et al. 2020). Furthermore, the involvement of *P. aeruginosa* in foodborne infections and food spoilage is also reported (Raposo et al. 2016).

In natural and artificial ecosystems, bacteria may adhere to surfaces and form a complex three-dimensional structure called biofilm (Donlan 2002). Bacterial cells in biofilm are enclosed in an extracellular polymeric substances (EPS) of proteins, polysaccharides, and nucleic acids that can serve as a barrier providing a resistance to various hostile conditions such as antibiotics, disinfectants, and other sanitizing conditions, and preventing thus their penetration into the deeper layers of biofilms. Therefore, those bacterial cells are more resistant to antibacterial agents or antibiotics than planktonic cells (Karygianni et al. 2020a). *E. faecalis* is frequently isolated from biofilms formed on the surfaces of various indwelling medical devices related to chronic infections (Kristich et al. 2004). In addition, Enterococcal biofilms formed in food processing environments are very difficult to eradicate, making them one of the most prevalent opportunistic pathogens and spoilage bacteria in meat products (Giaouris et al. 2014; Pesavento et al. 2014; Rizzotti et al. 2016; Liu et al. 2020b). *P. aeruginosa* also effectively colonizes various surfaces, including medical equipment (implants, urinary catheters, contact lenses, etc.) causing many chronic infections, and food industry equipment (tanks, mixing tanks and pipes) causing food spoilage (Quintieri et al. 2019; Thi et al. 2020).

Plant-derived essential oils are natural antimicrobial agents with effective antimicrobial activity against bacteria, fungi and viruses. Carvacrol [2-Methyl-5-(1-methylethyl) phenol], a volatile monoterpene, is a major component of many essential oils of the Labiatae family plants, including *Thymus, Origanum, Satureja,* and *Coridothymus* species. Carvacrol is classified as generally recognized as safe (GRAS) by the Food and Drug Administration for its uses in food as preservative and food flavoring ingredient (FDA 2013; Suntres et al. 2015). Carvacrol is known for its broad antimicrobial activity against foodborne or pathogenic microorganisms, including drug-resistant bacteria (Nostro et al. 2004; Esteban and García-Coca 2017). However, the application of carvacrol is limited due to its low stability, poor water solubility and high volatility (Yildiz et al. 2018).

Microencapsulation techniques have been widely used in the pharmaceutical and food industries to control the release of active molecules, improve the stability of formulations and mask flavors (Gibbs et al. 1999; Dias et al. 2015). In addition, microencapsulation may reduce the amounts of biocides used and thus decreases their negative environmental impacts. This technique can also impede the interactions of antimicrobials with biofilm EPS matrix, which can lead to repulsion or retention of the biocide and prevent their interactions with microbial cells, thus allowing for deep layer biofilm disinfection. Therefore, the microencapsulation of natural terpenes could also be a promising method to surmount their water immiscibility, volatility and cytotoxicity. Many microencapsulation techniques are commonly used, including spray-drying, extrusion, and coacervation (Gharsallaoui et al. 2007a).

In the current study, feed emulsions were first prepared using sodium caseinate as emulsifier and maltodextrins as drying matrix. Emulsions were then spray-dried to obtain dry carvacrol microcapsules. The antimicrobial activity of free and microencapsulated carvacrol was assessed against *P. aeruginosa* and *E. faecalis* biofilms performed on stainless steel. The main objective was to improve the efficiency of this antimicrobial agent against pathogenic bacterial biofilms through its spray-dried formulation, while reducing the amount used.

Material and Methods

Growth conditions and cell suspension preparation

P. aeruginosa (CIP 103467) and *E. faecalis* (isolated from French cheese) were used in this study. The strains were maintained in tryptic soy broth (TSB; Biokar Diagnostics, France) supplemented with 40% (v/v) glycerol at -80 °C. Prior to use, bacteria were pre-cultivated by inoculating 100 µL of the frozen strain cultures into 5 mL of TSB medium and incubating for 24 h at 37 °C. Then 100 µL of the pre-culture were used to inoculate 50 mL of TSB medium in 500 mL sterile flasks, and incubated for 16 h at 37 °C under shaking conditions at 160 rpm to prepare the culture. Cells were pelleted by centrifugation for 5 min at 5000 × *g*. Then, bacteria were washed twice with 20 ml of potassium phosphate buffer (PPB; 100 mM, pH 7) and finally re-suspended in PPB. The cells were dispersed by sonication at 37 kHz (Elmasonic S60H, Elma®) for 5 min at 20 °C.

Antimicrobial agents

Carvacrol (98% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO; Sigma-Aldrich, France) with a final concentration of 2% (v/v) was used to prepare F-CARV solution, while ME-CARV did not require DMSO since microcapsule already contain emulsifier that solubilize carvacrol.

Microencapsulation of carvacrol

Carvacrol emulsions were prepared by dissolving sodium caseinate in water while shaking at room temperature until complete hydration. The pH was then adjusted to 7 using HCl (0.1 or 1.0 mol L^{-1}) or NaOH (0.1 or 1.0 mol L^{-1}). Each emulsion was then mixed with a maltodextrin DE 19 solution (50% w/v) to obtain a feed emulsion with the following composition: carvacrol (1%), sodium caseinate (0.5%), and maltodextrins (20%). The pH of the feed emulsions was readjusted to 7 before analysis.

Feed emulsions were shaken for 30 minutes and subsequently spray-dried by using a lab-scale device equipped with a 0.5 nm nozzle atomizer (Mini Spray-Dryer Buchi B-290, Switzerland). The drying process operational conditions were as follow: outlet air temperature 80 ± 5 °C, inlet air temperature 180 ± 2°C, and feed flow rate 0.5 L h^{-1}. Powders were collected in separate sealed vessels after spray-drying and stored at 4 °C until testing. Reconstituted suspensions were then prepared by scattering weighted quantities (the same amount of dry matter as before spray-drying) of spray-dried powders in water and shaking for 1 h.

Zeta potential measurement

The electrical charge (ζ-potential) of the emulsions before and after spray-drying and reconstitution was determined using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). If needed, samples were diluted in water with the appropriate pH. For each test, at least three repetitions were performed. The average of ζ-potential (ZP) values was obtained from the instrument.

Scanning electron microscopy (SEM) analysis of microcapsules and cells

The internal and external structures of dried carvacrol microcapsules were examined using SEM (JEOL-JSM-7800FLV, Japan). To observe the external structure, a dry powder layer was simply attached to a sample holder with a double-sided adhesive (Agar Scientific Oxford). To study the internal structure, powders containing carvacrol microcapsules were smashed by moving a razor blade perpendicularly through a layer of microcapsules. The morphology of treated biofilm cells was also investigated using SEM. Briefly, bacterial cells were recovered after biofilm treatment by scraping the surface, aspirating and expelling with 6 mL of ultrapure water at least 10 times. Recovered cell suspensions were vortexed for 30 s, followed by sonication (5 min, 37 kHz) then diluted tenfold in Tryptone Salt broth (TS; Biokar Diagnostics, France). Samples (1 mL) were filtered using a polycarbonate membrane filter of 0.2 µm pore size (Schleicher & Schuell, Dassel, Germany) then fixed with cacodylate buffer 0.1 M, pH 7.0 (sodium cacodylate trihydrate $(CH3)_2AsO_2Na.3H_2O$) containing 2% glutaraldehyde, at 4 °C. Fixed bacterial cell samples were dehydrated in an upward series of ethanol (50, 70, 95, and 2×100% (v/v) ethanol) for 10 min at each concentration and critical point dried. Fixed bacterial cell or dried microcapsules samples were covered with a thin carbon film before scanning by SEM. Microscopy was performed at 3 kV.

Antimicrobial activity of carvacrol against planktonic cells

Determination of the minimal inhibitory of planktonic cells

The minimum inhibitory concentration (MIC) of F-CARV and E-CARV was determined using Mueller-Hinton Broth (MHB; Biokar Diagnostics, Pantin, France) by a microdilution growth inhibition assay using a Bioscreen C (Labsystems, Helsinki, Finland) that measures turbidity by vertical photometry. Briefly, 100 µL of double serial dilutions of F-CARV and E-CARV (from 10 to 0.156 mg mL^{-1}) were carried out in the 96-well microdilution plates. Subsequently, 100 µL of *P. aeruginosa* (CIP 103467) and *E. faecalis* suspensions (10^6 CFU mL^{-1}) were added. Bacteria were not added to negative control, while in positive controls, DMSO without antimicrobials was used for F-CARV and the same control without DMSO was used for ME-CARV. The plates were incubated in Bioscreen C at 37 °C with a continual shaking and the optical density (OD600 nm)

was read every 2 h for 24 h. MIC value was defined as the lowest concentration of the antimicrobial agent that prevents the bacteria from obvious growth in the microdilution wells after incubation.

Time kill assay

The time kill test was used to study the bactericidal effects of the antimicrobial agents. The experiment was performed according to Isenberg (2004) with some modifications. Briefly, bacteria were overnight cultured and transferred at MIC values to MHB, supplemented with F-CARV and ME-CARV, to obtain a final inoculum of 10^6 CFU mL^{-1}. Control containing DMSO was used for F-CARV and without DMSO for ME-CARV. Then, bacteria were incubated at 37 °C with shaking. At the selected time, 100 µL were taken, diluted serially, and plated on Mueller Hinton agar (MHA, Difco Pont-de-Claix, France). Plates were incubated at 37 °C for 24 h and the colony forming units (CFU) were enumerated. Tests were replicated three times.

Carvacrol-induced potassium ion leakage

Planktonic *P. aeruginosa* cells grown at 37 °C were concentrated to 10^{10} CFU mL^{-1} (5 000 × *g*, 15 min, 20 °C). Tenfold dilutions of the concentrated bacterial suspensions were prepared in 50 mM morpholinopropane sulfonic buffer (MOPS; Fisher scientific, Belgium) containing F-CARV and ME-CARV (at the MICs). K^+ concentrations were measured at the time 0, 5 and 10 min in a tenfold dilution of the concentrated bacterial suspension filtrate (0.2 µm, Sartorius™ Minisart™ NML Syringe Filters, France) before contact with the antimicrobial solutions. After the exposure of the bacterial suspension cells to the F-CARV and ME-CARV solutions, samples (4 mL) were filter sterilized at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 15, 20, 30 and 40 min. MOPS buffer with DMSO was used as control for F-CARV and without DMSO for ME-CARV. The concentration of K^+ in the filtrate samples was measured using a Varian SpectrAA 55/B atomic absorption spectrometer in flame emission mode (slit 0.7 nm high; wavelength 766.5 nm; air-acetylene flame).

Assessment of the mechanism of activity of carvacrol on *Pseudomonas aeruginosa* GFP

The experiment was performed according to Khelissa et al. (2021a) with some modifications. After overnight culture of *P. aeruginosa* GFP (ATCC ®10145GFP™) in TSB supplemented with 100 mg mL^{-1} ampicillin, cells were harvested by centrifugation (5 000 × *g*, 5 min, 20 °C), washed twice with HEPES buffer (5 mM, pH 7.2), and resuspended in HEPES buffer to obtain a concentrated inoculum (10^{10} CFU mL^{-1}). Tenfold dilutions of this concentrated inoculum were prepared in HEPES buffer and sterilized by filtration (Sartorius ™ Minisart™ NML 0.2 µm Syringe Filters, France) at 0, 5, and 10 min prior to exposure of the cells to carvacrol. These filtrates were used to determine the extracellular fluorescence intensity of GFP before antibacterial treatments. In order to expose cells to carvacrol, tenfold dilutions of the concentrated inoculum were prepared in HEPES buffer supplemented with F-CARV and ME-CARV (at the MICs). The samples were sterilized by filtration at 5, 10, 15, 20, 30 and 40 min after exposure of the cells to the antimicrobial solutions. HEPES buffer with DMSO was used as control for F-CARV and without DMSO for ME-CARV. To quantify GFP fluorescence, 200 µL of the filter samples were transferred to a 96-well microplate and measured by BioTek fluorescence spectrophotometer (BioTek Instruments SAS, France) with excitation at 485 nm and emission at 510 nm. The ratio of fluorescence intensity of the samples was plotted against contact time. The results represent the mean of three independent experiments.

In order to demonstrate that the increase in extracellular GFP fluorescence intensity indicates cell membrane damage and subsequent cell death, the percentage of viable cells in the treated suspension was calculated after staining with LIVE/DEAD® BacLight kit (Invitrogen Molecular Probes, USA). Briefly, after treatment of the suspension with the antimicrobial solutions (at very low concentrations to monitor the effect of carvacrol over time), 10^7 CFU mL^{-1} of the suspension were filtered with polycarbonate filter (0.2 µm-pore-size, Millipore, France) at 5, 10, 15, 20, 30 and 40 min and then stained in the dark for 10 min. Stained cells were rinsed with 1 mL of distilled water, and filters were kept in the dark to air-dry, then viable cells (green) and dead cells (red) were enumerated using epifluorescence microscope (Olympus BX43, Germany) over 50 microscopic fields. The percentage of viable cells was calculated

using the following formula: (number of green cells × 100 / number of total cells (red and green cells)). Results represent the average of three independent experiments.

Anti-Biofilm assessment using free and encapsulated carvacrol

Preparation of stainless steel slides

Stainless steel (SS) slides (304L, Equinox, France) of 41 mm diameter and 1 mm thickness were used for this study. Slides were immersed overnight in 95% ethanol (Fluka, Sigma-Aldrich, France), then washed with distilled water. The rinsed slides were immersed again in 1% DDM ECO detergent (ANIOS, France) for 15 min at 20 °C. The slides were afterwards washed vigorously with distilled water 5 times for 1 min, followed by three ultra-pure water washes (Milli-Q® Academic, Millipore, France) to completely remove detergent residues. Finally, SS slides were air-dried before sterilization by autoclaving at 121 °C for 20 min. The sterile slides were placed in a static biofilm reactor, called *NEC biofilm System*, for biofilm deposition. This system as previously described by Abdallah et al. (2015), is composed of several assembled SS parts and a rubber O-ring. Briefly, the SS lower part forms the circular base of the system, and the O-ring placed on the upper plate side is used to tightly match the SS slide. The SS cylinder with two holes is used to form a well for biofilm formation with oxygen supply. The clamp is used for sealing and the metal cover is used to maintain the sterility of the closed system. All parts were autoclaved at 121 °C for 20 min.

Biofilm formation assay

After placing sterile slides in the *NEC biofilm system*, 3 mL of bacterial suspension (10^7 CFU mL^{-1}) of *P. aeruginosa* and *E. faecalis* were deposited on the SS slides in each reactor and incubated at 20 °C for 60 min aiming the adhesion of bacterial cells to surfaces. Beyond this period, the 3 mL were discarded and the slides were carefully washed twice with 5 mL of PPB to remove non-adhered cells. Then, each slide with adhered cells was covered by 5 mL of TSB and the sealed systems were incubated for 24 h at 37 °C. After incubation, the old TSB medium was discarded and biofilms covering the SS slides were rinsed twice with 5 mL PPB to eliminate planktonic cells.

Rinsed slides were then used for the antibiofilm treatments, the quantification of biofilm biomass and the epifluorescence microscopy analysis.

Antibiofilm assay

In order to treat biofilms with antimicrobial agents, rinsed slides were placed horizontally in 3 mL of F-CARV or ME-CARV solution at ½ MIC and MIC concentrations of F- CARV for each strain (5 mg mL^{-1} and 2.5 mg mL^{-1} for *P. aeruginosa*, 0.625 mg mL^{-1} and 0.312 mg mL^{-1} for *E. faecalis*), and treated for 1, 5 and 15 min at 20 °C. Control 1 was performed by submerging the slides in 3 mL of TS; in control 2, the slides were immerged in 3mL of TS broth containing DMSO. After treatment, slides were removed from the disinfectant solution and soaked into 5 mL of neutralizing solution (containing a combination of Saponin (30 g L^{-1}), Sodium Thiosulphate (5 g L^{-1}), Tween 80 (30 g L^{-1}), L-Histidin (1 g L^{-1}), Lecithin (30 g L^{-1}), and TS broth (9.5 g L^{-1}) to block the antibacterial action (Toté et al. 2010). For the epifluorescence microscopy analysis, the slides were transferred to petri dishes and the biocide action was stopped by applying 3 mL of neutralizing solution on the top side.

For the quantification of biofilm biomass, slides were placed into 20 mL TS broth using a sterile 100 mL pot. Attached cells were detached by vortexing for 30 s, sonication for 5 min (37 kHz, 5 min, 25 °C) (Elmasonic S60H, Elma, Germany), followed by vortexing again for 30 s. Thereafter, serial dilutions were prepared in TS broth and plated onto Tryptic Soy Agar (TSA; Biokar Diagnostics, France) plates. The number of cells was counted on the plates after incubation for 24 h at 37 °C and the results were presented as log CFU mL^{-1}. Results represent the average of three independent experiments.

Epifluorescence microscopy analysis

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. After staining sessile cells, slides were thoroughly rinsed with distilled water and then kept in the dark to air dry. Thereafter, epifluorescence microscopy observations were performed using an epifluorescence

microscope (Olympus BX43, Germany). Green cells were designated as viable and red cells were considered as non-viable.

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 multiple comparisons test). Values of $p < 0.05$ were considered statistically significant.

Results

Zeta potential of carvacrol droplets

During the preparation of the carvacrol microcapsules, the pH of the emulsion was adjusted to 7. The measurement of the zeta potential of the dispersed droplets gave an average value of -24.39 mV. This negative value is explained by the fact that sodium caseinate was used as an emulsifier at a pH higher than the isoelectric point (pHi~4.5). Indeed, proteins show an overall negative charge when the pH exceeds the pHi because of the ionization of the carboxyl groups (COOH --> COO^-). With the aim of studying the effect of spray-drying process (thermomechanical treatment combining shearing and heating), the zeta potential was measured after reconstitution of the powder. The average value obtained was -23.57 mV, which proves that the presence of maltodextrins as a drying matrix makes it possible to preserve the structure of the protein interfacial membrane which surrounds the carvacrol droplets.

SEM morphology of spray-dried microcapsules

Fig 1 shows the scanning electron micrographs of microcapsules obtained by spray-drying emulsions of carvacrol in the presence of maltodextrins DE19 and sodium caseinate. The microcapsules were spherical and well-separated with non-uniform diameters (Fig 1a), a blunted shape, and generally bumpy surfaces, with the presence of some small shrinked particles having a rough surface (Fig 1b). The internal structure observations of the formed microcapsules showed the presence of a boundary air bubble (called void) in the center (Fig 1c). In addition, the wall

matrix of these microcapsules appeared thickened and typically hollow, with an obvious encapsulated core material retained within (Fig 1d).

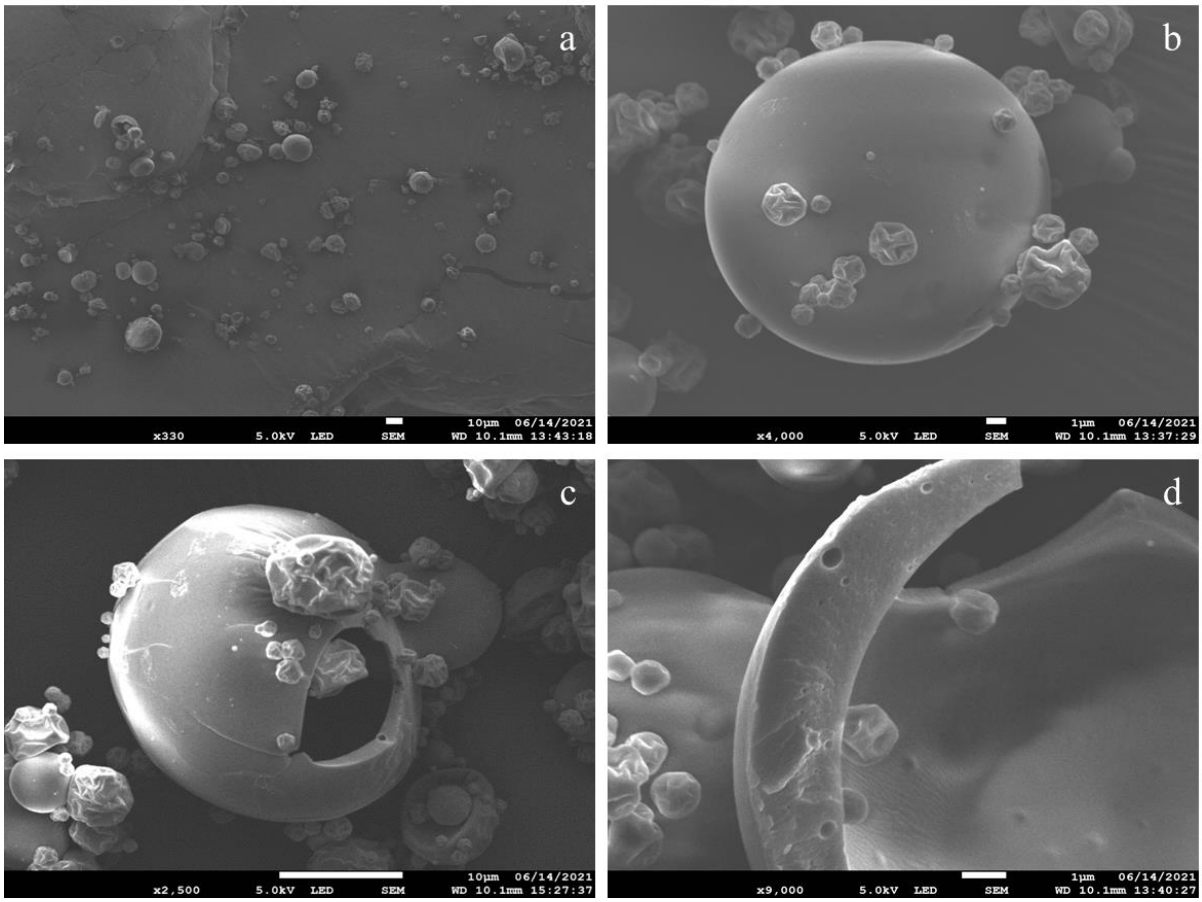


Fig 1. SEM images of carvacrol spray-dried microcapsules (ME-CARV). Overview of a multitude microcapsules (a) (330×); Outer morphology of ME-CARV microcapsules (b) (4000×); Inner structure of ME-CARV microcapsules (c) (2500×) and (d) (9000×)

Determination of the Minimal Inhibitory Concentrations (MIC) of free and encapsulated carvacrol

The MIC values of F-CARV were determined and compared with those of ME-CARV against *P. aeruginosa* and *E. faecalis* strains. Results showed that the use of DMSO, at a final concentration of 2% (v/v), to improve the miscibility of carvacrol in water, did not affect the growth of the strains studied (data not shown). This suggests that at this concentration DMSO

is not toxic to the bacteria cells studied. The MIC values of F-CARV and ME-CARV against *E. faecalis* were similar, corresponding to 0.625 mg mL^{-1} (Table 1). However, for *P. aeruginosa*, the MIC value of ME-CARV (5 mg mL^{-1}) was 4 times lower than that of F-CARV (1.25 mg mL^{-1}) (Table 1).

Table 1 Minimal inhibitory concentrations of free (F-CARV) and microencapsulated (ME-CARV) carvacrol against *P. aeruginosa* and *E. faecalis* (in mg mL^{-1})

Bacterial strains	F-CARV	ME-CARV
Pseudomonas aeruginosa	5	1.25
Enterococcus faecalis	0.625	0.625

Carvacrol time killing determination

The assessment of the time required to kill over than 99% of the total bacterial population of 10^6 log CFU mL^{-1} was performed by time kill assay. The control cell suspension of both *P. aeruginosa* and *E. faecalis* showed a bacterial population of approximatively 6 log CFU mL^{-1} ($p < 0.05$). When the target bacterial cells were exposed to the MIC of both F-CARV and ME-CARV a rapid killing of over than 99% of the total *P. aeruginosa* and *E. faecalis* populations was observed during the first 1 min and 5 min of exposure, respectively.

Assessment of the anti-biofilm activity of free and encapsulated carvacrol

The antibiofilm effect of F-CARV and ME-CARV was performed on *P. aeruginosa* and *E. faecalis* biofilms grown on SS for 24 h at 37 °C. This experiment aimed to assess the effect of contact time and carvacrol concentrations on the biofilm removal, as well as the difference in antibiofilm efficiency between F-CARV and ME-CARV.

P. aeruginosa biofilms presented a bacterial biomass of approximatively 7 log CFU mL^{-1}. The biomass reduction obtained after treatment by the F- CARV with ½ MIC value for 1 min was 2 log

CFU mL^{-1} ($p < 0.05$) (Fig 2A). This reduction was increased to 3 log CFU mL^{-1} ($p < 0.05$) after 5 min of treatment as well as after 15 min (Fig 2A). Using the MIC value, the reduction in biofilm biomass after 15 min of treatment by F-CARV reached 4.8 log CFU mL^{-1} ($p < 0.05$) (Fig 2A). However, the biomass reduction obtained with ME-CARV was more effective compared to F-CARV using ½ MIC and MIC concentration (Table 2). Results showed that the reduction was approximately similar after 1 min and 5 min treatment using ½ MIC and MIC concentrations (≈ 5 log CFU mL^{-1}) ($p < 0.05$) (Fig 2B). However, the results of Fig 2B showed that the treatment for 15 min using ½ MIC and MIC concentrations was the most effective treatment, since there were no colonies detected suggesting that more than 99% of biofilm cells were killed.

For *E. faecalis*, the biofilm presented a bacterial biomass of approximatively 7 log CFU mL^{-1}. After 1 and 5 min of treatment with ½ MIC value of F-CARV, there were no significant biofilm biomass reductions, whereas after 15 min of treatment, the biofilm biomass was reduced by 2.3 log CFU mL^{-1} ($p < 0.05$) (Fig 2C). Using MIC value, the antibacterial effect of F-CARV increased with treatment time and resulted in a reduction of 2.1 and 4.7 log CFU mL^{-1} ($p < 0.05$), after 5 and 15 min, respectively (Fig 2C). However, when *E. faecalis* biofilms were exposed to ME-CARV, reduction of biofilm biomass was more significant than F-CARV using ½ MIC and MIC concentrations, except after 1 min of treatment with ½ MIC concentration where there was no reduction in biofilm biomass compared to the control (Table 3). The reductions obtained after 5 and 15 min of treatment using ½ MIC concentrations were 2 and 3.3 log CFU mL^{-1} ($p < 0.05$), respectively (Fig 2D). This reduction was more significant using the MIC concentration and resulted in a reduction of 1.3, 4.3 and 5.5 log CFU mL^{-1} ($p < 0.05$), after 1, 5 and 15 min of treatment, respectively (Fig 2D).

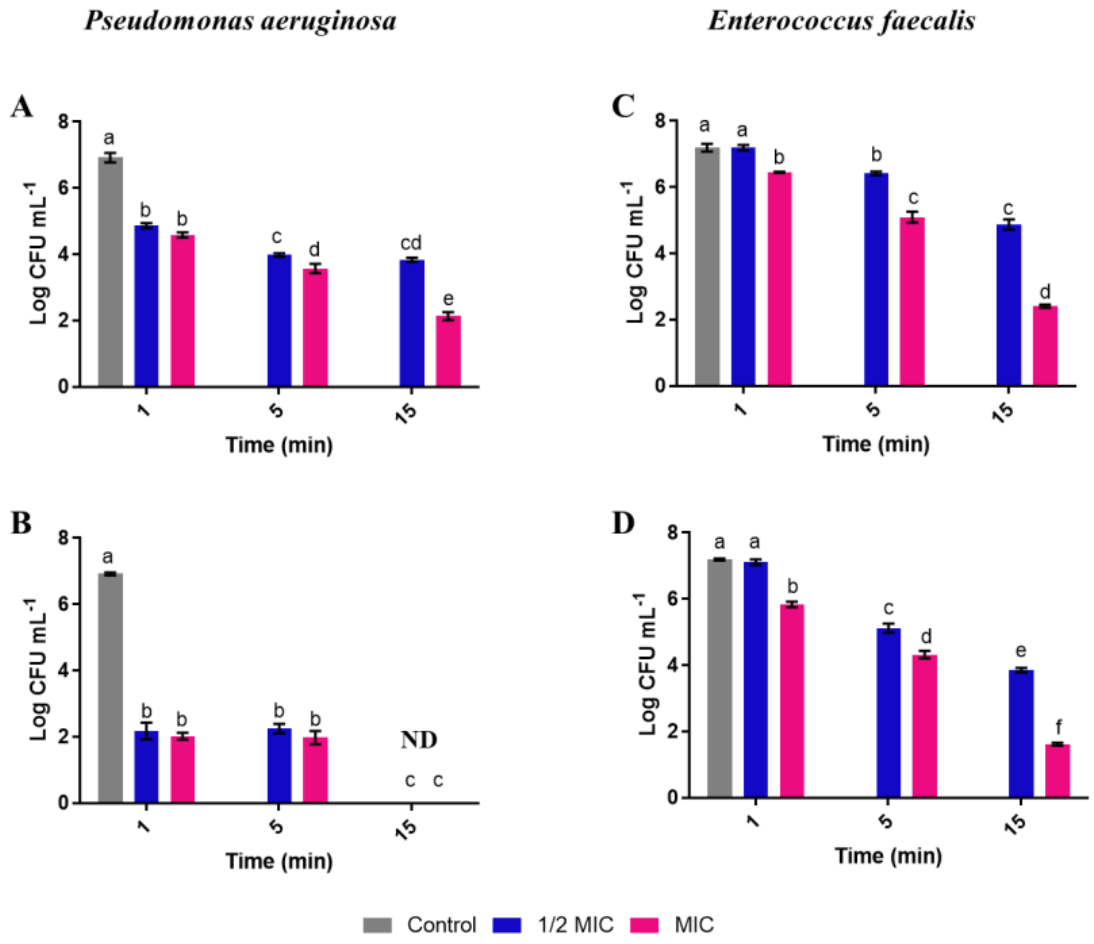


Fig 2. Effect of free (F-CARV) (A, C) and microencapsulated (ME-CARV) carvacrol (B, D) on the total biofilm biomass of *P. aeruginosa* (2.5 mg mL^{-1}, 5 mg ml^{-1}) and *E. faecalis* (0.312 mg mL^{-1}, 0.625 mg mL^{-1}). Results are expressed as the mean (± SD) of three independent experiments. Control represents biofilms treated with tryptone salt broth with DMSO for F-CARV and without DMSO for ME-CARV. ND: non detectable bacteria. Different letters at the top of the error bars (a, b, c, …) represent significant differences ($p < 0.05$); the same letters represent non-significant differences ($p > 0.05$)

Table 2 Statistical analysis of the results of *P. aeruginosa* biofilm treatment with free (F-CARV) and microencapsulated (ME-CARV) carvacrol. Different letters (A and B) between F-CARV and ME-CARV treatment represent significant differences ($p < 0.05$); the same letters represent non-significant differences ($p > 0.05$)

	½ MIC			**MIC**		
Time (min)	**1**	**5**	**15**	**1**	**5**	**15**
F-CARV	A	A	A	A	A	A
ME-CARV	A	B	B	B	B	B

Table 3 Statistical analysis of the results of *E. faecalis* biofilm treatment with free (F-CARV) and microencapsulated (ME-CARV) carvacrol. Different letters (A and B) between F-CARV and ME-CARV treatment represent significant differences ($p < 0.05$); the same letters represent non-significant differences ($p > 0.05$)

	½ MIC			**MIC**		
Time (min)	**1**	**5**	**15**	**1**	**5**	**15**
F-CARV	A	A	A	A	A	A
ME-CARV	B	B	B	B	B	B

Effect of free and encapsulated carvacrol on biofilm bacterial cells morphology

To investigate the effect of F-CARV and ME-CARV on bacterial cell morphology, the biofilm cells of *P. aeruginosa* and *E. faecalis* were analyzed using SEM. The electromicrographs obtained are shown in Fig 3. The TS-treated biofilm (negative control) of both bacterial strains showed intact cells with a normal and regular structure. However, cells treated with F-CARV and ME-CARV at the MICs showed different morphological changes and deformation of bacterial cell structures. For

P. aeruginosa, a complete cell shrinkage and deflation was observed after F-CARV and ME-CARV treatment, providing evidence of membrane destruction and leakage of the intercellular pool. Moreover, *E. faecalis* cells treated with both carvacrol treatments appeared irreversibly damaged, deformed, and had holes in their cell walls.

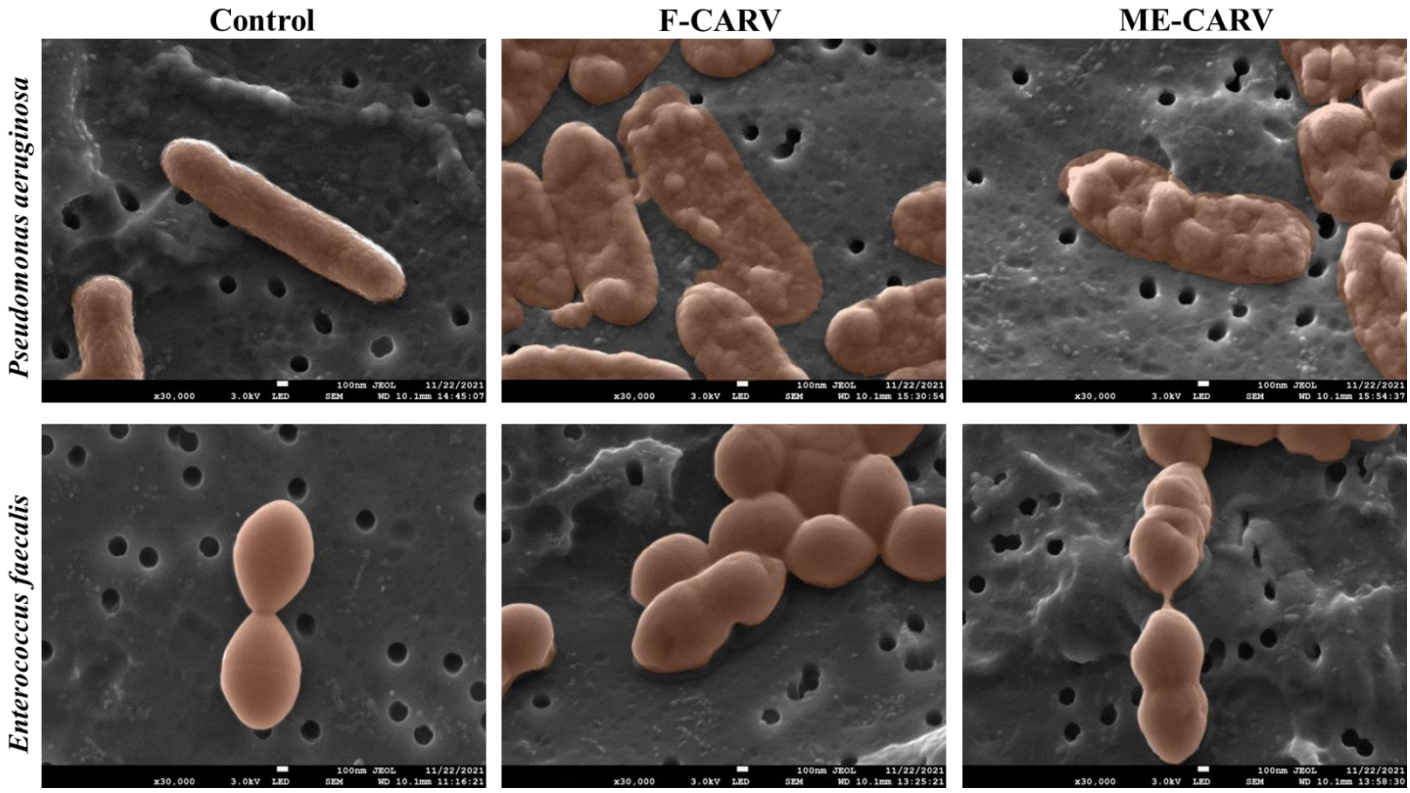


Fig 3. SEM micrographs of *P. aeruginosa* and *E. faecalis* biofilm cells after treatment with free (F-CARV) and microencapsulated (ME-CARV) carvacrol. The control represents biofilm cells treated with tryptone salt containing DMSO for F-CARV and without DMSO for ME-CARV

Impact of antimicrobial treatment on biofilm structure and cell viability

The 24-hour biofilms of *P. aeruginosa* and *E. faecalis* were stained with SYTO9 and propidium iodide (PI) after treatment with F-CARV and ME-CARV at the MICs and observed by epifluorescence microscopy (Fig 4). For both bacteria, results showed that the TS-treated control exhibited a dense biomass of viable biofilms mainly stained with SYTO9 (green bacteria) with very few dead bacteria stained with PI (red bacteria). After treatment with F-CARV and ME-CARV, results showed a representative decrease in SYTO9 staining and an increase in PI staining of the remaining biofilm. Furthermore, treatment with ME-CARV induced more cell death than F-CARV, presented by an increase in the PI-stained cell layer.

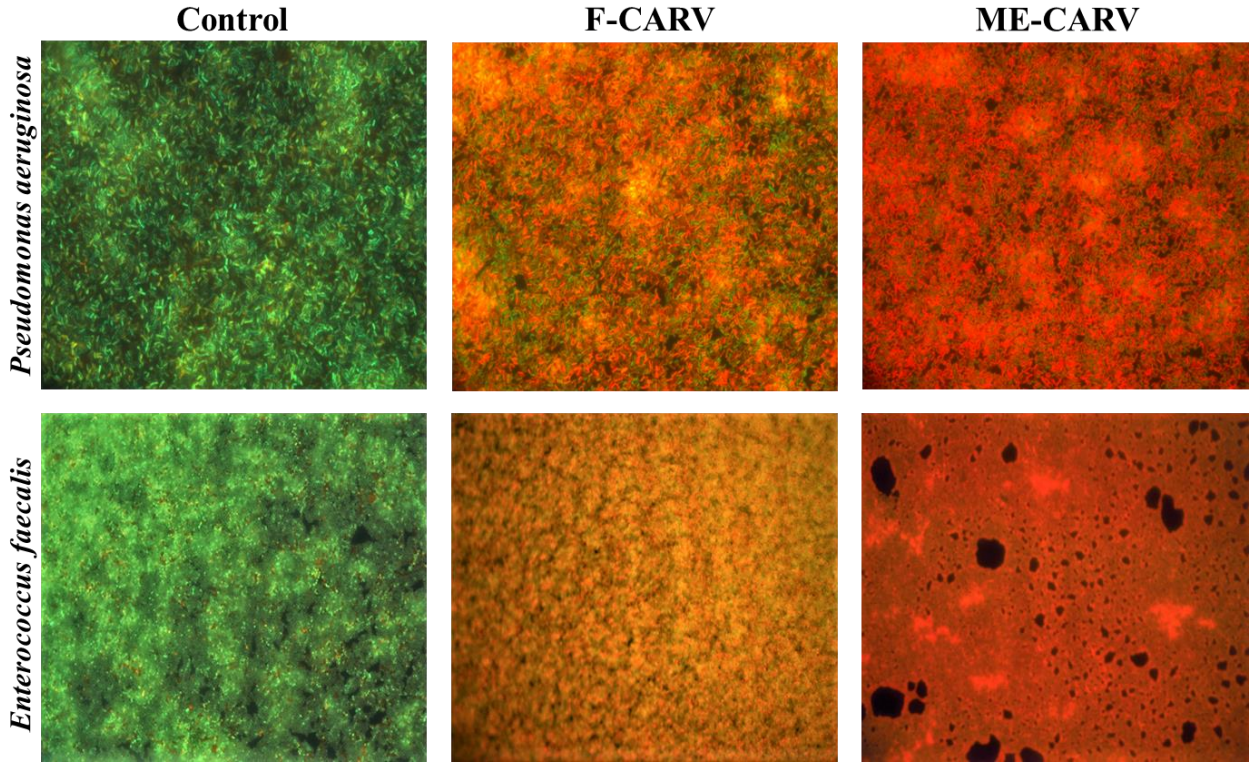


Fig 4. Fluorescence microscopic images of *P. aeruginosa* (A) and *E. faecalis* (B) biofilms after treatment with free (F-CARV) and microencapsulated (ME-CARV). Cells were visualized after staining with SYTO-9 and propidium iodide. Green cells represent living bacteria and red fluorescence represents dead bacteria. The control represents biofilm treated with tryptone salt broth

Effect of free and encapsulated carvacrol treatment on *Pseudomonas aeruginosa* and *Enterococcus faecalis* cytoplasmic cell membrane permeability

Effect of carvacrol on potassium gradient in *Pseudomonas aeruginosa* and *Enterococcus faecalis* cells

In order to investigate the effect of carvacrol on bacterial membrane permeability, the extracellular K^+ ions concentration was monitored. The results showed that after the addition of HEPES buffer (with or without DMSO), the concentration of extracellular K^+ ions remained stable (Fig 5). However, after exposure of *P. aeruginosa* and *E. faecalis* cells to F-CARV and ME-CARV at the MICs, the concentration of K^+ ions in the extracellular medium of both bacterial suspensions increased immediately within 30 s of the treatment, and remained almost stable after 5 min of treatment. Forty minutes after exposure of *P. aeruginosa* cells to F-CARV (5 mg mL^{-1}), the extracellular K^+ concentration reached 0.34 mg L^{-1}. This concentration was further increased (0.48 mg L^{-1}) after treatment with ME-CARV even using a 4-fold lower concentration (1.25 mg mL^{-1}) (Fig 5A). However, for *E. faecalis*, the extracellular K^+ concentration was similar (13.5 mg L^{-1}) after forty minutes of treatment with F-CARV (0.625 mg mL^{-1}) and ME-CARV (0.625 mg mL^{-1}) (Fig 5B).

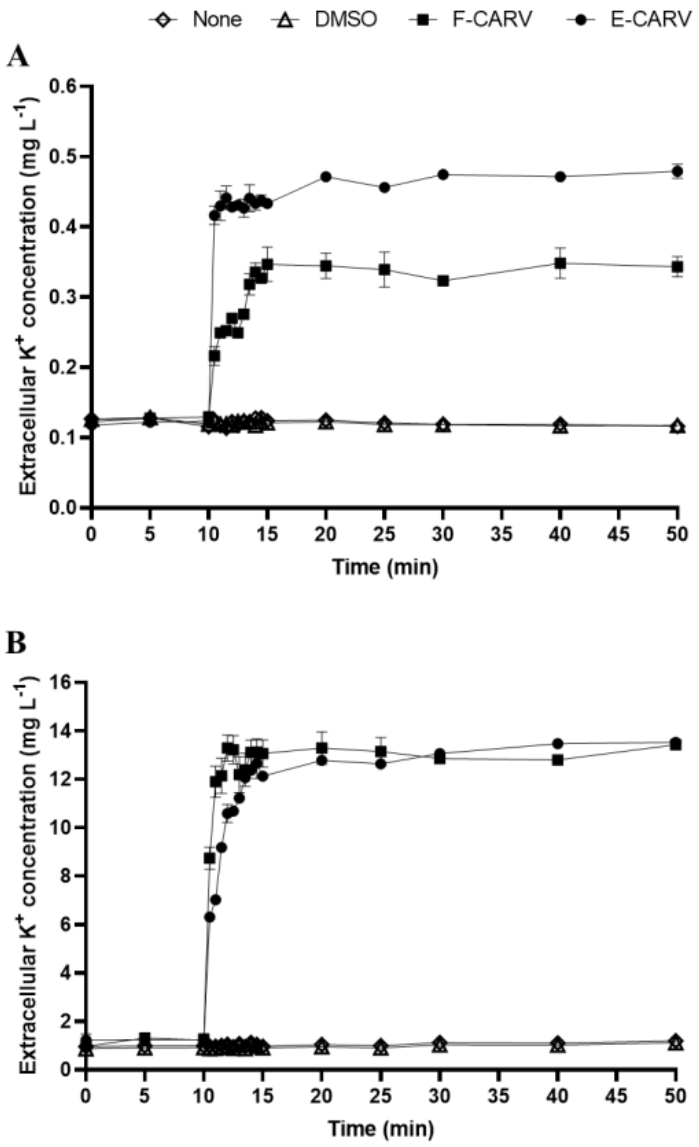


Fig 5. Kinetics of K^+ ion leakage from the intracellular medium of *P. aeruginosa* (A) and *E. faecalis* (B) cells after treatment with free (F-CARV) and microencapsulated (ME-CARV) carvacrol. The black arrow indicates the time of addition of F-CARV or ME-CARV. The concentrations of K^+ ions were measured using an atomic spectrometer and expressed as mg L^{-1}. Data are presented as the means (±SD) of three independent experiments. Control represents cells treated with HEPES buffer containing DMSO for F-CARV and without DMSO for ME-CARV

Membrane disruption by carvacrol results in release of low-molecular-weight proteins

To investigate the leakage of low-molecular-weight proteins, *P. aeruginosa* GFP strain was used. Fig 6 results showed that the negative control (with or without DMSO) had no effect on the extracellular fluorescence intensity of the bacterial population (Fig 6). By contrast, the GFP fluorescence intensity increased immediately after the addition of the carvacrol, demonstrating a significant instantaneous effect on membrane disruption (Fig 6). The results showed that this increase in GFP fluorescence intensity promoted by the treatment is time dependent. Indeed, after 5 min of bacterial treatment with F-CARV and ME-CARV, the GFP fluorescence intensity increased by 1.1 and 4.6-fold compared to the control, respectively. While after forty minutes of exposure of bacteria to F-CARV, it increased by 2.6-fold, and this increase was significantly higher in intensity after ME-CARV treatment and reached 6.5-fold (Fig 6).

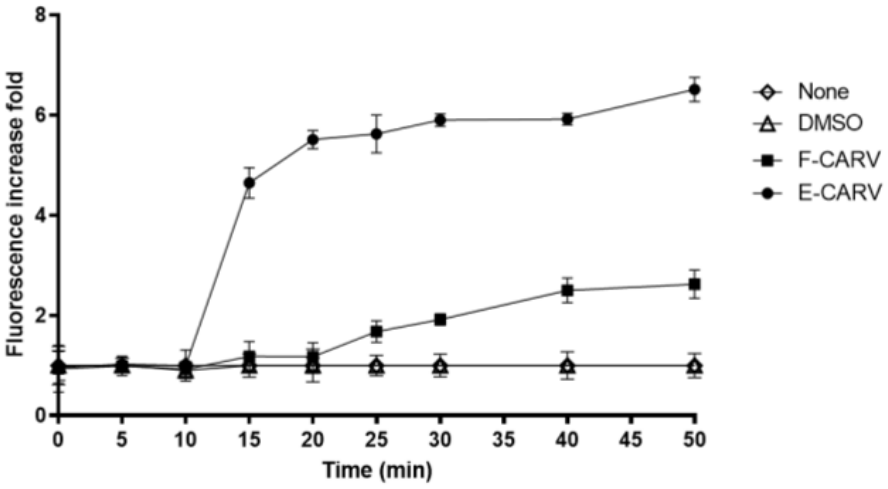


Fig 6. Assessment of cytoplasmic membrane permeability of *P. aeruginosa* GFP (ATCC ®10145GFP™) after treatment with F-CARV and ME-CARV. The black arrow indicates the time at which free (F-CARV) and microencapsulated (ME-CARV) carvacrol were added. The fluorescence of the GFP was measured using spectroscopy at an excitation and emission wavelength of 485 to 510 nm, respectively. Data are presented as means (±SD) of three independent experiments. Control represents cell treated with HEPES buffer containing DMSO for F-CARV and without DMSO for ME-CARV

Effect of carvacrol on *Pseudomonas aeruginosa* GFP cells viability

The number of viable cells as well as the intensity of GFP fluorescence were monitored after the exposure of *P. aeruginosa* GFP to a low concentration of the antimicrobial solution (0.2 mg mL^{-1}). The results showed that the percentage of viable cells of *P. aeruginosa* GFP before treatment was 82% (Fig 7A). In addition, Fig 7B shows that the control harbored predominantly green cells (viable cells). However, treatment of bacteria with carvacrol was shown to progressively reduce the percentage of viable cells and increase the intensity of extracellular GFP fluorescence over time (Fig 7A). Indeed, after 15 min of treatment, the percentage of viable cells was reduced to 40% and the intensity of GFP fluorescence was increased by 3.9-fold. Furthermore, after 40 min of exposure of bacteria to carvacrol, the percentage of viable cells was further decreased to 11% and the extracellular GFP intensity was raised by 6.2-fold (Fig 7A). Similarly, Fig 7B shows that after treatment of bacterial cells with the antimicrobial solution, the number of green cells (viable cells) decreased and the number of red cells (dead cells) increased significantly over time. These results emphasize that the increase in extracellular GFP fluorescence intensity indicates cell death.

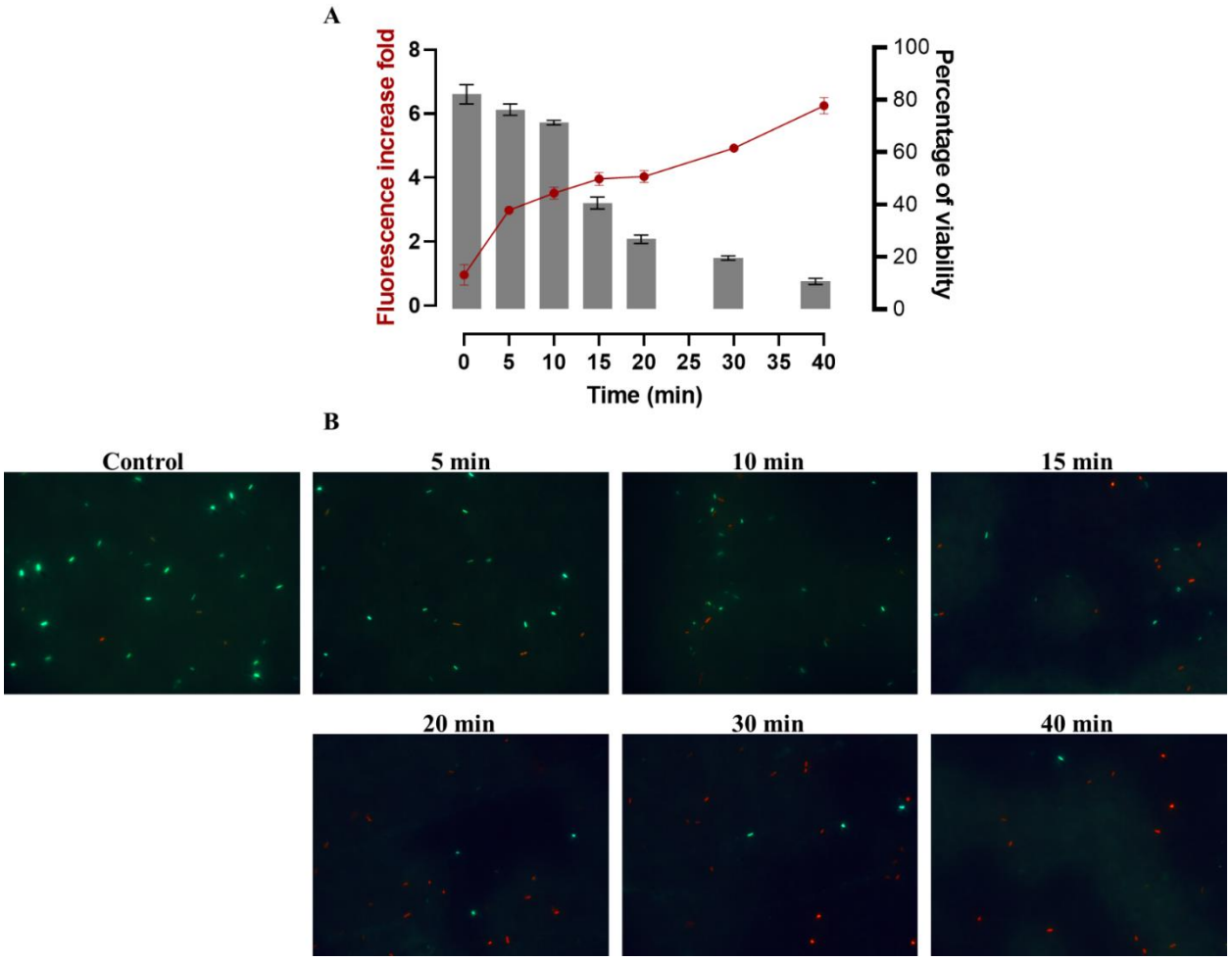


Fig 7. (A) Monitoring of extracellular GFP fluorescence intensity and cell viability of *P. aeruginosa* GFP over time after treatment with carvacrol at low concentration. (B) Epifluorescence microscopy visualization of *P. aeruginosa* GFP cells after staining with SYTO-9 (green fluorescence for living bacteria) and propidium iodide (red fluorescence for dead bacteria). Data are presented as the means (±SD) of three independent experiments. Control represents cells treated with HEPES buffer

Discussion

The use of natural plant-based substances, such as essential oils, has gained popularity as natural biocides alternative. This is due to their antimicrobial potential against a wide range of pathogenic bacteria whether in their planktonic or biofilm state, thus enabling the required treatment by overcoming the drawbacks of conventional antimicrobials used (Wińska et al. 2019). However, plant extracted compounds are generally not stable due to their volatility and vulnerability to photolysis and oxidation. In addition, the low solubility of these active compounds may reduce their antimicrobial efficacy (Shan et al. 2007; Gharsallaoui et al. 2007a; Liu et al. 2018). It is therefore important to find the optimal formulation to go through these challenges. In our study, sodium caseinate was chosen as an emulsifier due to its ability to form an interfacial membrane around carvacrol droplets and supplemented with maltodextrins as wall material to achieve the best encapsulation efficiency.

SEM was used to assess the microstructure of spray-dried carvacrol microcapsules. Fig 1 showed that the microparticles had a spherical shape and bumpy surfaces. The shrinkage that occurred during the drying and cooling procedures might be the cause of some of the irregular surfaces which is a characteristic of the spray-drying process (Botrel et al. 2012; Hijo et al. 2015). The micrographs of obtained microcapsules also showed that the surface indentation and roughness were more prominent in the small particles than in the large ones, suggesting that wall solidification occurred before the expansion of the microcapsules (Rosenberg and Young 1993). The study of the internal structure showed that carvacrol microcapsules were hollow, with a central void. The presence of air holes in the wall of microcapsules proved the presence of volatile compounds in the capsules. The voids might be the result of air expansion through spray drying process in the drops (Teixeira et al. 2004).

The MIC of F-CARV dissolved in DMSO against *P. aeruginosa* has shown a high value of 5 mg mL^{-1}. As shown previously, the MIC of carvacrol against *P. aeruginosa* was higher than that of the other bacteria, which could be assigned to the resistance of *P. aeruginosa* associated with its efflux pump mechanism and β-lactamase activity (Delgado et al. 2007; Tapia-Rodriguez et al. 2017). However, results showed that ME-CARV inhibited growth of bacteria at concentration 4-fold lower than those of F-CARV (1.25 mg mL^{-1}). In addition, it was reported that the antimicrobial activity

of carvacrol microencapsulated in hydroxypropyl-beta-cyclodextrin was higher than that of free carvacrol against both *Escherichia coli* and *Salmonella enterica* strains, indicating that encapsulation increased the water solubility and therefore the contact between carvacrol and bacteria in the medium (Kamimura et al. 2014). Our results also showed that the MIC values of the F-CARV and ME-CARV against *E. faecalis* were similar (0.625 mg mL^{-1}). Although microencapsulation did not decrease the MIC value of carvacrol, the antimicrobial activity of carvacrol was retained under appropriate processing conditions. Other studies showed that, even F-CARV showed similar and even stronger antimicrobial activity compared to ME-CARV. Nevertheless, the use of encapsulated carvacrol could be more interesting for food applications by masking the strong aroma of the compounds and ensuring a controlled release of carvacrol (Sun et al. 2019; Ayres Cacciatore et al. 2020). Furthermore, encapsulation is intended to protect the active compounds from environmental factors such as light, water, oxygen, pH, etc., thus maintaining their antibacterial action for a relatively longer period of time (Mozafari et al. 2008; Liolios et al. 2009). The time-kill assay is an appropriate and robust tool to gather information regarding the dynamic interaction between the bacterial strain and the antibacterial agent. Results showed that F-CARV and ME-CARV exhibited a significant inhibition of bacterial cells for both tested strains compared to their controls, as well as a rapid bacterial reduction (1 and 5 min for *P. aeruginosa* and *E. faecalis*, respectively). However, other studies reported that carvacrol did not showed rapid activity using the MIC value, and that it required 6 h to reduce the bacterial population of *Salmonella* Typhimurium below the detection limit, even when exposed to the 2 MIC value, no culturable cells were detected after 1 hour of exposure (Trevisan et al. 2018). The relatively fast reduction in bacterial cell count is considered as important as the compound's bactericidal nature, since more quickly the antimicrobial agent kills, more effectively it can inhibit the formation of biofilms (Kalita et al. 2015). The instantaneous bactericidal action of carvacrol observed in this study suggested that carvacrol may have affected the integrity of the bacterial membrane.

In addition, in order to study the effect of carvacrol against biofilm of bacterial strains, *NEC biofilm system* was used (Abdallah et al. 2015). This system is a static biofilm system, which offers simplicity in the experimental procedure (biofilm formation and antibiofilm testing) and allows the study of the efficacy of disinfectants on biofilms. Furthermore, this system allows an easier access

to the sessile cells, which permits the assessment of the biofilm formation and the disinfectant effectiveness through both cell counts and microscopic observations. The system also presents another advantage concerning its ability to receive all solid substrata, thereby enabling study of different solid surfaces (Abdallah et al. 2015). In this study, stainless steel materiel was used as the solid surface to perform biofilms of *P. aeruginosa* and *E. faecalis*. Stainless steel is a material to which Gram-negative and Gram-positive bacteria, including *Pseudomonas* and *Enterococcus* strains, can adhere in a short time (Hood and Zottola 1997; Barnes et al. 1999; Castro et al. 2018). Our findings showed that after 24 h of incubation, biofilms of both bacteria exhibited a bacterial biomass of approximately 7 log CFU mL^{-1}. Results showed that the applied carvacrol treatment resulted in a dose- and time-dependent reduction of total preformed biofilms. The biomass reduction of *P. aeruginosa* and *E. faecalis* biofilms after treatment with F-CARV and ME-CARV was enhanced by increasing the time treatment as well as the carvacrol concentration (Fig 2). Thus, for both biofilms, the most effective treatment using F-CARV was obtained after 15 min treatment with MIC concentration that reduced biofilm biomass by approximately 5 log CFU mL^{-1} ($p < 0.05$). In addition, the biofilm reduction obtained using ME-CARV after 15 min of treatment with MIC concentrations was 5.5 log CFU mL^{-1} for *E. faecalis*, and no bacterial counts were detected for *P. aeruginosa* ($p < 0.05$). Other studies showed that the use of higher concentrations of EOs result in severe cell membrane damage and a complete homeostasis disruption inducing cell death (Nazzaro et al. 2013a; Trevisan et al. 2018).

The increased efficacy of EOs with prolonged action time is due to a greater diffusion of the applied substances through the EPS matrix of the biofilm by prolonging the exposure time. This mode of action of EOs can be explained by the greater effectiveness of carvacrol after 15 min of exposure compared to a treatment with a short exposure time (1 min). Furthermore, our results showed that the antibacterial activity of ME-CARV against *P. aeruginosa* and *E. faecalis* biofilm cells was significantly greater than that of F-CARV using either ½ MIC or MIC values. Hence, the encapsulation of carvacrol reduced the concentration of applied carvacrol while enhancing the anti-biofilm effect compared to the F-CARV.

These results are concordant with the biofilm analysis by epifluorescence microscopy, which allowed the direct observation of biofilms before and after treatment with F-CARV and ME-

CARV. Results showed that images of TS-treated (control) *P. aeruginosa* and *E. faecalis* biofilms stained with SYTO9 and PI showed compact biofilms composed predominantly of live bacteria (green cells) with minimal areas of dead bacterial cells (red cells) (Fig 4). However, after treatment with both F-CARV and ME-CARV, the number of dead cells stained by PI increased. Moreover, treatment with ME-CARV induced a higher percentage of dead cells compared to F-CARV. Thus, similar to previous results, this direct analysis provided evidence that treatment with the capsules showed significantly higher efficacy than un-encapsulated EOs, supporting the hypothesis that the capsules' shell enhances the interaction with biofilms and induces effective penetration of the antimicrobials into the deep layers of biofilms (Li et al. 2015; Duncan et al. 2015).

Although various antimicrobial mechanisms have been described, bacterial cell walls and membranes are often considered the primary targets of EOs (Yap et al. 2021). Thus, the effect of F-CARV and ME-CARV treatment on cell membrane integrity was investigated by measuring the extracellular GFP fluorescence intensity and K+ ions concentration. The monitoring of the intensity of extracellular GFP fluorescence is a novel method developed Khelissa et al. (2021a) who reported that the leakage of GFP to the extracellular medium is an indication of membrane permeabilization in stressed microbial cells. In this study, it was observed that exposure of *P. aeruginosa* and *E. faecalis* to F-CARV and ME-CARV at the MICs increased the extracellular K^+ ions concentrations immediately within 30 s of treatment (Fig 5). In addition, the extracellular GFP fluorescence intensity was gradually increased after both carvacrol treatment using *P. aeruginosa* GFP strain (Fig 6). This shows that F-CARV and ME-CARV induced rapid release of potassium ions due to their small volume and progressive leakage of low-molecular-weight proteins from the cytoplasm over time because of their large volume. These results are in contradiction with our previous study, which showed that free and microencapsulated RUL3 did not induce protein leakage from the cytoplasm of *Escherichia coli* GFP (ATCC 25922GFP) (Khelissa et al. 2021a). The final extracellular GFP intensity after 40 min treatment of *P. aeruginosa* GFP with ME-CARV was 3.9-fold higher than that measured after treatment with F-CARV, even at a concentration of ME-CARV 4-fold lower than that of F-CARV (Fig 6). Thus, the results showed that microencapsulation enhances and reinforces the membrane disturbing action of carvacrol yet reducing the concentrations being used. Our finding also showed that the irreversible leakage of

the intra-cellular bacterial pooled material leads to cell death (Fig 7). Hence, the results highlighted that carvacrol is able to target the cytoplasmic membrane of bacteria and disrupt the integrity of its phospholipid bilayer. Many previous studies have shown that the primary target of carvacrol is the bacterial membrane, as carvacrol is an inherently hydrophobic monoterpene that readily penetrates bacterial cell membranes, resulting in a disruption of their integrity and a subsequent release of bacterial cell contents and thus cell death (Di Pasqua et al. 2007; Cristani et al. 2007; Khan et al. 2017b). In addition, SEM observations proved the damage to the cell membranes of both bacterial strains caused by carvacrol treatments (Fig 3). Our findings are in agreement with previous studies reporting that the spray-drying microencapsulation can be used as an effective tool to enhance the antibacterial agents' activities while reducing the required concentrations (Khelissa et al. 2021a; Khelissa et al. 2021b)

Conclusion

The present study investigated the microencapsulation of the volatile antibacterial agent carvacrol in a maltodextrin-sodium caseinate matrix using the spray-drying method. Our results suggested that the ME-CARV exhibited a higher antimicrobial activity compared to F-CARV, while reducing the amounts of carvacrol required. The results obtained have shown a promising prospect of using microencapsulated carvacrol, as an alternative to conventional sanitizing methods to fight biofilms in the food industry and medical environments. However, before the application of these encapsulated products as disinfectants, they must be approved and registered by regulatory agencies. Finally, microencapsulation by spray-drying could be used to encapsulate other products in order to combat biofilms in the artificial ecosystems, and even to combat biofilms *in vivo*.

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Chapter III

Article III-Article IV-Results

INTRODUCTION

The formation of biofilms on medical and food settings is a recurring problem for public health. Indeed, biofilms constitute a reservoir of pathogens that are continuous sources of serious infections worldwide. Different strategies have recently been proposed to control biofilms, including chemical removal (e.g. detergents, biocides and surfactants), mechanical removal (e.g. thawing, freezing, sonication and scraping) and biological removal (e.g. natural compounds from plants, bacteriophages, antimicrobial surfaces and enzymes) (Borges et al. 2020). Each method includes several techniques and has advantages and disadvantages that provide different effects in the control of biofilms (Simões et al. 2010; Chen et al. 2013; Borges et al. 2016; Chen et al. 2018; Shariati et al. 2019). However, the complete eradication of biofilm by the sole use of these methods has proven difficult to achieve due to the strong protection of the biofilm cells by the EPS matrix. This matrix serves as an initial protective barrier for biofilm cells, blocking xenobiotic and antimicrobial access to biofilm cells and providing protection against environmental stresses, making the biofilm up to 1,000 times more resistant to antimicrobial agents than its planktonic counterparts (Tan et al. 2018). It is therefore crucial to apply a combination of various strategies to reach different targets in the biofilm. The combined use of two or more biofilm control methods, known as Hurdle technology, is a newly developed and potentially effective strategy to remove biofilm cells from abiotic surfaces (Khan et al. 2017b). The synergistic effect of Hurdle technology in reducing biofilm from abiotic surfaces has been proven in numerous studies (Ban and Kang 2016; Jung et al. 2018; Lim et al. 2019; Hussain et al. 2019). Accordingly, combining enzymes with biosourced antimicrobials would provide a promising approach to control biofilms in such a way that the enzymes destroy and destabilize the biofilm EPS matrix, so that matrix-protected cells become more effectively removed by the antimicrobials.

Enzymes act by weakening the physical integrity of the biofilm by breaking down the various structures that form the EPS matrix. EPS consists of different multi-structural components, that are mainly derived from carbohydrates, polysaccharides, proteins, glycoproteins, lipids, nucleic acids, phospholipids and glycolipids, among others (Liu et al. 2004). Hence, the application of enzymes targets the components of the EPS, transforming them into smaller units. These smaller units can then be transferred across cell membranes and metabolized by the cell (Molobela et al. 2010). The

EPS matrix is heterogeneous and different from biofilm to another and some studies indicate that carbohydrates are the main components of the EPS, while other studies underline the abundance of proteins (Zhang et al. 2005; Orgaz et al. 2007; Borges et al. 2020). Several studies have demonstrated the high destabilizing and destructive efficacy of protease-based enzymes against the biofilm matrix of several bacterial strains (Brindle et al. 2011; Fagerlund et al. 2016; Mohamed et al. 2018; Lim et al. 2019; Jee et al. 2020).

In addition, the use of natural antimicrobial substances, such as EOs, have attracted public attention due to their high efficacy, safety and non-toxicity (Nazzaro et al. 2013b) . Carvacrol, a monoterpenoid phenol and the major component of *Origanum vulgari* EO, could be used as an antifungal and anti-inflammatory agent and exhibits a broad antimicrobial spectrum (Burt 2004; Ahmad et al. 2011; Lima et al. 2013; Esteban and García-Coca 2017). Furthermore, several previous studies have demonstrated its antibiofilm activity on various surfaces (Amaral et al. 2015; Trevisan et al. 2018; Walczak et al. 2021). Nevertheless, essential oils are generally volatile and highly vulnerable to oxidation and photolysis. Furthermore, the low solubility of these active compounds may minimize their antimicrobial efficacy (Shan et al. 2007; Gharsallaoui et al. 2007b; Liu et al. 2018). In addition, enzyme activity is strongly influenced by environmental factors and is optimal under restricted conditions (Cordeiro and Werner 2011). Other disadvantages of using enzymes are the high cost and self-degradation, which leads to instability. Therefore, as a solution, the enzymes and carvacrol were stabilized by encapsulation. Encapsulation can improve the anti-biofilm activity of these active compounds, increase their stability, and may be cost effective if the stability achieved is sufficiently maintained for repeated use.

In this context, the third chapter of this thesis was devoted to the study of the potential activity of Hurdle technology using proteolytic enzymes (pepsin and trypsin) and an essential oil (carvacrol) to combat *P. aeruginosa* and *E. faecalis* biofilms. For this purpose, the first part investigated the potential efficacy of free pepsin and trypsin to degrade *P. aeruginosa* and *E. faecalis* biofilms and their synergistic effect when combined with free carvacrol. The minimum dispersive concentrations and contact times of the enzymes were determined against biofilms grown on polystyrene surfaces. Similarly, the minimum inhibitory concentrations and contact times of carvacrol were determined against both bacterial strains in suspension. In addition, the dispersive

activity of enzymes and the antimicrobial activity of carvacrol, as well as the synergistic effect of these two active components against bacterial biofilms grown on stainless steel surfaces, were demonstrated using direct microscopic analysis and indirect cell counting. Otherwise, the second part aimed to present the effect of encapsulated pepsin and trypsin in degrading *P. aeruginosa* and *E. faecalis* biofilms and their potential synergistic effect with encapsulated carvacrol. The encapsulation was performed by spray-drying method in order to enhance the stability of these active agents and improve their biological activity. Moreover, to investigate whether encapsulation enhances, retains, or decreases the anti-biofilm activity of these active components, this study also examined the dispersive activity of encapsulated enzymes and the antimicrobial activity of encapsulated carvacrol using direct and indirect analysis and their synergistic effect when used in combination.

Pepsin and trypsin treatment combined with carvacrol: an efficient strategy to fight *Pseudomonas aeruginosa* and *Enterococcus faecalis* biofilms

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Abstract

Biofilms consist of microbial communities enclosed in a self-produced extracellular matrix which is mainly responsible of biofilm virulence. Targeting this matrix could be an effective strategy to control biofilms. In this work, we examined the efficacy of two proteolytic enzymes, pepsin and trypsin, to degrade *P. aeruginosa* and *E. faecalis* biofilms and their synergistic effect when combined with carvacrol. The minimum dispersive concentrations (MDCs) and the contact times of enzymes were determined against biofilms grown on polystyrene surfaces. Both proteolytic enzymes markedly detached *P. aeruginosa* and *E. faecalis* biofilms after 1 h of contact at a concentration of 1 mg mL^{-1}, without affecting the growth of planktonic cells. Moreover, the minimal inhibitory concentrations (MICs) and contact times of carvacrol were determined against both bacterial suspension strains. For biofilms grown on stainless steel surfaces, the combined pepsin or trypsin treatment with carvacrol showed more significant reduction of both biofilms compared to carvacrol treatment alone. In addition, this reduction was more substantial after sequential treatment of both enzymes in the order trypsin-pepsin, followed by carvacrol treatment

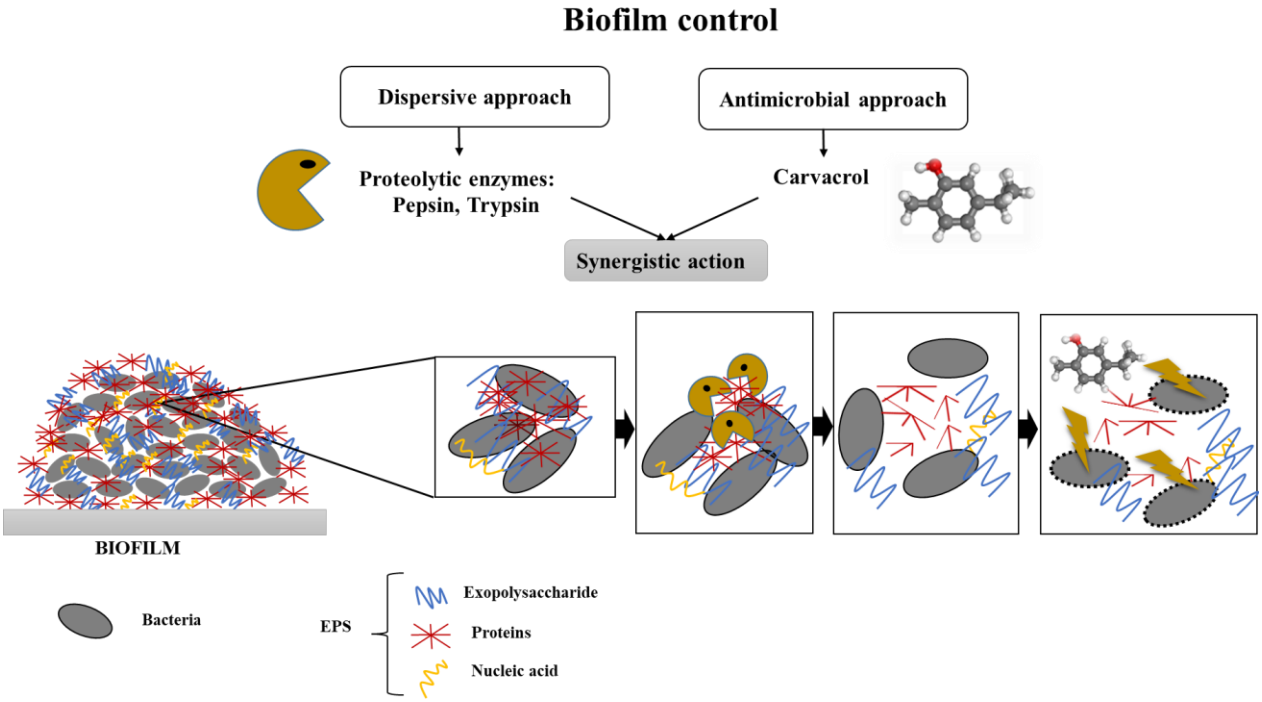
with a maximum reduction of 4.7 log CFU mL^{-1} ($p < 0.05$) for *P. aeruginosa* and 3.3 log CFU mL^{-1} ($p < 0.05$) for *E. faecalis*. Such improved efficiency was also obvious in the epifluorescence microscopy analysis. These findings demonstrate that the combined effect of the dispersive activity of protease and the antimicrobial activity of carvacrol could be a prospective approach for controlling *P. aeruginosa* and *E. faecalis* biofilms.

Key points

- *In the medical field, biofilms are of particular interest as they can cause serious problems such as chronic infections.*
- *The structurally and functionally complex extracellular polymeric matrix plays a key role in the emergent properties of biofilms.*
- *The combined treatment using enzymes and essential oils is a very effective strategy to eradicate biofilms by targeting the extracellular polymeric matrix and embedded bacterial cells.*

Keywords Biofilm; Pepsin; Trypsin; Carvacrol; *Pseudomonas aeruginosa*; *Enterococcus faecalis*.

Graphical abstract



Introduction

Biofilms are structured microbial associations attached to surfaces and enclosed in extracellular polymeric matrix (EPS) (Lee et al. 2017). The main property of biofilms is their high resistance to antimicrobials and other types of stress compared to the cells under planktonic state; This make biofilms very difficult to eradicate (Khelissa et al. 2019). Biofilms can be found on many types of abiotic surfaces in the medical field, causing serious problems such as chronic infections, as they can reserve and disperse several infectious agents (Hall-Stoodley et al. 2004; Alav et al. 2018).

Pseudomonas species are ubiquitously present in the environment, with some causing infections in both plants and animals (Crone et al. 2020). Among *Pseudomonas* species, *Pseudomonas aeruginosa* is a prevalent opportunistic human pathogen that can cause a wide range of acute and chronic life-threatening infections, in particular in patients with suppressed immune systems. *P. aeruginosa* is of specific importance because it is the main cause of mortality and morbidity for patients with cystic fibrosis and one of the main health-care associated pathogens that affect hospitalized patients, while being inherently resistant to a large array of antibiotics (Moradali et al. 2017). *P. aeruginosa* is able to produce numerous virulence factors, which expression is regulated by complex systems of signal transduction as a response to environmental stresses, such as biofilm formation (An et al. 2019).

Enterococci are opportunistic pathogens frequently isolated from the normal flora of the human oral cavity, gastrointestinal tract and genital tract of female. Enterococci are known to readily attach to diverse medical devices and form biofilms (Fisher and Phillips 2009). Among the enterococcal species, *Enterococcus faecalis* is the most prevalent health-care associated pathogen and commonly causes bacteremia, urinary tract infections, infections in abscesses, peritonitis, endocarditis and decubitus and foot ulcers. This bacterium is involved in 80-90% of all health care-associated enterococcal infections (Shridhar and Dhanashree 2019).

The bacterial biofilm matrix, is composed of three main categories: exopolysaccharides, extracellular and associated cell surface proteins/adhesins, and extracellular DNA. It generally represents 90% or more of the dry biofilm weight, and it is involved in the attachment of cells to surfaces and the maintaining of structural integrity and hydration of biofilm. Furthermore, the resistance of biofilm-structured bacteria against antibiotics and other components of antimicrobial

agents is mainly supported by the EPS matrix that limit the transport of biocides within the biofilm (Al Kassaa et al. 2019; Karygianni et al. 2020b). Therefore, a new approach for the effective inactivation of biofilm bacterial cells is needed to control biofilms.

Strategies using matrix-degrading enzymes have been investigated to disrupt EPS in biofilms. Mohamed et al. (2018) demonstrated that among different biological enzymes tested, proteolytic enzyme (bromelain) was the best for achieving inhibition and eradication of *Klebsiella pneumoniae* biofilms. Lequette et al. (2010) evaluated papain, serine protease, α-amylase, β-glucanase and cellulase for removing biofilms formed by different bacterial species currently present in food processing chains, and demonstrated different efficacy of these enzymes. They also confirmed that the use of enzymes-combination that target multiple EPS components, improves the effectiveness of an enzymatic remover against multi-species biofilms. Also, Fagerlund et al. (2016) applied protein K, dispersin, and trypsin to control biofilms of various staphylococcal species, and confirmed the high efficiency of biofilm removal by these enzymes.

When dispersing biofilm with enzymes, the released cells and microaggregates can contaminate new areas and restart the biofilm development cycle. Thus, enzymatic treatment should be used in combination with a killing step to treat biofilm contamination. Recently, strategies using the combination of EPS-degrading enzymes with antimicrobial agents, have been investigated for potential application in biofilm treatment (Kim et al. 2013; Wang et al. 2016; Rodríguez-López et al. 2017; Zhou et al. 2018; Lim et al. 2019; Baidamshina et al. 2021). EPS-degrading enzymes can disperse bacteria embedded in biofilms for more efficient disinfection when combined with biocide agents. Targeting matrix may also perturb the viscoelastic properties to additionally reduce biofilm cohesion and improve antimicrobial access and efficiency (Jiang et al. 2020). Such approach using proteolytic enzymes that target matrix proteins, combined with an antimicrobial agent, has not been sufficiently investigated for effective inactivation of *P. aeruginosa* and *E. faecalis* cells in biofilms. Thereby, in this study, two proteolytic enzymes, pepsin and trypsin, targeting matrix proteins, were investigated for their potential to degrade *P. aeruginosa* and *E. faecalis* biofilms and the synergistic effect when combined with carvacrol, a natural monoterpenoid phenol that exhibits a broad antimicrobial and anti-biofilm activity.

Materials and Methods

Bacterial strains, reagents and cell suspensions preparation

The microorganisms used were *P. aeruginosa* (CIP 103467) and *E. faecalis* (isolated from French cheese). The strains were stored at -80 °C in tryptic soy broth (TSB; Biokar Diagnostics, France) supplemented with 40% (v/v) glycerol. Bacteria were pre-cultivated by inoculating 100 µL of the frozen strains culture into 5 mL of TSB medium and incubating for 24 h at 37 °C. Then, to prepare culture, 100 µL of the pre-culture was used to inoculate 50 mL of TSB medium, and incubated for 16 h at 37 °C with agitation at 160 rpm. After overnight culture, cells were pelleted by centrifugation (5,000 ×*g*, 5 min, 20 °C), then harvested cells were washed twice with 20 mL of potassium phosphate buffer (PPB; 100 mM, pH 7). Finally, cells were resuspended in PB and dispersed by sonication at 37 kHz (Elmasonic S60H, Elma®) for 5 min at 20 °C. These suspensions were used for the preparation of bacterial suspensions of defined concentration required for each experiment. Pepsin was obtained from MP Biomedicals (Strasbourg, France). Trypsin and carvacrol (98% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycine HCl buffer (100 mM, pH 3) was used to prepare pepsin solution, phosphate buffer (100 mM, pH 7.6) was used to prepare trypsin solution, and dimethyl sulfoxide (DMSO) with a final concentration of 2% (v/v) was used to prepare carvacrol solution.

Bacterial susceptibility assay

Broth microdilution method was used to determine the minimal inhibitory concentrations (MICs) of carvacrol against both bacterial strains. Bacterial suspensions were adjusted to 10^6 CFU mL^{-1} using sterile Müller-Hinton broth (MHB; Biokar Diagnostics, Pantin, France). Firstly, 100 µL of MHB was added to each well of microtiter plates. Then, twofold serial dilutions of carvacrol solution supplemented with dimethyl sulfoxide (DMSO; Sigma-Aldrich, France) were prepared in the microtiter plates over the range of 0.312 to 10 mg mL^{-1}. Finally, 100 µL of the bacterial suspensions were added to each well. Bacteria were not added to the negative control, while antimicrobial was not added to the positive control containing DMSO. Plates were incubated in Bioscreen C at 37 °C with a continual shaking and the OD600 nm was read every 2 h during 24 h.

MIC value was considered as the lowest concentration that inhibit the obvious growth of bacteria in the wells after incubation.

Time-Killing assessment

The time-killing assay was used to investigate the bactericidal effects of carvacrol against planktonic cells of *P. aeruginosa* and *E. faecalis*. The experiment was carried out according to Isenberg (2004) with some modifications. Briefly, *P. aeruginosa* (CIP 103467) and *E. faecalis* were grown overnight then transferred to MHB, supplemented with carvacrol at MIC value for each strain, to obtain a final inoculum of 10^6 CFU mL^{-1}. Medium containing DMSO was used as a control. Then, bacteria were incubated under shaking at 37 °C for 1 min, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 24 h, respectively. At the selected time, 100 µL were taken, serially diluted, and plated onto Mueller Hinton agar (MHA, Difco Pont-de-Claix, France). Plates were incubated at 37 °C for 24 h and colony forming units (CFU) were counted. The tests were repeated three times.

Assessment of the enzymatic effects on biofilm on polystyrene surface

Biofilm formation was performed on polystyrene surfaces and quantified by crystal violet assay as previously described with some modifications (Lim et al. 2019). Briefly, 200 µL of the bacterial strain of 10^7 CFU mL^{-1} was inoculated into a 96-well microtiter plate and incubated at 37 °C for 24 h to allow biofilm formation. Then, wells were rinsed once by depositing and aspirating 200 µL of PB, treated with 200 µL of enzymes (final concentrations 10^{-4} - 10 mg mL^{-1}), or 200 µL of TSB (control), and incubated at 37 °C for 30 min, 1 h and 2 h. After incubation time, the enzyme-containing medium was discarded by pipetting and the wells were rinsed once with PB, fixed with 96% ethanol for 15 minutes and stained with 1.5% (v/v) crystal violet for 20 min. Afterwards, unbound dye was removed by washing three times with PB and dye bounding to the bacterial cells was solubilized in 200 µL of 33% (v/v) acetic acid. Then 100 µL of the destaining solution was moved to a new 96-well plate and absorbance was measured at 595 nm using the Synergy HTX multimode microplate reader (BioTek, France). The resulting OD values were determined by subtracting OD value of negative control (treated with TSB only) from OD values of treated

samples, and the percentage of biofilm reduction was calculated. The minimum dispersive concentration (MDC) of enzymes that is effective against both biofilm strains, and the time of action required to disperse biofilms were determined. All tests were repeated three times for each strain.

The bactericidal effects of both enzymes on planktonic cells of *P. aeruginosa* (CIP 103467) and *E. faecalis* were evaluated in MHB. Accordingly, 100 µL of the bacterial strains of a final concentration of 10^6 CFU mL^{-1} were added to a 96-well microtiter plate containing enzymes. Then, microtiter plates were incubated in Bioscreen C at 37 °C with continuous shaking and the OD600 nm was measured for 24 hours. The effect of enzymes on the bacterial growth was assessed by reference to the well-described turbidity. This test was repeated three times.

Combined treatment for removal of biofilms on stainless steel

Biofilm formed on stainless steel surfaces

Circular stainless steel (SS) coupons (304 L, Equinox, France) of 41 mm in diameter and 1 mm thick, were used as a surface for biofilm formation. Before use, coupons were soaked in 95% ethanol (Fluka, Sigma-Aldrich, France) overnight, then washed with distilled water. After rinsing, coupons were immersed in 1% DDM ECO detergent (ANIOS, France) for 15 minutes at 20 °C. Then, coupons were washed vigorously with distilled water five times for 1 min, and again three times with ultrapure water (Milli-Q® Academic, Millipore, France) to totally remove detergent residues. Finally, coupons were air-dried before autoclaving at 121 °C for 20 min. The sterile coupons were placed in a sterile static biofilm system, called *NEC biofilm system*, as previously described by Abdallah et al. (2015). Biofilm formation was initiated by depositing 3 mL of bacterial suspension (10^7 CFU mL^{-1}) of *P. aeruginosa* (CIP 103467) and *E. faecalis* on the sterile SS coupons in each reactor and incubated at 20 °C for 1 h under static conditions to permit bacterial cell adhesion. Afterwards, coupons were rinsed twice with PB to remove non-adhering cells. Coupons were then covered with 5 mL of TSB medium and the sealed systems were incubated for 24 h at 37 °C. After incubation, the old TSB medium was discarded and the coupons covered with biofilm were washed twice with PB to remove planktonic cells. Rinsed coupons were used for quantification of biofilm biomass, antibiofilm testing, and epifluorescence microscopy analysis.

For the quantification of biofilm biomass, attached cells were detached in 20 mL of TS broth using a sterile 100 mL pot. The pots were vortexed for 30 s, sonicated for 5 min (37 kHz, 5 min, 25 °C) (Elmasonic S60H, Elma, Germany), and subsequently vortexed for 30 s. Thereafter, serial dilutions were prepared in TS broth and plated on Tryptic Soy Agar (TSA; Biokar Diagnostics, France) plates then incubated at 37 °C. After 24 h of incubation, the number of cells was enumerated and the results are presented in log CFU mL^{-1}. Results represent the average of three independent experiments.

Biofilm removal by single and combined treatment

The single and combined treatment of enzymes and antimicrobial was evaluated against bacterial biofilms by cell counting. For enzymatic treatment, biofilms were treated with 3 mL of pepsin and trypsin solutions at a final concentration of MDCs (1 mg mL^{-1}), individually or sequentially, and incubated at 37 °C for 1 h. For carvacrol treatment, biofilms were placed in 3 mL of carvacrol solutions at a final concentration of ½ MICs (2.5 mg mL^{-1} for *P. aeruginosa* and 0.312 mg mL^{-1} for *E. faecalis*), and incubated at 20 °C for 1 min for *P. aeruginosa* and 5 min for *E. faecalis*. For combined treatment of enzymes and carvacrol, biofilms were treated with enzymes, individually or sequentially, followed by carvacrol using the same concentrations and incubation times as above. The antimicrobial action of carvacrol was stopped by immersing slides in 5 mL of neutralizing solution (Toté et al. 2010). Sessile cells were detached and enumerated as described above. Biofilms treated with glycine HCl and phosphate buffer without enzymes served as enzyme controls, and biofilm treated with DMSO served as carvacrol control. Results represent the mean of three independent experiments.

Epifluorescence microscopy imaging

After treatment of biofilms with enzymes and carvacrol (as described above), biofilms were stained with the LIVE/DEAD BacLight kit (Invitrogen Molecular Probes, USA) for 15 minutes in the dark according to the manufacturer's instructions. Then, coupons were rinsed with distilled water, kept in the dark to air dry, and observed under an epifluorescence microscope (Olympus BX43, Germany). Green cells were designated as viable and red cells were considered as nonviable.

Scanning electron microscopy analysis (SEM)

In order to investigate the effect of enzymes and carvacrol on bacterial cells morphology, the treated and untreated biofilm bacterial cells were observed using SEM (JEOL-JSM-7800FLV, Japan). After biofilm treatment with different compounds, cells were recovered and diluted tenfold in TS. One milliliter of the diluted cells was filtered using 0.2 µm pore size polycarbonate membrane filter (Schleicher & Schuell, Dassel, Germany) then fixed for 4 h at 4 °C with cacodylate buffer 0.1 M, pH 7.0 (sodium cacodylate trihydrate $(CH3)_2AsO_2Na.3H_2O$) containing 2% glutaraldehyde. Fixed cells were dehydrated by submerging filter in an ascending series of ethanol (50, 70, 95, and 2×100% (v/v) ethanol), for 10 min at each concentration and critical point dried. Samples were covered with a thin carbon film and observed with microscope at 3 KV.

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

Antimicrobial activity of carvacrol against planktonic cells of *Pseudomonas aeruginosa* and *Enterococcus faecalis*

The antimicrobial activity of carvacrol was assessed against both bacterial strains. Fig. 1 showed that the use of DMSO, at a final concentration of 2% (v/v) to improve the water miscibility of carvacrol, was not toxic for the studied bacterial strains. The results showed that the strains growth was not affected in the presence of DMSO as shown in Fig. 1. The MIC value of carvacrol against *P. aeruginosa* strain was 5 mg mL^{-1} (Fig. 1a). *E. faecalis* strain was more sensitive to carvacrol with a MIC value equal to 0.625 mg mL^{-1} (Fig. 1b).

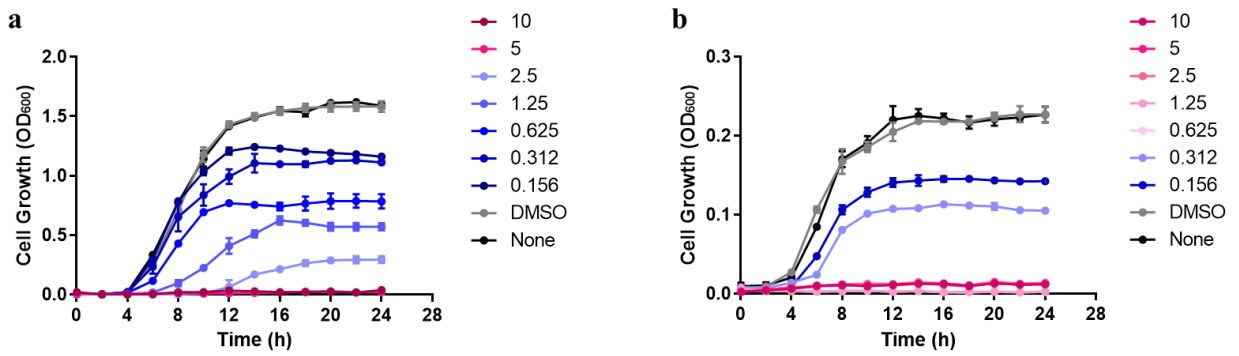


Fig. 1 Minimal Inhibitory Concentration (MIC) of carvacrol against planktonic cells of *P. aeruginosa* (a) and *E. faecalis* (b). Bacterial turbidity was measured at OD600 nm using a spectrophotometer at intervals of 2 h over a 24-h incubation period with different concentrations of carvacrol in three independent experiments. Control containing DMSO was used

In time killing assay, a bacterial population of approximatively 6 log CFU mL^{-1} was exposed to carvacrol MIC (Fig. 2). The results showed that by using the MIC values of carvacrol for each strain, no visible cells were detected after only 1 minute of treatment for *P. aeruginosa* strain (Fig. 2a) and 5 minutes of treatment for *E. faecalis* strain (Fig. 2b).

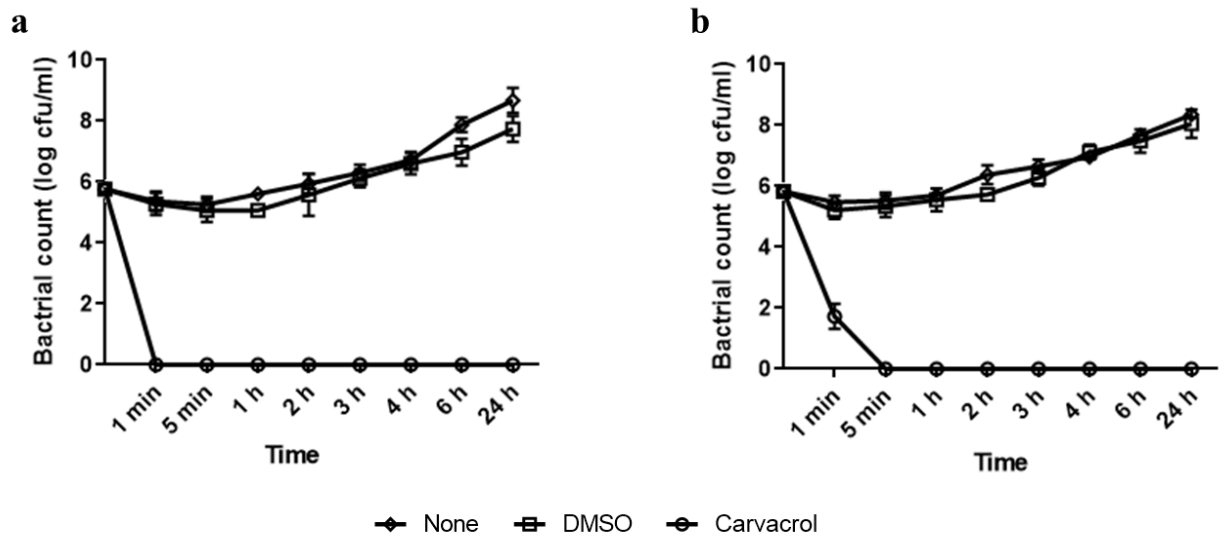


Fig. 2 Time-kill curve assay from agar plate count method of carvacrol at MICs against planktonic cells of *P. aeruginosa* (a) and *E. faecalis* (b). Control containing DMSO was used. Results are expressed as mean (±SD) of three independent experiments. "0" in the scale represents "below the detection limit"

Assessment of the minimal dispersive concentration and enzyme-action-time on biofilm developed on polystyrene microtiter plates

Our results showed that pepsin and trypsin disassemble 24 hours-biofilms of *P. aeruginosa* and *E. faecalis* developed in 96-well polystyrene plates in a dose- and time-dependent manner (Fig. 3). Specifically, after 1 h of treatment, pepsin and trypsin at 1 mg mL^{-1} detached 41 % and 50 % of *P. aeruginosa* biofilm, respectively (Fig. 3a-b), and 50 % and 48 % of *E. faecalis* biofilm, respectively (Fig. 3c-d). Results also showed that the use of extended dispersion time (2 h) and higher concentration (10 mg mL^{-1}) of pepsin and trypsin, resulted in similar dispersion of both bacterial strain' biofilms, and that the enzymes did not remove completely the biofilms from the polystyrene surface, regardless of the treatment time and enzymes concentration used.

In order to study the effect of pepsin and trypsin on the growth of bacterial strains, *P. aeruginosa* and *E. faecalis* were incubated in the presence of enzymes. The results showed that the growth of

both bacterial strains was not affected in the presence of the enzymes at concentrations up to 1 mg mL^{-1} ($p > 0.05$) (Fig. 4).

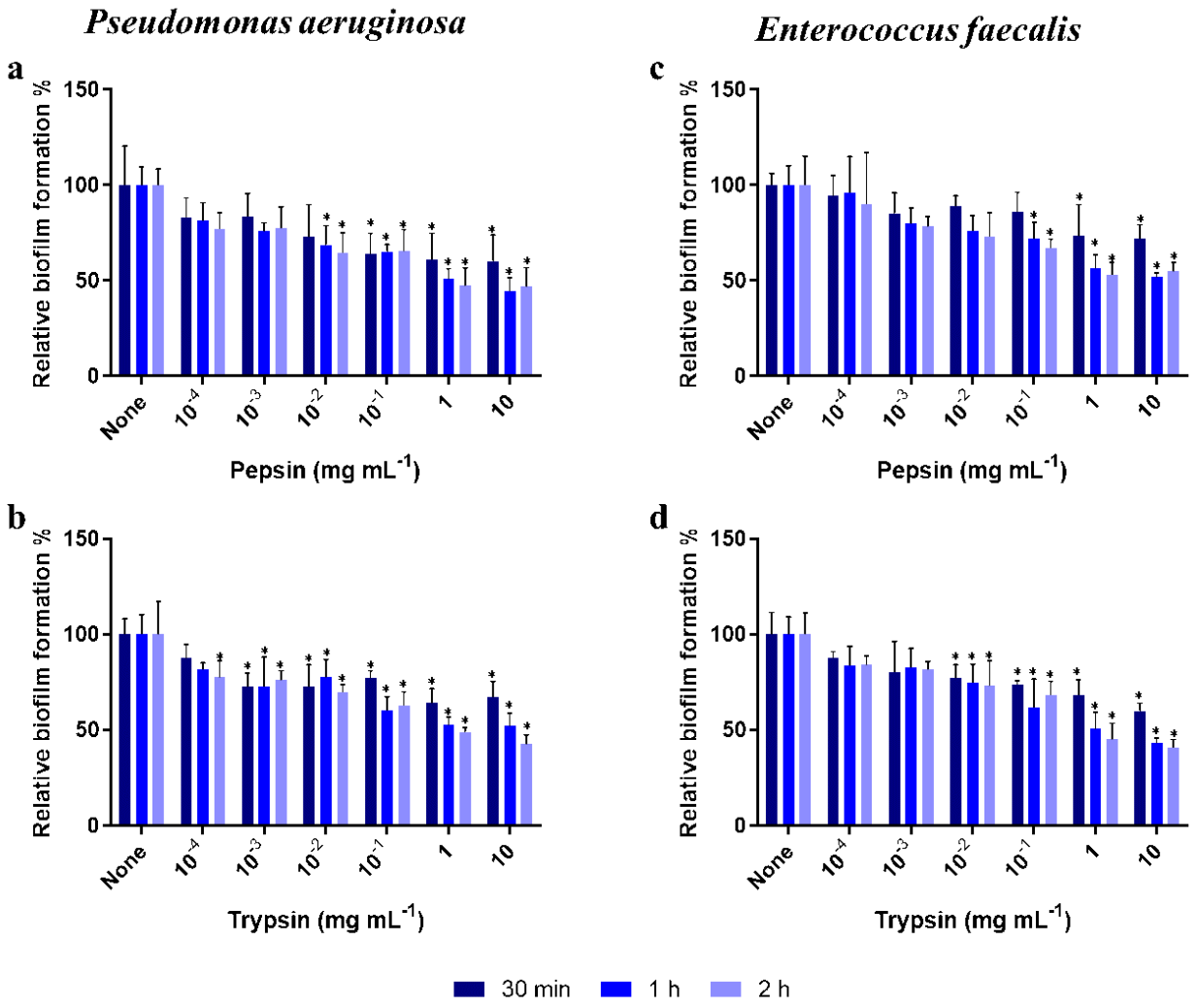


Fig. 3 Dispersal of established *P. aeruginosa* (a, b) and *E. faecalis* (c, d) biofilms by pepsin (a, c) and trypsin (b, d) using different concentrations (10^{-4} – 10 mg mL^{-1}) and different times of action (30 min - 1h - 2h). Biofilm dispersion was studied in 96-well polystyrene plates at 37 °C. Total biofilm formation was measured at OD600. Results are presented as the means (±SD) of three independent experiments. *$p < 0.05$ indicates significant difference compared with control using Tukey's

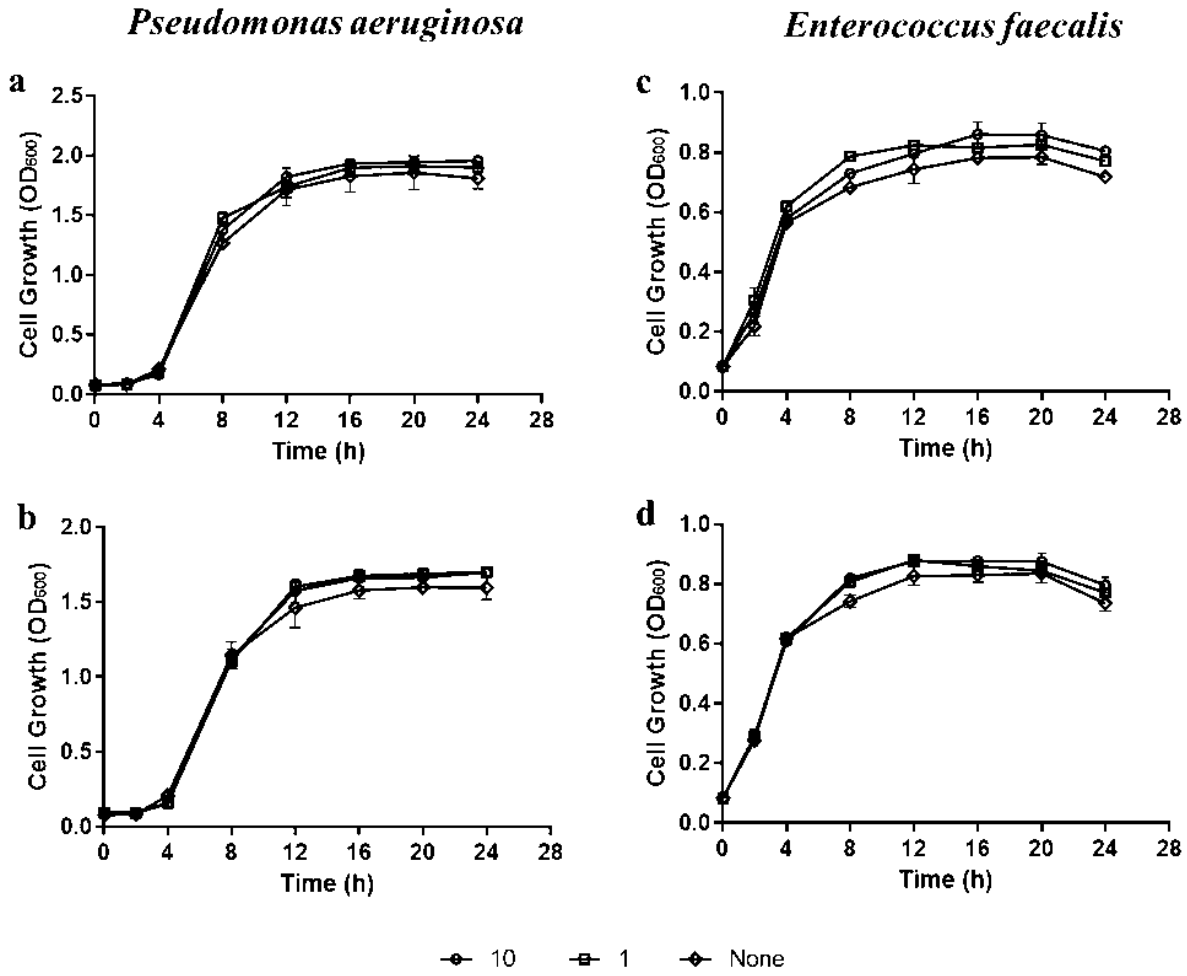


Fig. 4 The growth of *P. aeruginosa* (a, b) and *E. faecalis* (c, d) cells in the presence of pepsin (a, d) and trypsin (b, d) cultured at 37 °C for 24 h with shaking. Bacterial turbidity was measured at OD600 nm using a spectrophotometer at intervals of 2 h over a 24- h incubation period with different concentrations of enzymes in three independent experiments

Quantitative assessment of the combined effect of enzymes and carvacrol on biofilm developed on stainless steel

The single and combined antibiofilm effect of enzymes and carvacrol against the preformed biofilm of *P. aeruginosa* and *E. faecalis* strains, grown on stainless steel coupons, was studied using culturable count assay. The biofilms of both bacterial strains exhibited a bacterial biomass of approximately 7 log CFU mL^{-1} (Fig. 5). After 1 h of enzymatic treatment using pepsin or trypsin at the MDC level (1 mg mL^{-1}), individually or sequentially, a limited biofilm removal of both bacterial strains was shown (Fig. 5). However, *P. aeruginosa* and *E. faecalis* biofilms treated with carvacrol at ½ MICs (2.5 mg mL^{-1} and 0.3125 mg mL^{-1}, respectively) were significantly reduced. *P. aeruginosa* biofilm was reduced by 2 log CFU mL^{-1} ($p < 0.05$) after 1 min of treatment (Fig. 5a), and *E. faecalis* biofilm was reduced by 1 log CFU mL^{-1} ($p < 0.05$) after 5 min of treatment (Fig. 5b).

For *P. aeruginosa* biofilm, the combined treatment using enzymes followed by carvacrol showed a substantial reduction in the culturable cells (Fig. 5a). Interestingly, treatment with pepsin followed by carvacrol reduced biofilm biomass by 3.7 log CFU mL^{-1} ($p < 0.05$). After combined treatment using trypsin followed by carvacrol, the reduction of biofilm biomass was 2.8 log CFU mL^{-1} ($p < 0.05$). This reduction was more significant after sequential treatment with both enzymes followed by carvacrol treatment. Specifically, there was a synergistic significant inactivation of 4.7 log CFU mL^{-1} ($p < 0.05$) after treatment in the order trypsin, pepsin, and carvacrol (Fig. 5a).

For *E. faecalis* biofilm, the combined treatment of enzymes followed by carvacrol showed a significant inactivation with a maximum reduction of approximatively 2 log CFU mL^{-1} ($p < 0.05$) after treatment using pepsin followed by carvacrol (Fig. 5b). Moreover, the sequential treatment of both enzymes, in the order pepsin-trypsin or trypsin-pepsin, followed by carvacrol treatment showed a notable and almost equal reduction in biofilm biomass of 3.3 log CFU mL^{-1} ($p < 0.05$) approximatively (Fig. 5b).

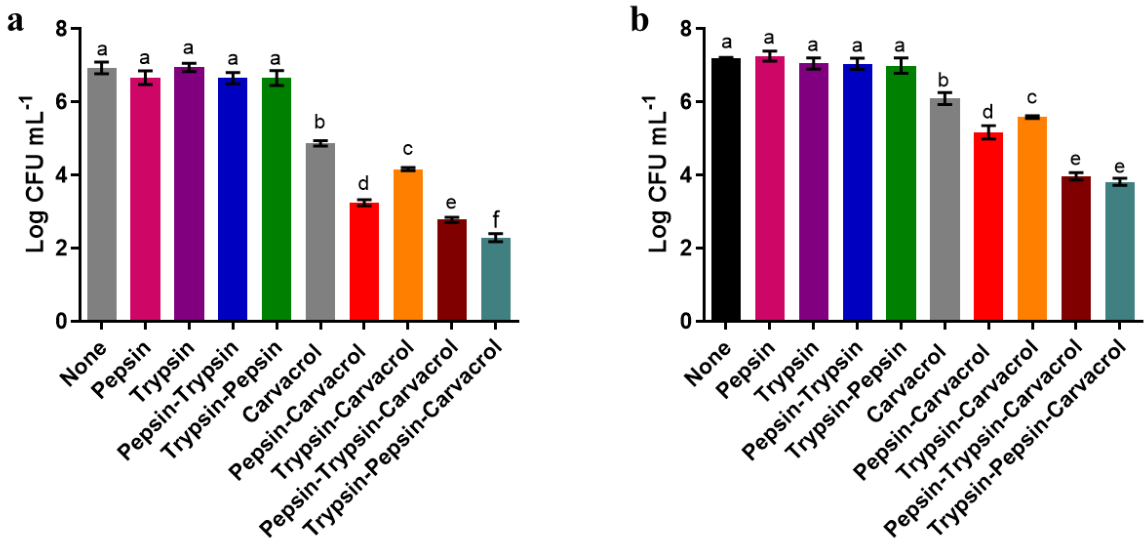


Fig. 5 The reduction (in log CFU mL^{-1}) of *P. aeruginosa* (a) and *E. faecalis* (b) biofilms after treatment with pepsin, trypsin, or sequential treatment of both at concentration of 1 mg mL^{-1} for 1 h each, or followed by treatment with carvacrol at ½ MICs of each strain for 1 min for *P. aeruginosa* and 5 min for *E. faecalis*. Biofilms were developed on stainless steel surface at 37 °C for 24 h. Results are presented as means (±SD) of three independent experiments. Different letters (a-d) indicate significant differences ($p < 0.05$) using Tukey's test

Qualitative assessment of the effect of enzymes and carvacrol on biofilms viability

Biofilms of *P. aeruginosa* and *E. faecalis* were stained with SYTO9 and propidium iodide (PI) and observed by epifluorescence microscopy after treatment (Fig. 6, Fig. 7). Results showed that the TS-treated controls of both bacteria, exhibited a thick biofilm of viable cells mainly stained by SYTO9 (green bacteria) with a few numbers of dead bacteria stained by PI (red bacteria). After treatment with pepsin or trypsin alone at the MDC values, the figures showed a reduction in biofilm biomass compared to the control, with a predominant SYTO9 staining (Fig. 6, Fig. 7). This reduction was more significant after sequential pepsin and trypsin treatment which showed a scattered bacterium with the presence of some non-dispersed bacterial clusters for *P. aeruginosa* biofilm (Fig. 6). Furthermore, for *E. faecalis* biofilm a thin layer of biofilm remaining was shown after this sequential treatment (Fig. 7). Results also showed that there was no significant difference in dispersion efficiency regardless the order of enzymes used.

After carvacrol treatment, the results showed a significant decrease in SYTO9 staining and increase in PI staining of the superficial layer of thick biofilm for both bacterial strains (Fig. 6, Fig. 7). However, after combined treatment using enzymes followed by carvacrol, the results showed a substantial reduction in biofilm biomass as well as the number of viable cells. This combined treatment showed the same biofilm reduction using either pepsin or trypsin followed by carvacrol for *E. faecalis* strain (Fig. 7). However, for *P. aeruginosa*, results showed that the combined treatment using pepsin followed by carvacrol induced a more substantial reduction in biofilm biomass than the combined trypsin and carvacrol treatment (Fig. 6). Furthermore, after sequential enzymatic treatment, in the order pepsin-trypsin or trypsin-pepsin, followed by carvacrol, the biomass of both biofilms and the number of viable cells were further decreased, and the remaining thin biofilms were predominantly stained by PI (Fig. 6, Fig. 7).

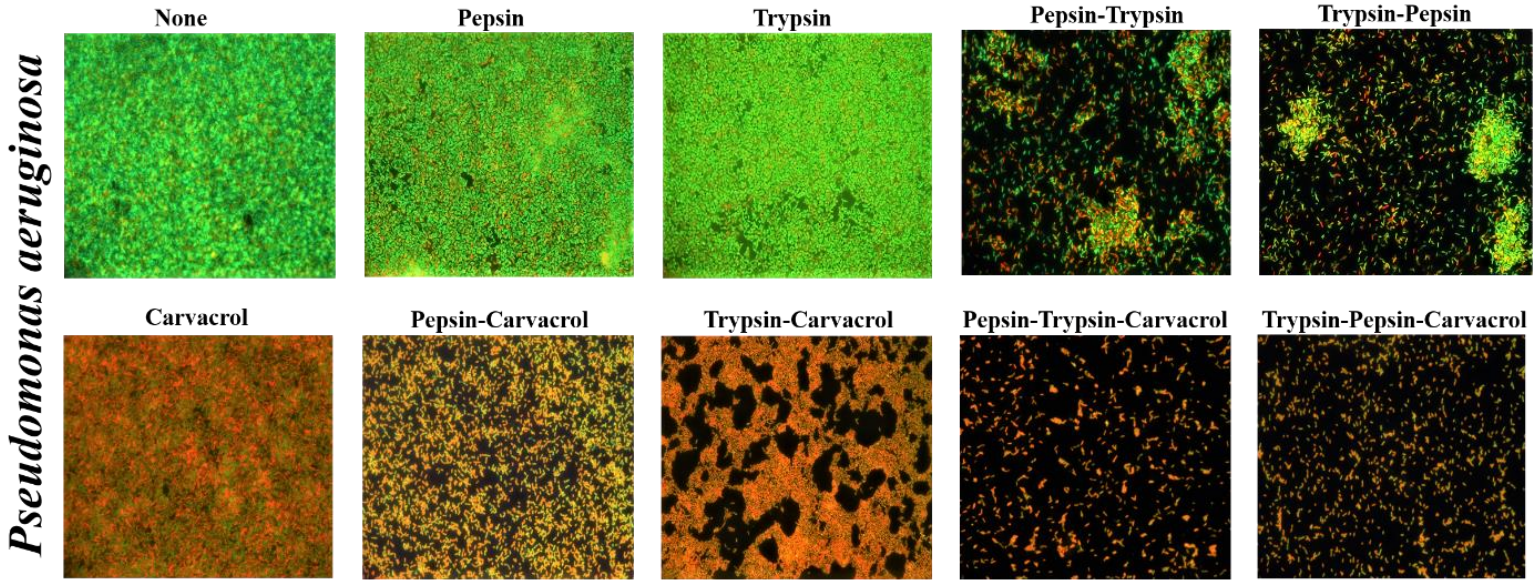


Fig. 6 Epifluorescence microscopic images of *P. aeruginosa* biofilms after treatment with pepsin, trypsin, or sequential treatment of both at concentration of 1 mg mL^{-1} for 1 h each, or followed by treatment with carvacrol at ½ MIC for 1 min. Cells were visualized after staining with SYTO-9 (green fluorescence for living bacteria) and propidium iodide (red fluorescence for dead bacteria). Control represents biofilm treated with tryptone salt broth

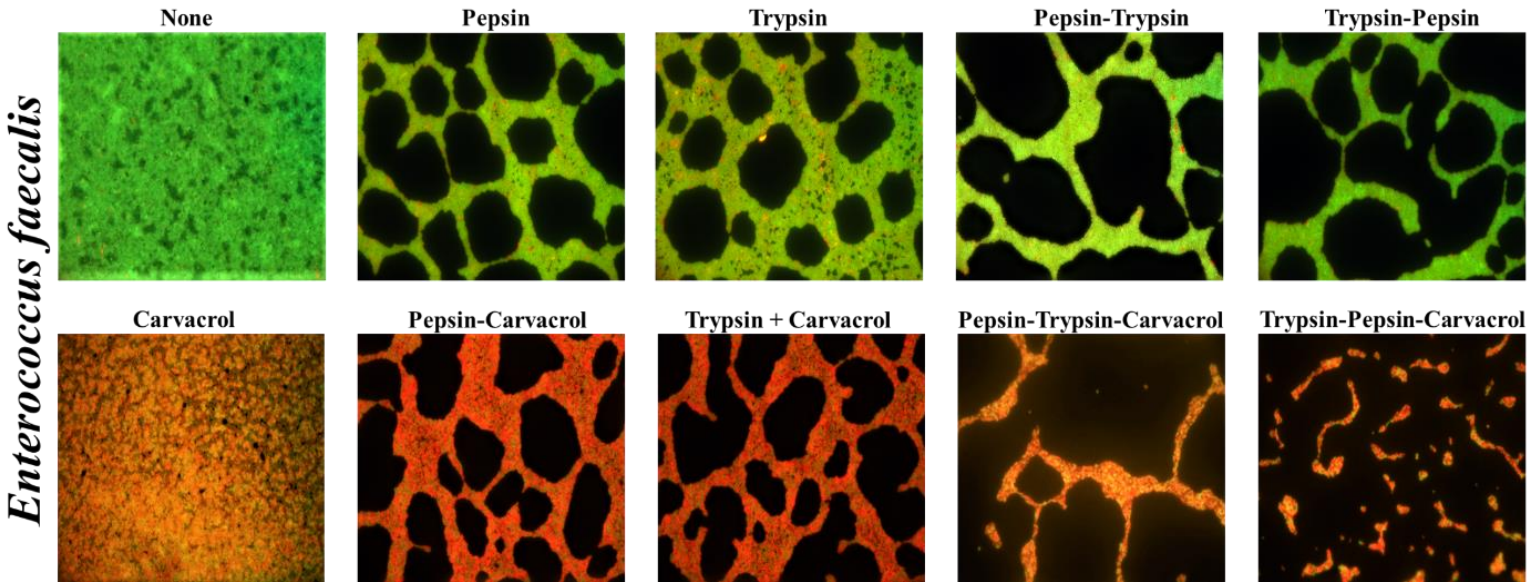


Fig. 7 Epifluorescence microscopic images of *E. faecalis* biofilms after treatment with pepsin, trypsin, or sequential treatment of both at concentration of 1 mg mL^{-1} for 1 h each, or followed by treatment with carvacrol at ½ MIC (0.312 mg mL^{-1}) for 5 min. Cells were visualized after staining with SYTO-9 (green fluorescence for living bacteria) and propidium iodide (red fluorescence for dead bacteria). Control represents biofilm treated with tryptone salt broth

Effect of enzymes and carvacrol on the morphology of biofilm cells

The structural morphology of the biofilm cells treated with pepsin, trypsin, and carvacrol was investigated using SEM and compared with untreated cells (Fig. 8). Fig. 8 shows that untreated *P. aeruginosa* and *E. faecalis* cells had normal bacilliform and coccoidal forms, respectively. Furthermore, enzyme treatment showed no alteration in their cell morphology and the bacterial cells remained intact. However, distorted and rough surface cells were observed in both biofilms after carvacrol treatment. For *P. aeruginosa*, a complete constriction and deflation of the cells was observed after carvacrol treatment. In addition, *E. faecalis* cells were also shown to be injured and deformed with a hollow cell wall after this treatment.

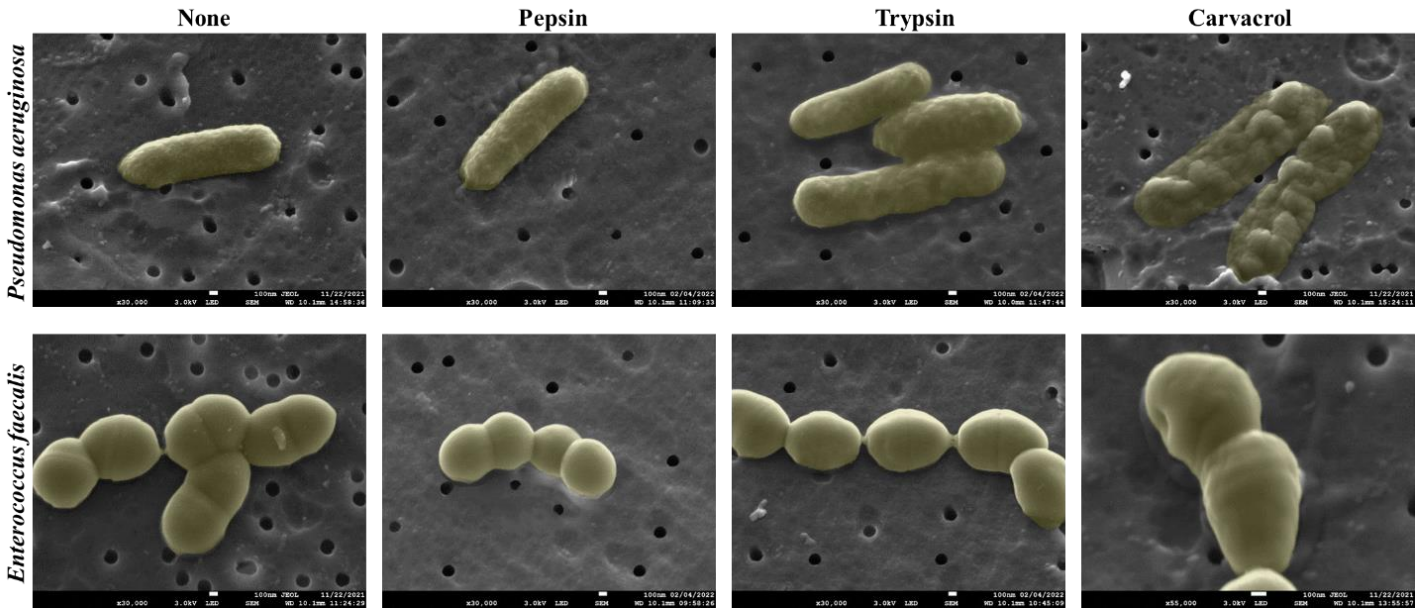


Fig. 8 SEM micrographs of *P. aeruginosa* and *E. faecalis* biofilm cells after treatment with pepsin and trypsin at the minimal dispersive concentration (1 mg mL^{-1}), and carvacrol at the MICs. Control represents biofilm cells treated with tryptone salt

Discussion

The EPS matrix serves as a 3D scaffold that maintains mechanical stability, cohesion and protection against antimicrobial therapies and host effectors (Jiang et al. 2020). The use of matrix-degrading enzymes has recently been investigated for potential applications in biofilm control due to their ability to disrupt biofilm matrix and return bacteria to the more vulnerable planktonic state. The disruption of this matrix can also deplete the viscoelastic properties to further weaken biofilm cohesion and improve antimicrobial efficacy (Meireles et al. 2016). Thus the use of enzymes in combination with biocides will enhance the accessibility of biocides to matrix-embedded biofilm cells, and therefore reducing the dose of disinfectants needed and decreasing environmental pollution (Rodríguez-López et al. 2017). In this study, we assess pepsin and trypsin for their effectiveness in disrupting preformed biofilm of *P. aeruginosa* and *E. faecalis*. These two proteolytic enzymes are mammalian digestive enzymes, widely available and produced on an industrial scale for food applications.

The biofilm matrix-proteins play an important role in biofilm stability and architecture in several bacterial strains (Taglialegna et al. 2020). Recently, proteases have been widely reported to be among the main enzymes used for their anti-biofilm effect (Molobela 2010; Brindle et al. 2011; Fagerlund et al. 2016; Mohamed et al. 2018; Lim et al. 2019). Several studies have demonstrated the high efficacy of trypsin in disrupting and controlling biofilm formation by *P. aeruginosa, Streptococcus mitis, Actinomyces radicidentis* and *Staphylococcus epidermidis* (Chaignon et al. 2007; Patterson et al. 2007; Niazi et al. 2014; Banar et al. 2016; Fagerlund et al. 2016). Other research has demonstrated the high potential of pepsin to remove multi-species biofilms (Marcato-Romain et al. 2012). The proteins in the EPS matrix are considered as essential components of *P. aeruginosa* and *E. faecalis* biofilms, contributing to the pathogenesis and maintenance of the biofilm (Matsukawa and Greenberg 2004; Tendolkar et al. 2004; Borlee et al. 2010; Zhang et al. 2015; Taglialegna et al. 2020).

The outer membrane proteins such as OmpA have been identified as the most abundant proteins in the biofilm of *P. aeruginosa* and play a crucial role in biofilm formation and stability (Schooling and Beveridge 2006; Toyofuku et al. 2012; Zhang et al. 2015). In addition, Taglialegna et al. (2020) have shown that enterococcal surface protein (Esp), a Bap-orthologous protein (biofilm-associated protein) widely produced by *E. faecalis*, contributes prominently in matrix construction and biofilm formation. Our results on the biofilm detachment grown on polystyrene microtiter strongly suggested that the used proteolytic enzymes interact with these major protein components of *P. aeruginosa* and *E. faecalis* biofilms. Furthermore, results showed that these enzymes were able to destroy the biofilm after 1 hour of exposure, which is in agreement with other findings that have used one hour of enzyme treatments to remove bacterial biofilms (Lequette et al. 2010; Banar et al. 2016; Lim et al. 2019).

The single and combined effect of enzymes and carvacrol were studied against *P. aeruginosa* and *E. faecalis* biofilms growing on stainless steel surfaces using cell counts and microscopic analysis. The MDC values and contact times of both enzymes were used for the enzymatic treatments. As well as the ½ MIC values and contact times of carvacrol against bacterial strains were used for antimicrobial treatments since the ½ MIC was sufficient for a combined treatment of carvacrol and enzymes, providing a good demonstration of enzymes action. The use of MIC values resulted in a

reduction of the biofilm biomass in a similar manner to the combined use of enzymes and carvacrol (data not shown). Thus, these results provide a good demonstration that enzymes were able to reduce the concentration of biocide used.

After treatment with enzymes, individually or sequentially, the number of culturable cells in *P. aeruginosa* and *E. faecalis* biofilms was not significantly affected. However, the epifluorescence analysis demonstrated that pepsin and trypsin were able to disrupt biofilm of both bacterial strains, and this dispersion was more significant after sequential treatment using the two enzymes. The difference in the dispersal pattern between the two biofilm strains observed may be explained by the fact that *P. aeruginosa* is a bacterial strain that can exist singly. In contrast, *E. faecalis* is a bacterium that does not exist alone, it is always grouped in chains or pairs. The results also showed that enzymes were effective in disrupting biofilms without causing cell death as the remaining biofilms were predominantly stained by SYTO9. These microscopic observations explain why the number of culturable cells was not affected after enzymatic treatments since enzymes are catalytic agents that do not exhibit antimicrobial activity and therefore destabilize biofilms without affecting cell viability. In addition, SEM analysis showed that both enzymes did not affect the morphological structure of *P. aeruginosa* and *E. faecalis* biofilm cells (Fig. 8). Furthermore, our results are in agreement with the bactericidal assessment of the enzymes, which showed that the growth of bacterial strains was not affected in the presence of pepsin and trypsin. Our results are consistent with a previous study showing that the use of proteolytic enzyme, such as proteinase K for individual or sequential treatment with other types of enzyme, did not significantly affect the number of viable culturable cells in *Escherichia coli* O157:H7 biofilm grown on stainless steel surface, and that proteinase K had no effect on the growth rate or viability of *E. coli* cells (Lim et al. 2019). However, Zhang et al. (2016) demonstrated that the presence of trypsin hindered the growth of *Streptococcus dysgalactiae* (ATCC12388) and *Streptococcus agalactiae* (CVCC586). In addition, chymotrypsin and the trypsin/chymotrypsin complex have shown variable inhibitory effects on several bacterial strains, as they can hydrolyze the outer membrane proteins of bacteria, cause damage to the surface structures integrity, and result in leakage of intracellular material. Carvacrol is a monoterpene phenol abundantly found in the essential oils of many aromatic plants, widely known for its broad antimicrobial activity against foodborne or pathogenic microorganisms

(Esteban and García-Coca 2017; Mauriello et al. 2021). The antimicrobial activity of carvacrol is mainly due to the destruction of microbial integrity, causing cell death (Zhang et al. 2018; Fang et al. 2019; Mauriello et al. 2021). In addition, carvacrol can interact with the DNA of microorganisms, affecting the gene expression of bacteria and thus reducing their virulence factors such as toxin production and biofilm formation (Siroli et al. 2018; Ghafari et al. 2018; Liu et al. 2019). Our study demonstrates that after carvacrol treatment, the number of culturable cells of both biofilm strains were significantly reduced. In addition, epifluorescence microscopy observations showed that the number of PI-stained cells (dead cells) in both biofilms was significantly increased after carvacrol treatment (Fig. 6, Fig. 7). SEM electromicrographs also showed that carvacrol induced many cell surface deformations and abnormalities in the both biofilm bacterial cells. These results clearly demonstrate the strong antimicrobial activity of carvacrol. Several previous studies have shown the high efficacy of carvacrol in controlling biofilms grown on stainless steel surfaces (dos Santos Rodrigues et al. 2017; Tapia-Rodriguez et al. 2017; Engel et al. 2017; Trevisan et al. 2018). However, previous studies have shown that the use of an essential oil alone did not cause the complete disruption of *P. aeruginosa* (Kalia et al. 2015) and *E. faecalis* biofilms (Negreiros et al. 2016). Additionally, many studies demonstrate that the biofilm matrix principally contributes to the increased tolerance and antibiotic resistance of biofilms compared to planktonic cells (Flemming et al. 2016; Fulaz et al. 2019; Pinto et al. 2020). Based on these studies, it is considered that the reduced disinfection efficiency of essential oils may be due to protective barrier effect of the EPS matrix. Hence, the degradation of EPS matrix would be a suitable strategy to improve the effectiveness of essential oils.

Our results demonstrated that carvacrol, when combined with proteolytic enzymes, exhibits increased biofilm disruption, since *P. aeruginosa* and *E. faecalis* biofilms were reduced more by pepsin or trypsin treatment followed by carvacrol treatment than biofilms treated with carvacrol alone. This reduction of both biofilms was significantly more important after sequential enzymes treatment followed by carvacrol treatment compared to treatment using a single enzyme followed by carvacrol. Such increased efficiency is also evident in the epifluorescence microscopy analysis which showed that the combined treatment using enzymes, individually or sequentially, followed by carvacrol, exhibited a synergistic effect of the dispersive activity of enzymes and the

antimicrobial activity of carvacrol since biofilms were more reduced in terms of biomass and viability. It was confirmed from our results that pepsin or trypsin treatment combined with carvacrol can synergistically improve biofilm disinfection. In addition, Cui et al. (2016) showed that *E. coli* biofilm was greatly reduced by essential oil in the presence of protease. Other studies have been performed using proteases and antimicrobials in tandem. They have shown that protein removal destabilizes and weakens the biofilm matrix and thus improves the susceptibility of Gram-negative and Gram-positive pathogens to antimicrobials (Rodríguez-López et al. 2017; Lim et al. 2019; Saggu et al. 2019; Baidamshina et al. 2021).

Pepsin and trypsin are both mammalian digestive enzymes requiring specific amino acids in the polypeptide chain to hydrolyze proteins. Pepsin splits the polypeptide chain of at least six different amino acids between two hydrophobic, preferentially aromatic, residues, whereas, trypsin cleavage requires the presence of specific basic amino acids, such as lysine or arginine (Marcato-Romain et al. 2012). Based on this specificity, pepsin showed more interesting results in reducing both treated biofilms when combined with carvacrol especially for *P. aeruginosa* biofilm (Fig. 5a). This improved action was also demonstrated in epifluorescence microscopy analysis showing that the biofilm removal of *P. aeruginosa* was more significant after combined treatment using pepsin with carvacrol than trypsin enzyme (Fig. 6). Similarly, Marcato-Romain et al. (2012) also demonstrated that pepsin was significantly more effective than trypsin in disrupting multi-species industrial biofilms. Furthermore, the difference in the efficiency of inactivation of *P. aeruginosa* biofilm depending on the order of treatment of the two proteolytic enzymes prior to treatment with carvacrol suggests that this order is important for efficient inactivation of *P. aeruginosa* biofilm cells (Fig. 5a). It might also be a reflection of the spatial or structural distribution of biofilm components.

Indeed, these two proteolytic enzymes can cause structural defects in the biofilms and degrade the barrier properties by possible interacting with proteins including outer membrane proteins for *P. aeruginosa*, and Esp for *E. faecalis*, thus facilitating the penetration of carvacrol and reducing the survivability of the cells. Hence, our findings suggest that proteins can be good targets for removal to allow effective penetration of disinfectants such as carvacrol to inactivate *P. aeruginosa* and *E. faecalis* cells in the biofilm.

In fact, approaches using enzymes to control biofilms have drawbacks in terms of cost, high dependence of activity on environmental factors and stability, as they can self-degrade causing instability (Cordeiro and Werner 2011). In addition, the high volatility and low water solubility of essential oils can minimize the antibacterial activity of these components (Dorman and Deans 2000; Liu et al. 2018). Therefore, as a solution, enzymes and carvacrol can be stabilized by encapsulation into abiotic materials. Encapsulation can enhance the anti-biofilm activity of these active agents, improve their stability and may be cost-effective if the stability achieved is sufficiently maintained for repeated use.

Conclusion

The complexity of the EPS matrix provides a preferential environment for the sessile cells embedded in biofilm, making them more resistant to various stresses, and thus more difficult to eradicate. Matrix-degrading enzymes that target different components of the biofilm matrix are being increasingly investigated for application to control biofilms. Although some enzymes are efficient as dispersing agents, it is also evident that a combination of enzymes with an antimicrobial can act in a synergistic manner to control microbial biofilms. Thus, our biofilm treatment strategy was based on the combined effect of the dispersive activity of protease, targeting the matrix-associated proteins, and the antimicrobial activity of carvacrol, targeting the biofilm embedded cells. Our findings demonstrated that *P. aeruginosa* and *E. faecalis* biofilms were further reduced after treatment using enzymes in combination with carvacrol compared to carvacrol treatment alone. Furthermore, sequential treatment of pepsin and trypsin followed by carvacrol can synergistically inactivate cells in both biofilms. The combined use of these natural agents would further reduce the use of chemical agents, energy costs and water consumption for biofilm control. This work suggests that the combined treatment using enzymes and essential oil may be a promising technology for the eradication of microbial biofilm infections.

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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 state plays a major role in the resistance and virulence factors 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. Epifluorescence microscopy analysis allowed visualization of the dispersive activity of microencapsulated proteases and the lethal activity of ME-CARV against bacterial biofilms. Moreover, cell count analysis proved the synergistic activity of combined enzymes and carvacrol treatment, since biofilms were further reduced after combined treatment than that of ME-CARV or enzymes alone. Furthermore, our findings showed that sequential treatment of the two proteases followed by ME-CARV resulted in more efficient biofilm removal. This study indicates

that the combination of microencapsulated proteases with ME-CARV could be useful for effective control of *P. aeruginosa* and *E. faecalis* biofilms.

Keyword: Biofilm; microencapsulation; proteases; carvacrol; *Pseudomonas aeruginosa*; *Enterococcus faecalis.*

Introduction

One of the major problems of industrial, domestic and collective hygiene is the ability of pathogenic bacteria to adhere to surfaces and form biofilms. *Pseudomonas aeruginosa* has become an important model organism for the study of bacterial biofilm formation. This bacterium is an opportunistic pathogen for humans that can induce life-threatening infections in patients with compromised immune systems (Moradali et al. 2017). In addition, the implication of *P. aeruginosa* in foodborne infections and food spoilage has been also reported (Raposo et al. 2016). Another opportunistic biofilm-forming pathogenic bacterium is *Enterococcus faecalis*, which can survive under arduous conditions, including high concentrations of salt and a wide range of temperature (10 °C to 45 °C) (Arias and Murray 2012). It is widely spread in nature and in the gastrointestinal tract of humans, animals and insects and represents an important pathogen of health-care associated infections (Tornero et al. 2014; Shridhar and Dhanashree 2019).

In biofilms, bacterial cells secret extracellular polymeric substances (EPS) consisting mainly of polysaccharides, proteins, DNA and lipids, and form a viscous film enclosing the bacterial cells (Simões et al. 2010; Flemming and Wingender 2010). The structurally and functionally complex structure of EPS matrix plays a major role in biofilm formation, survival, and development. It provides not only a barrier protection against external stresses, but also a nutrient and enzyme source, and an intercellular connective. Furthermore, the specific characteristics of matrix ensure the high tolerance and antimicrobial resistance of biofilms (Pinto et al. 2020). Consequently, a new strategy for the efficient inactivation of bacterial cells in biofilms is needed. Recently, approaches using enzymes that degrade EPS matrix have been investigated to remove biofilms (Kim et al. 2013). Many studies have demonstrated the high destructive and destabilizing power of protease-based enzymes against the biofilm matrix of several bacterial strains (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 may often be insufficient to eradicate the entire biofilm. In addition, treatment with enzymes may result in contamination of other areas with the dispersed bacteria and thus allow the redevelopment of neoformed biofilms. It is therefore essential to use a combination of enzymes with other active agents (Hurdle technology) in order to attack different targets in biofilms (EPS matrix and biofilm cells). The combination of enzymes with biobased antimicrobials would be a promising approach to control biofilms in such a way that the enzymes destroy and destabilize the EPS matrix of the biofilm, so that the cells protected by the matrix are more effectively killed by the antimicrobials. Numerous studies have shown that the use of EPS-degrading enzymes in combination with biocides is a promising approach 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). Currently, biosourced antimicrobial substances have attracted the attention of the public because of their high efficacy, safety, and non-toxic effect (Nazzaro et al. 2013). Carvacrol, a monoterpenoid phenol, is a major component of *Origanum vulgari* essential oil (EO), and can be found in several other EOs such as *Thymus vulgaris* and *Satureja bachtiaria* (Trevisan et al. 2018). Carvacrol could be used as an anti-inflammatory and antifungal agent (Ahmad et al. 2011; Lima et al. 2013), and has a broad antimicrobial spectrum (Nostro et al. 2004; Burt 2004; Esteban and García-Coca 2017). Several studies have demonstrated its antibiofilm 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 solubility of these active compounds can also minimize their antimicrobial efficacy (Shan et al. 2007; Gharsallaoui et al. 2007; Liu et al. 2018). In addition, the activity of enzymes is highly influenced by environmental factors such as temperature and pH, and is only optimal under restricted conditions. Another disadvantage of using enzymes is the self-degradation, causing instability (Cordeiro and Werner 2011).

Strategies such as encapsulation of these active molecules could be a good tool to overcome these issues. Microencapsulation is a good tool to increase stability and reduce water immiscibility of EOs and to 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 physico-chemical interactions with the biofilm matrix components, often associated with the decrease of 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 realized 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 *E. faecalis* (isolated from French cheese) and *P. 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 (Baupte, 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). Trypsin, carvacrol (98% purity), analytical grade imidazole ($C_3H_4N_2$), 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 pepsin solution, potassium phosphate buffer (PPB; 100 mM, pH 7.6) was used to prepare trypsin solution, and distilled water was used in the preparation of the all-other solutions.

Microencapsulation of enzymes and carvacrol

Imidazole-acetate buffer was used as a buffer for enzyme microencapsulation. Imidazole-acetate buffer solutions (5 mmol L^{-1}) were obtained by dispersing specified amounts of imidazole and acetic acid in distilled water. To prepare carvacrol emulsions, carvacrol and sodium caseinate were dissolved in water then mixed with maltodextrin powders and homogenized (IKA T 25 Ultra Turrax) at 18 000 rpm during 5 min to obtain spray-drying feed emulsions. To prepare enzyme solutions, pepsin or trypsin was mixed with pectin and maltodextrin powders in imidazole-acetate buffer. 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 lab-scale device equipped with a 0.5 nm nozzle atomizer (Mini Spray-Dryer Buchi B-290, Switzerland). 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.

Zeta potential analysis

The electric charge of enzymes at different pH values and that of carvacrol emulsion droplets at pH 7 were measured by Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). If required, the samples were diluted with water or imidazole-acetate buffer of corresponding pH. For all tests, at least three repetitions were performed. The average of the Zeta potential values (ZP) was obtained from the instrument.

Scanning electron microscope observation of particles

To analyze the inner and outer structures of capsules prepared, the scanning electron microscope (SEM-JEOL-JSM-7800FLV, Japan) was used. The outer structures of the capsules containing carvacrol and enzymes was studied by deposing a simple layer of each dry capsule to a sample holder with a double-sided adhesive (Agar Scientific Oxford). For the study of the inner structure of the capsules, 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. Samples were critical point dried and coated with a thin carbon film before SEM analysis. Microscopy was at 3 kV.

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 were 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, Elma®).

Stainless steel (SS) slides (304L, Equinox, France) of 41 mm diameter and 1 mm thickness were used for deposition of the biofilm. 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 minutes 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.

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 (10^7 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 encapsulated carvacrol and enzymes for treatment of biofilms

The effect of a single and combined antimicrobial and enzymes treatment against 24-hour-old *P. aeruginosa* and *E. faecalis* biofilms was evaluated by quantification of biofilm cells assay. For enzymes treatment, rinsed biofilms were treated with 3mL 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 ½ Minimal inhibitory concentration (MIC) of ME-CARV for each strain) and incubated for 1 min for *P. aeruginosa* and 5 min for *E. faecalis* at 20 °C (corresponding to the times kill of ME-CARV). 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 control for enzymes, and biofilm treated with tryptone salt broth (TS) served as 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, 5 min, 25 °C) (Elmasonic S60H, Elma, Germany). Then the pots were vortexed again for 30 s. Each sample was serially diluted in TS broth, plated on Tryptic Soy agar (TSA; Biokar Diagnostics, France), and incubated for 24 h at 37 °C to count culturable cells attached to the slides. Results represent the mean of three independent experiments.

Epifluorescence microscopy analysis

In order 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, 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 by tryptone salt broth. The treated biofilms were stained with 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 for at 37 °C for each treatment. Control biofilms were treated by tryptone salt broth. Treated biofilms were stained with Acridine Orange 0.01% (w/v) (Sigma Aldrich, Saint-

Quentin Fallavier, France) (AO) for 15 min in the dark. After staining of sessile cell biofilms, the slides were rinsed with ultrapure water and allowed to air dry in the dark. Then, visualization by epifluorescence microscopy was performed using an epifluorescence microscope (Olympus BX43, Germany). For Live Dead staining, green cells were considered as viable cells and red cells as dead cells.

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

To evaluate the charge of enzymes under bacterial cell and biofilm treatment conditions, the zeta

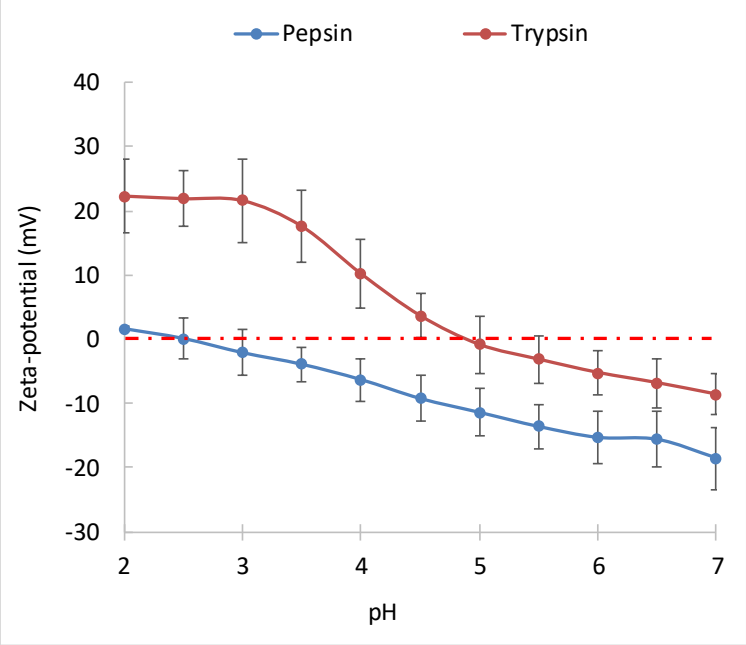

Fig 1. Zeta-potential of pepsin and trypsin in imidazole–acetate buffer at different pH.

potential of enzymes as a function of pH was measured to determine the pH value to be selected for each buffer. Using buffer with a pH higher than isoelectric point of the enzyme ensures that the enzyme will be negatively charged and therefore soluble in this buffer. The results (Figure 1) showed that the zeta potential values of the molecules were highly dependent on pH, and that the charge of the molecule shifts from positive to negative with increasing pH. The point at which the overall charge of the molecule is neutral was approximatively 5 for trypsin and 2.5 for pepsin. This difference in electrical charge between the 2 enzymes could have different consequences on the interactions between the enzymes and the target biofilms. Regarding carvacrol, 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 zeta potential measured for carvacrol emulsion was -24.39 mV.

Morphology and structure of spray-dried microcapsules

Figure 2 shows the scanning electron microscopy micrographs of microcapsules obtained by spray-drying carvacrol and enzymes solutions. The observation of the outer structure of microcapsules showed that all microcapsules consisted of spherical and well-separated particles with non-uniform diameter (Figure 2Aa, Figure 2Ba and Figure 2Ca). In addition, Figure 2Ab, Figure 2Bb and Figure 2Cb show that all microcapsules had blunt shape and generally embossed surface, with the presence of a few small shrunken particles with rough surfaces. The observation of the inner structure show that microcapsules had a central void (Figure 2Ac, Figure 2Bc and Figure 2Cc). Moreover, Figure 2Ad shows that carvacrol microcapsules had a thick, rough and hollow wall matrix with an obvious encapsulated core material retained inside. However, Figure 2Bd and Figure 2Cd show that the microcapsules of enzymes (ME-PEP, ME-TRYP) had a thick, smooth and homogeneous wall matrix.

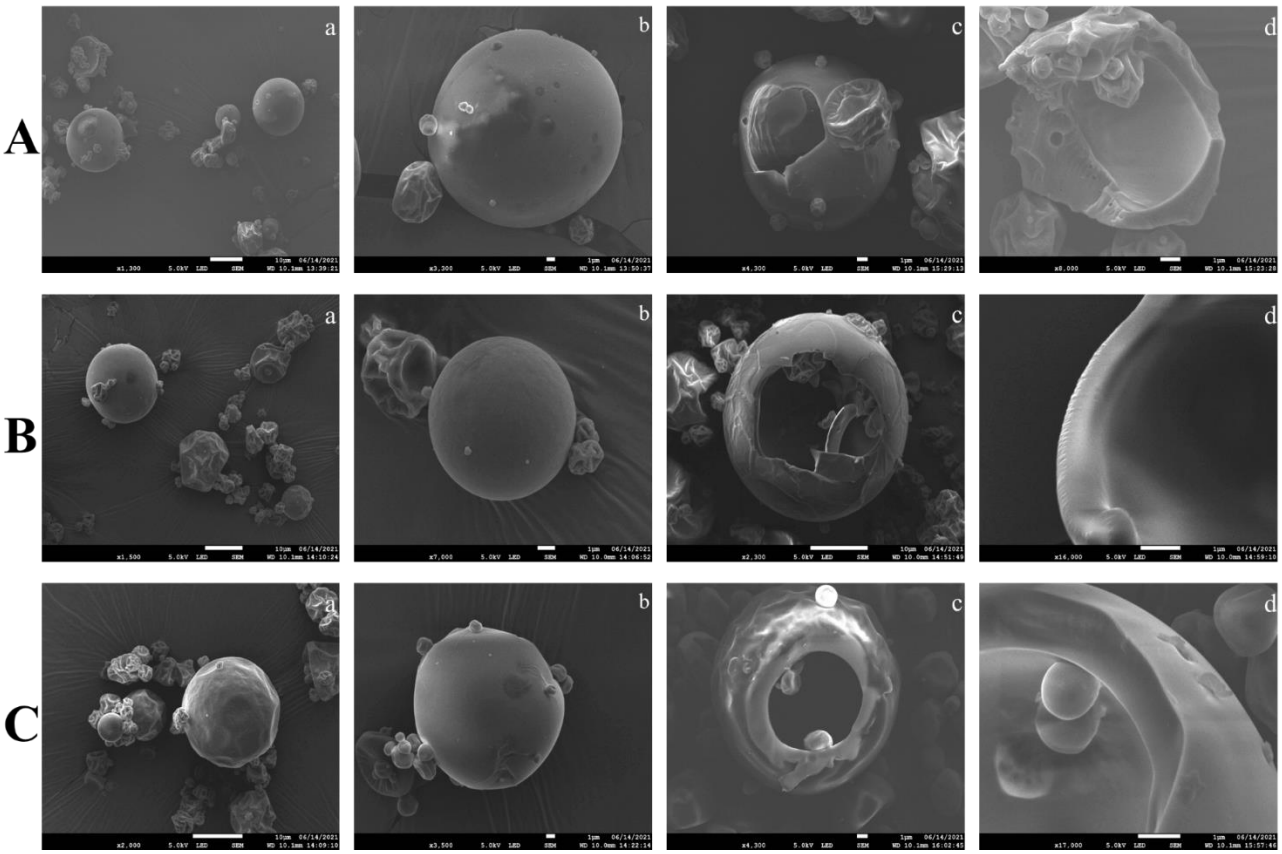

Fig 2. Scanning electron micrographs of Carvacrol (A), Pepsin (B) and Trypsin (C) spray-dried microcapsules. Overview of a multitude microcapsules (a); Outer morphology of microcapsules (b); microcapsule void (c) and Inner structure of microcapsules (d).

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. Therefore, experiments have been conducted to decrease the concentration of carvacrol at a minimum level but still active towards *P. aeruginosa* and *E. faecalis* biofilms. The results demonstrated that the use of ½ MIC for each strain showed a significant reduction of biofilms. Hence, the antimicrobial activity of ME-CARV using ½ MIC for each strain was investigated by direct analysis using epifluorescence microscopy. *P. aeruginosa* and *E. faecalis* biofilms were stained with SYTO9 and propidium iodure (PI). For both bacteria, results showed that the control treated with TS presented a biofilm composed of a biomass 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 against biofilms (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 3).

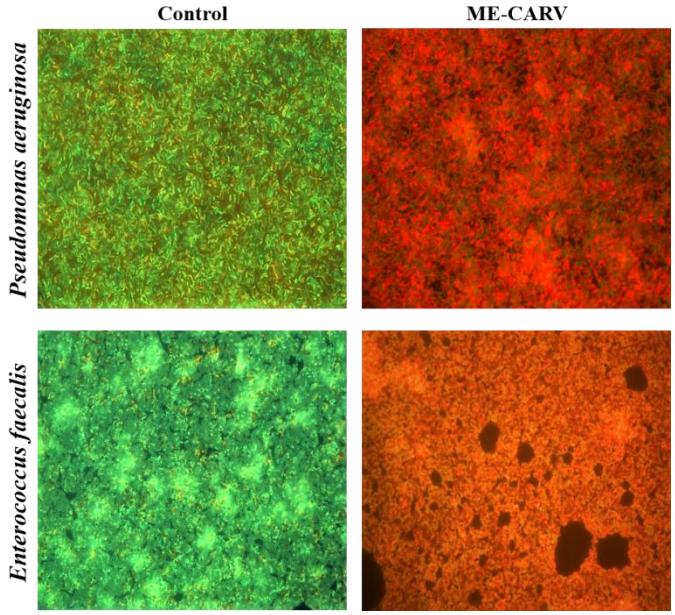

Fig 3. Epifluorescence microscopy images of *P. aeruginosa* and *E. faecalis* biofilms stained by Live Dead kit after treatment by microencapsulated carvacrol (ME-CARV). Control biofilms were treated by tryptone salt broth.

Dispersive enzymes activity determination

To study the dispersive activity of the two microencapsulated enzymes, after individual or sequential treatment, the biofilms were stained by AO and observed by epifluorescence microscopy. Figure 4 shows that the control biofilm of *P. aeruginosa* and *E. faecalis* exhibited a thick biofilm 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 biomasses compared to the control, as shown in Figure 4. This reduction was more significant after treatment of biofilms with enzymes sequentially (ME-PEP followed by ME-TRYP or ME-TRYP followed by ME-PEP) which showed a thin layer of biofilm remaining of both bacterial strains (Figure 4).

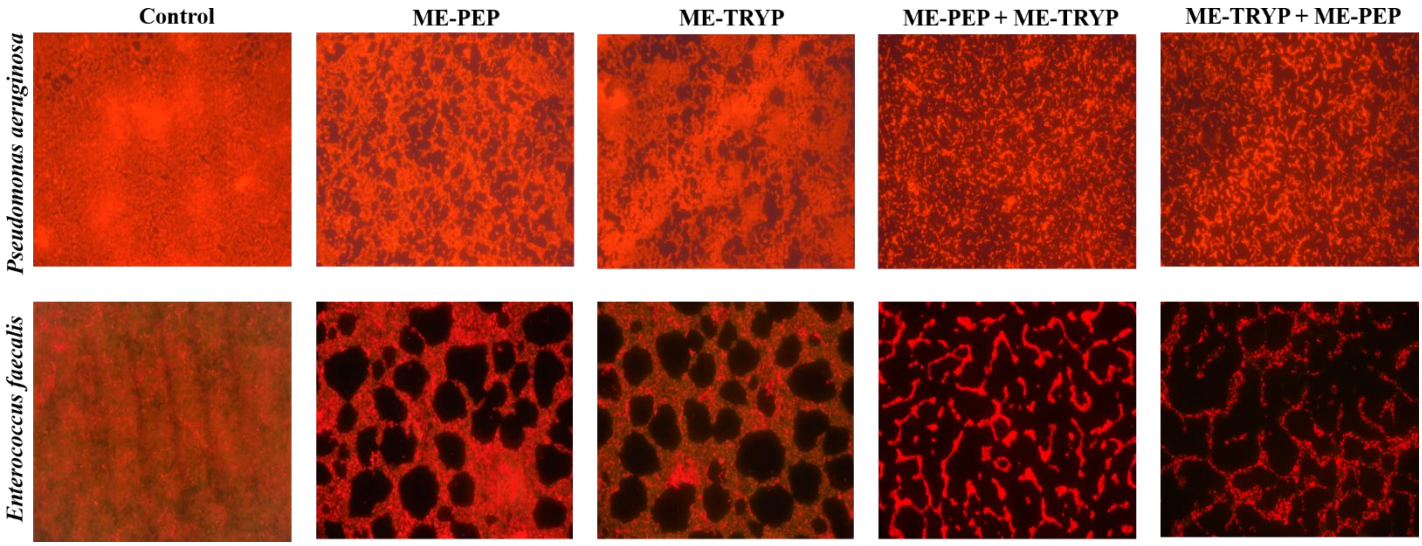

Fig 4. Epifluorescence microscopy images of *P. aeruginosa* and *E. faecalis* biofilms stained by Acridine orange after treatment by microencapsulated pepsin (ME-PEP) and trypsin (ME-TRYP), individually or sequentially. Control biofilms were treated by tryptone salt broth.

Single and combined treatment of microencapsulated enzymes and carvacrol

Pseudomonas aeruginosa case

The antibiofilm effect of ME-CARV (0.625 mg mL^{-1} for *P. aeruginosa* and 0.312 mg mL^{-1} for *E. faecalis*), ME-PEP (1 mg mL^{-1}) and ME-TRYP (1 mg mL^{-1}) against the two bacterial biofilms grown on stainless steel for 24 hours at 37 °C was studied using culturable counts. *P. aeruginosa* biofilm exhibited bacterial cell population of approximatively 7 log CFU mL^{-1} (Figure 5A). Results showed that, 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 showed a significant reduction of 2.2 log CFU mL^{-1} ($p < 0.05$) (Figure 5A). When using the combined treatment of ME-PEP or ME-TRYP followed by ME-CARV, the reduction in viable cells was more significant and reached 4.2 log CFU mL^{-1} and 3.8 log CFU mL^{-1} ($p < 0.05$), respectively (Figure 5A). 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 5A). Interestingly, a different sequential enzyme treatment in the order ME-PEP, ME-TRYP, followed by ME-CARV was also efficient, but by a less biofilm reduction which was of 4.5 log CFU mL^{-1} ($p < 0.05$). However, no significant reduction in biofilm cells was revealed after the two different treatments without ME-CARV ($p < 0.05$) (Figure 5A).

Enterococcus faecalis case

The biofilm of *E. faecalis* strain presented bacterial cell population of 7 log CFU mL^{-1} (Figure 5B). 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 5B). The combined treatment using ME-PEP followed by ME-CARV showed an improved biofilm reduction by 2.8 log CFU mL^{-1} (Figure 5B). 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 5B). 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 showed a significant and similar reduction in biofilm cells of 4 log CFU mL^{-1} ($p < 0.05$) approximatively (Figure 5B).

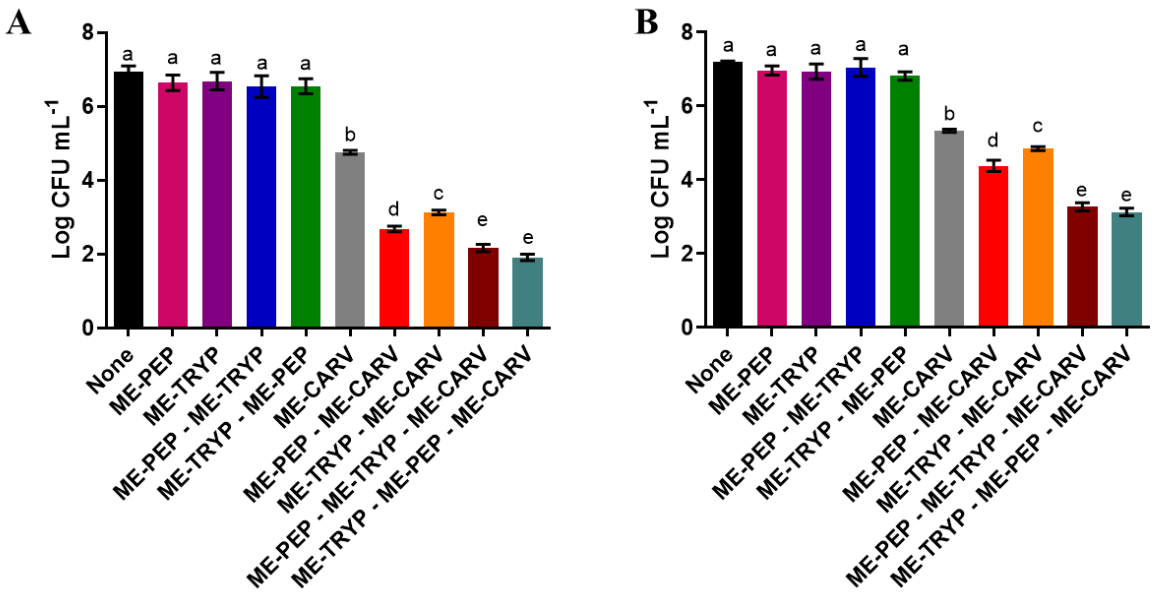

Fig 5. Number of viable cells in *P. aeruginosa* (A) and *E. faecalis* (B) biofilms after treatment with microencapsulated pepsin (ME-PEP) and trypsin (ME-TRYP), individually or sequentially, or combined treatment with microencapsulated carvacrol (ME-CARV). Results represent the average (±SD) of three independent experiments. The letters (a-d) indicate significant differences ($p < 0.05$) using Tukey's test.

Discussion

The EPS matrix is the protective barrier of biofilm against external agents, such as conventional detergents or disinfectants. Several enzymes are capable of disrupting the biofilm matrix. These biofilms-enzymes dispersing are more effective when combined with antimicrobial agents to kill the bacteria enclosed in the EPS matrix. In this study, we investigated the anti-biofilm effect of combined treatment using microencapsulated enzymes such as pepsin and trypsin with carvacrol,

a natural antimicrobial. Microencapsulation is a powerful tool used to separate active compounds from their environment by trapping them in microcapsules. It 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). Sodium caseinate has been selected for carvacrol microencapsulation due to its ability to form a negatively charged interfacial membrane in order to stabilize the emulsion (Gharsallaoui et al. 2007). On the other hand, pectin was used for enzymes microencapsulation in order to increase the resistance of the wall material by reinforcing its structure. The wall material is mainly composed of maltodextrins and the presence of pectin would help to obtain maximum microencapsulation efficiency (Gharsallaoui et al. 2007).

The SEM observation was used to study the outer and inner structures of carvacrol and enzymes spray-dried microcapsules. Results showed that all microparticles 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 microparticles, which enhances the protection and retention of the active materials (Carneiro et al. 2013). In addition, some carvacrol and enzymes microparticles have an irregular surface that may be caused by shrinkage during the drying and cooling procedures (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 prior to 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, results show that the carvacrol microcapsules were hollow, indicating that the volatile active compound was adhered to the surface as small droplets imbedded in the matrix of the wall materials. However, the enzyme microcapsules had no holes because enzymes are not volatile compounds and their location does not appear as a hole in the SEM micrographs. The obtained results are in accordance 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; Khelissa et al. 2021b).

In this study, we assessed the efficacy of microencapsulated proteolytic enzymes, pepsin and trypsin for the disruption of pre-existing *P. aeruginosa* and *E. faecalis* biofilms. These enzymes 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 such as *Staphylococcus lentus Staphylococcus cohnii , Staphylococcus saprophyticus* and *P. aeruginosa* can be degraded by trypsin (Banar et al. 2016; Fagerlund et al. 2016). Other study demonstrated the potential of pepsin to destabilize multi-species biofilm (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) report that enterococcal surface protein (Esp), a biofilm-associated protein (Bap) known as Bap-orthologous protein, is widely produced by *E. faecalis* and contribute significantly to matrix building and biofilm formation.

The enzymes 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 efficient tool to eradicate biofilm adhered to abiotic surfaces (Orgaz et al. 2007; Elchinger et al. 2015; Baidamshina et al. 2021). In order 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

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 show the dispersed activity of the enzymes, however, the results of cell counts show that treatment with enzymes, individually or sequentially, did not significantly affect the biofilm cell count ($p < 0.05$). 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 and therefore the number of viable cells were not significantly affected.

Essential oils, such as carvacrol, has 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. These alternative biocides provide the required treatment by overcoming the disadvantages of conventional antimicrobials used (Wińska 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, Li, et al. 2016), as well as reduce volatility and cytotoxicity, and enhance stability and solubility in water (Bilia et al. 2014). In order to analyze the antimicrobial activity of ME-CARV, biofilms of *P. aeruginosa* and *E. faecalis* were stained using the Live Dead kit and then observed by epifluorescence microscopy. Results show that after treatment of both bacterial biofilms with ME-CARV, the number of dead cells stained by PI increased significantly compared to the control biofilms. These results are consistent with the biofilm count analysis, which shows the significant reduction in the number of viable cells after ME-CARV treatment for both bacteria. These findings proved the high antimicrobial power 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 have also demonstrated (Chifiriuc et al. 2012; Bilcu et al. 2014; Duncan et al. 2015; Jamil et al. 2016; Khelissa et al. 2021c).

The use of EPS-degrading enzymes combined with biocides has been widely investigated recently 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 biofilm will be increasingly attacked by the 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, 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, Ma, et al. 2016). Lim et al. (2019) have also demonstrated that the sequential treatment with multiple enzymes followed by antimicrobial was more effective in removing biofilm developed on stainless steel surfaces than the single enzyme treatment (Lim et al. 2019). Hence, the results obtained suggest that the degradation of 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 by 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 this, 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 associated infections. Although several EOs have been investigated for their antibiofilm properties, the present study is a first report on the synergistic combination of microencapsulated proteolytic enzymes and a microencapsulated EO for increased efficacy in biofilm disruption. 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 time 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 microcapsule would also be required to allow simultaneous activity when used for biofilm control.

Conclusion

The objective of this study was to analyze the combined effect of microencapsulated enzymes and antimicrobials, on the removal of biofilm by a dual action, matrix destabilization and bacterial lethality. In brief, enzymes and carvacrol were successfully microencapsulated in different types of microcapsules, using spray-drying method, and their anti-biofilm action against biofilms of *P. aeruginosa* and *E. faecalis* was demonstrated. The microencapsulation serves to protect the antibiofilm agents from environmental stresses and to improve their activity and interactions with the biofilm matrix. 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 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 control biofilms produced by *P. aeruginosa* and *E. faecalis* in medical and food associated environments.

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General Conclusion and Perspectives

GENERAL CONCLUSION AND PERSPECTIVES

Microorganisms have the ability to multiply, adhere to abiotic surfaces, and produce a matrix of EPS, thus forming biofilms. The formation of biofilms can occur on almost at any types of surfaces (abiotic and biotic) and can leads to serious problems in the food and healthcare fields. In the food industry, different micro-organisms can contaminate surfaces depending on the food handled or processed and forms biofilms. Contamination of processed and distributed foods is a public health hazard causing serious foodborne infections, and results in considerable economic loss to the industry and healthcare sectors (Chatterjee and Abraham 2018). Furthermore, biofilms are the main cause of healthcare associated infections (HCAIs). They can develop on several medical devices and also cost the healthcare sector many billions each year (Bryers 2008). The multiple cell and EPS layers give biofilms a complex and compact structure that impedes the penetration of antimicrobial agents, reducing their ability to reach the inner layers, and rendering them inefficient. Thus, when microorganisms develop biofilms, they become highly resistant to disinfection procedures and antimicrobial display, revealing a MIC value up to 1000 times higher than their planktonic counterparts and making their control difficult to achieve (Araújo et al. 2011). Besides, due to the heterogeneity/complexity of these communities, the application of one single strategy is deemed insufficient. In this context, there is a need to develop new strategies, new antimicrobials, and techniques to fight biofilms. Promising approaches can be applied using "green" and bio-based products, such as enzymes. Enzymes are used in many areas of research and are known for their ability to degrade key constituents of the biofilm matrix (Mechmechani et al. 2022). Moreover, the combination of enzymes with other active agents (Hurdle technology) to control biofilms has been recognized to improve their activity. In this regard, our work investigated the effect of the combined use of enzymes and essential oil to combat biofilms. Two bacterial strains were selected for this study: *P. aeruginosa* and *E. faecalis* regarding their involvement in both healthcare and foodborne infections. Stainless steel, a surface commonly found in food and medical equipment, was also used as a substrate for bacterial adhesion and biofilm formation tests.

EOs of plant origin have been used for thousands of years in field of ethnomedicine as natural antiviral and antimicrobial agents. Their antimicrobial activity is related to the alteration of the microbial cell wall, which leads to cell lysis with leakage of the cell contents and inhibition of the

proton motive force. Their advantages arise from the fact that microbial resistance is much less probable than with chemical substances, ease of preparation, high biodegradability and biocompatibility (Chorianopoulos et al. 2008). Besides their antimicrobial properties, EOs also show anti-inflammatory effects. Moreover, their anti-biofilm activity has attracted the attention of researchers over the past ten years (Nuță et al. 2021). However, the use of EOs and their application present many challenges such as stability, volatility and poor water solubility issues that can reduce their activity. For this reason, an encapsulation approach has been proposed to overcome these issues and control their release (Nguyen et al. 2021). The first axis of our work was conducted on the single use of carvacrol, a widely available essential oil, to fight biofilms of *P. aeruginosa* and *E. faecalis*. In this part, we focused on the antimicrobial activity of carvacrol prior to its combined use with enzymes. In addition, in this study, microencapsulation of carvacrol by spray-drying was performed to enhance its biological activity and improve its stability. The physicochemical properties and microscopic morphology of the obtained capsules were characterized. In addition, the mode of action of F-CARV and ME-CARV against bacterial strains was studied. Moreover, the anti-biofilm activity of F-CARV and ME-CARV was also investigated against both biofilms adhered to stainless steel surfaces. Our results underlined that the MIC value of ME-CARV (1.25 mg mL^{-1}) was 4-times lower than F-CARV (5 mg mL^{-1}) against *P. aeruginosa*, while it remained the same against *E. faecalis* (0.625 mg mL^{-1}). Furthermore, ME-CARV showed enhanced anti-biofilm activity compared to F-CARV regardless of the contact time and concentration used and was able to reduce *P. aeruginosa* biofilm below the detection limit and *E. faecalis* biofilm by 5.5 log CFU mL^{-1} after 15 min of treatment. This improved antimicrobial activity was also demonstrated by epifluorescence microcopy analysis. The results also showed that F-CARV and ME-CARV destabilize the bacterial cell membrane, leading to the leakage of intracellular pool and subsequent cell death. These results indicate that carvacrol exhibited a strong antimicrobial effect against both bacterial biofilms. Moreover, our results demonstrated that spray-drying could be used as an effective approach to enhance the activity of carvacrol against biofilms, while reducing the concentrations required for disinfection of abiotic surfaces. These results suggest that the use of microencapsulated EOs to combat biofilms could serve as an alternative approach to conventional sanitation methods. However, before the application of these active anti-biofilm agents as

disinfectants in the food and medical sectors, they must be registered and approved by regulatory agencies. Moreover, it is recommended that the effects of storage time and manipulation practices on the retention of encapsulated EOs should be studied. Further research on sensory analysis of encapsulated EOs could be carried out to ensure consumer satisfaction and economic benefits. In addition, assessment of product hygroscopicity under varying humidity conditions is necessary to support prediction of storage life as well as selection of appropriate packaging materials. Finally, further exploration of new wall materials should also be sought to expand the application of encapsulated EOs towards environmental durability and cost effectiveness.

Enzymes are widely known and used in many industrial applications (drugs, food, baking, detergents, textiles, animal feed and beverages) (Srey et al. 2013). More recently, the application of enzymes to eradicate biofilms from industrial surfaces is increasing and developing (Xavier et al. 2005). This new trend is driven by a conscious society that increasingly demands "green" alternatives with a minimal impact on the environment. Enzymes typically target EPS, as it forms biofilms and influences their structure, shape and resistance to all types of stresses. The application of enzymatic treatment to eradicate biofilms from abiotic surfaces has already proven effective by degrading the key constituents of the biofilm matrix (Borges et al. 2020). Such strategies are related to the intrinsic properties and composition of the biofilm (Donlan 2002). This green anti-biofilm agent can be used in combination with biocides, reducing their concentrations required without compromising the inactivation of microorganisms. Since enzymes target EPS to remove and/or inactivate biofilms, they also improve accessibility of disinfectants to biofilm deep layer. In this regard, after having well demonstrated the high antimicrobial activity of carvacrol, the second axis of our work was devoted to the effect of the combined use of this antimicrobial with proteolytic enzymes, pepsin and trypsin, to combat of *P. aeruginosa* and *E. faecalis* biofilms. In this study, the MDCs and the contact times of enzymes were determined against biofilms grown on polystyrene surfaces. Both proteolytic enzymes clearly detached *P. aeruginosa* and *E. faecalis* biofilms after 1 h of contact at a concentration of 1 mg mL^{-1}, without affecting the planktonic cells growth. Moreover, our results showed that the combined treatment using pepsin or trypsin with carvacrol exhibited a more significant reduction of both biofilms grown on stainless steel surfaces compared to the carvacrol treatment alone. Furthermore, this reduction was greater after sequential treatment

of the two enzymes, followed by carvacrol with a maximum reduction of 4.7 log CFU mL^{-1} for *P. aeruginosa* and 3.3 log CFU mL^{-1} for *E. faecalis*. Such improved efficiency has also been proven by epifluorescence microscopy analysis. These results support the hypothesis that the use of enzymatic treatment prior to disinfection improves the activity of the antimicrobial agents since *P. aeruginosa* and *E. faecalis* biofilms were further reduced by carvacrol after pepsin and trypsin treatment. Indeed, these two proteolytic enzymes may provoke structural defects biofilms and degrade barrier properties by possibly interacting with matrix-associated proteins, thus facilitating the penetration of carvacrol and reducing the survival capacity of cells. The combined use of these natural agents would further reduce the use of chemical agents, energy costs and water consumption for biofilm control.

In fact, approaches using enzymes for biofilm control have disadvantages regarding cost, a significant dependence of activity on environmental factors, and stability, as they can self-degrade, resulting in instability (Cordeiro and Werner 2011). Moreover, the successful disruption of biofilm is limited by the delayed penetration and poor retention of enzymes (Liu et al. 2020a). In addition, as previously mentioned, the high volatility and low water solubility of essential oils can reduce the antibacterial activity of these compounds. As a solution, enzymes and carvacrol were stabilized by encapsulation on abiotic materials in order to improve their stability and biological activity and to ensure efficient penetration of these active compounds into the biofilm deep-layer. In order to demonstrate that the dispersive activity of protease and the antimicrobial activity of carvacrol were maintained and even enhanced after spray-drying microencapsulation, the same activity tests were repeated using these encapsulated active agents. Our results showed that the combined use of microencapsulated pepsin or trypsin combined with ME-CARV resulted in a more significant reduction in biofilm biomass compared to their free counterparts. Indeed, after treatment of *P. aeruginosa* biofilm with free pepsin or trypsin combined with free carvacrol, the reduction obtained was 3.7 and 2.8 log CFU mL^{-1}, respectively. These reductions were more significant using encapsulated pepsin or trypsin combined with ME-CARV, which were 4.2 and 3.8 log CFU mL^{-1}, respectively, even by using ME-CARV concentration 4-times lower than that of F-CARV. In addition, the sequential use of the two free proteolytic enzymes combined with carvacrol, in the order trypsin-pepsin-carvacrol, reduced the biofilm biomass by 4.7 log CFU mL^{-1}. This reduction

was quite comparable using the same sequential treatment combined with ME-CARV which was approximatively 5 log CFU mL^{-1}. Furthermore, after *E. faecalis* biofilm treatment using free pepsin combined with carvacrol, the reduction of biofilm biomass obtained was 2 log CFU mL^{-1}, approximatively. This biofilm was further reduced using encapsulated pepsin with ME-CARV witch was 2.8 log CFU mL^{-1}. Moreover, sequential use of the two encapsulated enzymes combined with ME-CARV reduced the biofilm biomass by approximatively 4 log CFU mL^{-1}, which was more significant than the same treatment using free enzymes and carvacrol (3.3 log CFU mL^{-1}). These results provide evidence that encapsulation of enzymes and carvacrol can improve the biological activity of these active compounds to reduce *P. aeruginosa* and *E. faecalis* biofilms. Although, for some treatments, results showed no or slightly greater reduction in biofilm biomass using encapsulated enzymes rather than free enzymes, encapsulation could nevertheless serve to improve the stability of these enzymes under different environmental conditions.

In order to extend this work, the static biofilm reactor should be upgraded to a dynamic reactor that would help to better represent the biofilms expected on contaminated industrial surfaces. In addition, the 24-h-old biofilms should be compared to 48-h-old biofilms to determine wich type of biofim is more resistant to our treatments. It would be interesting also to complete this work by analyzing the EPS components of *P. aeruginosa* and *E. faecalis* biofilms before and after enzymatic treatment using LC-MS to understand which types of proteins pepsin and trypsin target in dispersing biofilm. In addition, detailed knowledge of the major matrix components and their proportions under practical industrial conditions would be a good strategy for selecting more appropriate and specific enzymes to eradicate these biofilms. Additionally, optimizing all factors that may affect the efficacy of anti-biofilm compounds, such as the pH of the surrounding environment, types of encapsulated compounds, bacterial species, and surface characteristics, may be a promising strategy to improve biofilm eradication. Further studies in order to determine whether immobilization of enzymes and carvacrol improve or affect their storage stability should be also conducted. Finally, research on co-encapsulation of enzymes and antimicrobials in the same type of capsules would be needed to allow simultaneous activity when used for biofilm control.

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Title: Hurdle technology using microencapsulated proteolytic enzymes and microencapsulated carvacrol to fight pathogenic bacterial biofilms

Abstract: The ambient operating environments in the food and medical sectors allow bacteria to adhere and develop on the substrates, resulting in the growth of resistant pathogenic bacterial biofilms. These pathogenic structures are involved in several foodborne diseases and health-care associated infections. Consequently, to combat this public health burden, several strategies have recently been proposed which include chemical and mechanical removal. This work presents the different factors that influence bacterial adhesion and biofilm formation on abiotic surfaces, as well as biofilm resistance to disinfectants. The different strategies for biofilm prevention and eradication are described. Microencapsulation using spray-drying method for the formulation of anti-biofilm active components as a tool to ensure their stability and improve their biological activities are also presented. In this context, a study was conducted using carvacrol, a natural antimicrobial agent, to control biofilms of *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Indeed, these two bacteria are responsible for several infections worldwide due to their persistence on abiotic surfaces in hospitals and food processing industries. Furthermore, in order to enhance the antimicrobial activity of carvacrol and reduce its volatility and low solubility in water, feed emulsions were prepared with sodium caseinate and maltodextrins and then spray-dried to obtain dry carvacrol microcapsules. The results showed that carvacrol had a strong antimicrobial activity against both bacterial biofilms. Furthermore, our findings revealed that microencapsulation by spray-drying significantly increased the antimicrobial activity of carvacrol while reducing the amounts used. Indeed, microencapsulated carvacrol was able to reduce biofilm below the detection limit for *Pseudomonas aeruginosa* and 5.5 log CFU mL^{-1} for *Enterococcus faecalis* after 15 min of treatment. However, the complete removal of biofilms from abiotic surfaces in medical and food sectors has proven difficult with the single use of disinfection strategy due to the high protection of the biofilm cells by the extracellular polymeric matrix. This matrix provides an initial protective barrier for the biofilm cells, and makes biofilms highly resistant to antimicrobial agents. The effectiveness of hurdle technology in removing biofilms using different strategies is discussed in this work. One of the hurdle technology approaches is the use of matrix-degrading enzymes that can disperse bacteria embedded in biofilms for more efficient disinfection when combined with biocide agents. Indeed, two proteolytic enzymes, pepsin and trypsin, targeting matrix proteins, have been studied for their potential to degrade biofilms of *Pseudomonas aeruginosa* and *Enterococcus faecalis* and their synergistic effect when combined with carvacrol. The direct analysis using epifluorescence microscopy allowed visualization of the dispersive activity of proteases and the lethal activity of carvacrol against the two bacterial biofilms. In addition, the combined pepsin or trypsin treatment with carvacrol showed more significant reduction of both biofilms compared to carvacrol treatment alone. Moreover, this reduction was more substantial after sequential treatment of both enzymes followed by carvacrol. However, the enzyme activity is highly influenced by environmental factors and is only optimal under restricted conditions. Another disadvantage of using enzymes is self-degradation, leading to instability. Indeed, protease microcapsules containing pepsin or trypsin complexed with pectin and maltodextrin have been prepared. The combined use of these miocrocapsules with microencapsulated carvacrol was also investigated in this study against *Pseudomonas aeruginosa* and *Enterococcus faecalis* biofilms. The results showed that enzyme microcapsules were also able to enhance the antimicrobial properties of encapsulated carvacrol with a retained and even improved activity compared to free enzymes and carvacrol. The physicochemical properties and the microscopic morphology of the realized capsules allowed to a better understanding of the mechanism of action of these microcapsules.

Titre: La technologie des barrières utilisant des enzymes à pouvoir déstructurant et du carvacrol microencapsulés pour lutter contre les biofilms de bactéries pathogènes

Résumé: L'environnement opératoire dans les secteurs alimentaire et médical permet aux bactéries de se fixer et de se développer sur les surfaces, ce qui entraîne la formation de biofilms bactériens pathogènes et résistants. Ces structures pathogènes sont responsables de nombreuses maladies d'origine alimentaire et d'infections associées aux soins. Par conséquent, pour lutter contre ce problème de santé publique, plusieurs stratégies ont récemment été proposées, notamment l'élimination chimique et/ou mécanique. Ce travail présente dans une première partie bibliographique les différents facteurs qui influencent l'adhésion bactérienne et la formation de biofilms sur des surfaces abiotiques, ainsi que la résistance des biofilms aux désinfectants. La microencapsulation par la méthode de séchage par atomisation pour la formulation de composants actifs anti-biofilm en vue d'assurer leur stabilité et améliorer leurs activités biologiques est également présentée. Dans ce contexte, l'étude menée a pour objectif d'utiliser le carvacrol, un agent antimicrobien naturel, pour contrôler les biofilms de *Pseudomonas aeruginosa* et *Enterococcus faecalis*. En effet, ces deux bactéries sont responsables de nombreuses infections dans le monde en raison de leur persistance sur des surfaces abiotiques dans les hôpitaux et les industries agroalimentaires. Par ailleurs, afin de renforcer l'activité antimicrobienne du carvacrol et de réduire sa volatilité et sa faible solubilité dans l'eau, des émulsions ont été préparées avec du caséinate de sodium et des maltodextrines, puis séchées par atomisation pour obtenir des microcapsules de carvacrol sèches. Les résultats ont montré que le carvacrol exerce une forte activité antimicrobienne contre les deux biofilms bactériens. De plus, nos résultats ont révélé que la microencapsulation par séchage par atomisation améliore d'une manière significative l'activité antimicrobienne du carvacrol tout en réduisant les quantités utilisées. En effet, le carvacrol microencapsulé a été capable de réduire le biofilm en dessous de la limite de détection pour *Pseudomonas aeruginosa* et de 5.5 log CFU mL^{-1} pour *Enterococcus faecalis* après 15 min de traitement. L'efficacité de la technologie hurdle pour éliminer les biofilms en utilisant différentes stratégies est discutée dans ce travail. Une des approches de la technologie hurdle est l'utilisation d'enzymes qui peuvent dégrader la matrice et disperser les bactéries intégrées dans les biofilms pour une désinfection plus efficace lorsqu'elles sont combinées avec des agents biocides. En effet, deux enzymes protéolytiques, la pepsine et la trypsine, ciblant les protéines de la matrice, ont été étudiées pour leur potentiel de dégradation des biofilms de *Pseudomonas aeruginosa* et *Enterococcus faecalis* et leur effet synergique lorsqu'elles sont combinées au carvacrol. L'analyse directe par microscopie à épifluorescence a permis de visualiser l'activité dispersive des protéases et l'activité létale du carvacrol contre les deux biofilms bactériens. En outre, le traitement combiné avec la pepsine ou la trypsine et le carvacrol a entraîné une réduction plus significative des deux biofilms par rapport au traitement avec le carvacrol seul. De plus, cette réduction était plus importante après un traitement séquentiel avec les deux enzymes suivi d'un traitement avec du carvacrol. Cependant, l'activité enzymatique est fortement influencée par les facteurs environnementaux et n'est optimale que dans des conditions restreintes. Un autre inconvénient de l'utilisation des enzymes est l'auto-dégradation, qui entraîne leur instabilité. En effet, des microcapsules de protéase contenant de la pepsine ou de la trypsine en présence de la pectine et de maltodextrines ont été préparées. L'utilisation combinée de ces miocrocapsules avec du carvacrol microencapsulé a également été étudiée dans cette étude contre les biofilms de *Pseudomonas aeruginosa* et *Enterococcus faecalis*. Les résultats ont montré que les microcapsules d'enzymes étaient capables de renforcer l'activité antimicrobienne du carvacrol microencapsulé avec un maintien et même une amélioration d'activité biologique par rapport aux enzymes et au carvacrol libres. Les propriétés physicochimiques et la morphologie microscopique des capsules obtenues ont permis de mieux comprendre le mécanisme d'action de ces microcapsules.