UNIVERSITÀ DEGLI STUDI DI NAPOLI
"FEDERICO II"

PhD Thesis

"Innovative food safety and hygiene management systems in the agri-food industry"

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List of abbreviations

$\Delta\lambda_{max}$	Maximum shift of wavelength
*a**	redness
AMP	antimicrobial peptide
Amp	Ampicillin
AmpC	serine-betalactamases
ANOVA	Analysis of variance
AP	active packaging
APC	Aerobic plate count
Ar	argon
Arg	arginine
ATR-FTIR	Fourier transform infrared spectroscopy measurements in attenuated total reflection mode
a_w	activity water
*b**	yellowness
BIC	Biofilm Inhibitory Concentration
B_{max}	maximum specific binding
CAMP	cationic antimicrobial peptide
CD	circular dichroism spectroscopy
CETC	Type Culture Collection
CFU	colony-forming units
CIA	critically important antimicrobials
CL	1′,3′-bis[1,2-dioleoyl-*sn*- glycero-3-phospho]-glycerol (Cardiolipin, 18:1)
C_L	phosphorus concentration (µg/µL)
CLSI	Clinical and Laboratory Standards Institute
C_P	peptide concentration (µg/µL)
D-PBS	Dulbecco's Phosphate Buffered Saline
ddH2O	double-distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPG	dioleoylphosphatidylglycerol
DRBC	Dichloran Rose Bengal Chloramphenicol Agar
DTT	Dithiothreitol
ECA	Water contact angles
ECDC	European Centre for Disease Control
EFSA	European Food Safety Authority
EO	Essential oil
ESBL	extended-spectrum beta-lactamase
EU	European Union
EUMOFA	European Market Observatory for Fisheries and Aquaculture Products
FAO	Food & Agriculture Organization
FBO	Food Business Operator
FBS	fetal bovine serum
FDA	Food and Drug Administration
Fr	fraction
GRAS	Generally Recognized as Safe
HDP	host-defense peptide

HL 1018-K6	PET discs functionalized with 1018-K6 peptide
HL CTR	not functionalized PET discs
HPLC	high-performance liquid chromatography
HR	Relative humidity
ICMSF	International Commission on Microbiological Specifications for Food
ISO	International Organization for Standardization
K	PETs
KAA	Kanamycin Aesculin Azide
K_d	equilibrium dissociation constant
K_p	partition coefficient
K_{sv}	Stern–Volmer constant
L^*	lightness
LAB	Lactic Acid Bacteria
Lan	lanthionine
Lys	lysine
MBC	minimum bactericidal concentration
MeLan	methyllanthionine
MH	Mueller–Hinton
MIC	minimum inhibitory concentration
MLV	multilaminar vesicles
MP	MTP1-PETs
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	Member States
MSSA	methicillin-susceptible *Staphylococcus aureus*
MTP1	mitochondrial-targeted peptide 1
MWCO	Molecular weight cut-off
Na	Nalidixic acid
NCS	newborn calf serum
NR	Neutral Red
NRL	National Reference Laboratory
NRU	Neutral Red Uptake
OD	Optical Density
PA	polyamide
PB	sodium phosphate buffer
PC	L-α-lysophosphatidylcholine
PCA	Plate count agar
PE	polyethylene
PE	L- α-phosphatidylethanolamine
PEs	plant extracts
PET	polyethylene terephthalate
PG	phosphatidylglycerol
PMSF	Phenylmethylsulfonyl fluoride
POPE	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol
PP	polypropylene
PS	polystyrene
PS	phosphatidyl serine
PTC	Psychotropic bacteria Count
PUFAs	n-polyunsaturated fatty acids

PVC	polyvinylchloride
PW	Peptone Water
Q	concentration of soluble quencher
RF	Radio frequency
RIE	Reactive Ion Etching
RP-HPLC	reverse-phase high-performance liquid chromatography
SDS	Sodium Dodecyl Sulfate
SEM	scanning electron microscopy
SM	N-Acyl-D-sphingosine-1-phosphocholine
SM-ID2	chromID Salmonella agar
SS	sirloin steaks
SSM	sirloin steak muscles
SSOs	specific spoilage organisms
STEC	Shiga toxin-producing *Escherichia coli*
Strep	Streptomycin
Sul	Sulfamethoxazole
SUVs	small unilamellar vesicles
TBX	Triptone Bile X-glucuronide Agar
Tet	Tetracycline
TFA	Trifluoroacetic acid
TPA	Texture profile analysis
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
Trp	Tryptophan
TSB	Tryptic Soy Broth
TVC	Total Viable Count
VOCs	volatile organic compounds
VRBG	Violet Red Bile Glucose Agar
VRBL	Violet Red Bile Lactose Agar
WHO	World Health Organization
WRAP	Waste & Resources Action Programme
YLL	Years of Life Lost

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Abstract

Food sector contributes significantly to the financial statements of the European Union (EU), despite the economic losses caused by the contamination of the foods, which are exposed to various hazards along the food chain. The microbiological contamination plays an important role in the food spoilage and foodborne diseases, causing public health and economic damage. Indeed, in the EU, around 90 million tons of food are wasted annually, with associated burden of microbial spoilage estimated to affect more than a quarter of the total weight. Furthermore, the European Food Safety Authority has attributed to pathogenic bacteria the role of mail culprit agents of human diseases linked to the ingestion of contaminated food. These microorganisms are also partly responsible of the spread of the antimicrobial resistance phenomenon, which has become one of the major global public health concerns.
In this context, the general aim of the thesis was to search for molecules capable of replacing the chemical additives and conventional antibiotics, and develop an "end-product" with antimicrobial activity ready to be put on the food market.
Ideally, the novel preservatives should have broad spectrum of action, be non-toxic, be active a low concentration, no affect organoleptic properties of food, and have a good ratio cost-effectiveness. Plants and their extracts, and antimicrobial peptides (AMPs) are gaining attention due to their naturalness, high bioavailability, and innumerable antimicrobial properties.

In **Chapter 1**, *Loranthus europaeus*, well-known medicinal plant, was chosen to evaluate its potential antimicrobial action. To this aim, different protocols were performed to selectively extract protein compounds, from *L. europaeus* yellow fruits, and evaluate the antimicrobial activity against four phytopathogenic fungi (*Aspergillus niger*, *Alternaria* spp., *Penicillium* spp., *Botrytis cinerea*) and a number of foodborne bacterial pathogens (*Listeria monocytogenes*, *Staphylococcus aureus* strains, *Salmonella* Typhimurium and *Escherichia coli*) by using serial dilutions and colony formation assays. Results evidenced no antifungal activity but a notable bactericidal efficiency of a crude protein extract against two foodborne pathogens, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values between 0.2 and 0.5 mg/mL, being *S. aureus* strains the most susceptible bacteria. Moreover, a strong bactericidal activity against *S. aureus* M7 was observed by two partially purified protein fractions of about 600 and 60 kDa molecular mass in native conditions. Therefore, these plant protein extracts could be used as natural alternative preventives to control food poisoning diseases and preserve foodstuff avoiding health hazards of chemically antimicrobial applications.

Chapter 2 aimed at evaluating the antimicrobial and antibiofilm activity of the innate defense peptide, named 1018-K6, against *Salmonella enterica*. A total of 42 strains, belonging to three different subspecies and 32 serotypes, were included in this study. The antibiotic resistance profile of all the strains and the cytotoxic effects of 1018-K6 on mammalian fibroblast cells were also investigated. Results revealed that MIC (minimum inhibitory concentrations) and MBC (minimum bactericidal concentrations) values were in the ranges of 8–64 μg/mL and 16–128 μg/mL, respectively, although most strains (97%) showed MICs between 16 and 32 μg/mL. Moreover, sub-inhibitory concentrations of 1018-K6 strongly reduced the biofilm formation in several *S. enterica* strains, whatever the initial inoculum size. The results showed that 1018-K6 was able to control and manage *S. enterica* growth with a large potential for applications in the fields of active packaging and disinfectants.

To this purpose, the goal of **Chapter 3** was to investigate the effects of a food contact surface of polyethylene terephthalate (PET) functionalized with a previously characterized antimicrobial peptide mitochondrial-targeted peptide 1 (MTP1), in reducing the microbial population related to spoilage and in extending the shelf- life of different types of fresh foods such as ricotta cheese and

buffalo meat. Modified polymers were characterized concerning the procedure of plasma-activation by water contact angle measurements and Fourier transform infrared spectroscopy measurements in attenuated total reflection mode (ATR-FTIR). Results showed that the MTP1-PETs provided a strong antimicrobial effect for spoilage microorganisms with no cytotoxicity on human cell line. Finally, the activated polymers revealed high storage stability and good reusability.

Chapter 4 aimed to evaluate the efficacy of packaging in polyethylene terephthalate (PET) functionalized with the antimicrobial peptide 1018-K6 to extend the *shelf life* of fish burgers of dolphinfish (*Coryphaena hippurus*). At 1, 3, 5 and 7 days, microbiological analyses, pH, a_w (activity water) and the sensory analyses were carried out.
Sensory results showed that samples packed with functionalized packaging were considered acceptable two days longer than the control group. Microbiological data underlined an evident antimicrobial activity of the active packaging at 5 days against coliforms, Enterobacteriaceae, *Pseudomonas* spp., *E. coli* and fecal streptococci, with a reduction of about 1 Log (CFU/g) compared to the control group.

The advantage to use natural antimicrobial compounds is also due to their poor link with the common bacterial resistance than conventional drugs. To this purpose, a better comprehension of the mechanisms of action of the antimicrobial peptide is essential for promoting their use in the food industry.

Although antimicrobial activity of 1018-K6 towards both Gram-positive and Gram-negative bacteria responsible for foodborne diseases has been extensively demonstrated, the mechanism of action at the molecular level is still unclear. In **Chapter 5,** mechanistic studies (binding saturation assay, and fluorescence methodologies) were carried out using model membranes and bacterial membranes extracted from pathogenic bacteria isolated in various food matrices. Results reveal that the antimicrobial activity of 1018-K6 is connectable to its affinity for bacterial membranes. The study highlights differences in peptide-lipid interaction and partitioning between mimetic membranes of bacteria, suggesting that peptides have to establish more deeper interactions for binding to Gram-positive membrane that could be favored with arginine (Arg) residues. Moreover, the peptide ability to interact with bacterial mimetic liposomes was confirmed by the formation of vesicular aggregates at ratios of lipid to peptide lower than 80. In conclusion, it is worth pointing out the higher affinity of 1018-K6 for bacterial membranes than those of the respective multilaminar vesicles (MLVs). Thus far, the knowledge gathered on the mechanism of action of this peptide shows a high specificity against anionic liposomes and provides valuable information on the time of action. By calculating that the peptide needs only about 30 minutes or 2 hours of contact to enter in the membranes of Gram-negative and Gram-positive, respectively, the prospect of using 1018-K6 in the formulation of active packaging becomes more concrete.

The antimicrobial packaging is offering a viable solution to tackle the economic and safety issues above mentioned, by extending the shelf-life and improving the quality and safety of fresh products. Overall, this thesis provided valuable information to develop alternative antimicrobial packaging for enhancing and extending the microbial quality and safety of perishable foods during storage by using natural compounds.

Riassunto

Il settore alimentare contribuisce in modo significativo al bilancio dell'Unione Europea, nonostante le ingenti perdite economiche correlate ai fenomeni di contaminazione alimentare. Tra i diversi pericoli a cui sono esposti gli alimenti dalla produzione primaria al consumo, la contaminazione microbiologica riveste un ruolo di primaria importanza poiché responsabile di danni alla salute pubblica e all'economia. Ai microrganismi sono, infatti, imputabili sia i fenomeni di deterioramento, che limitano la durabilità degli alimenti, sia numerose malattie di origine alimentare nell'uomo. Si stima, infatti, che ogni anno in Europea vengono sprecati circa 90 milioni di tonnellate di cibo e che le contaminazioni di origine microbiologica ne siano la causa nel 25 % dei casi. Inoltre, secondo il Report annuale redatto dall'EFSA (European Food Safety Authority) i batteri patogeni sono i principali responsabili di malattie a trasmissione alimentare. Gli stessi sono anche in parte responsabili della diffusione del fenomeno della resistenza agli antimicrobici, divenuto uno dei maggiori problemi di salute pubblica mondiale.
In questo contesto, l'obiettivo generale di questa tesi è stato la ricerca di nuove molecole in grado di sostituire gli additivi chimici e gli antibiotici convenzionali e sviluppare un "prodotto finale" provvisto di attività antimicrobica pronto per essere immesso sul mercato dell'industria agro-alimentare.
Idealmente, i nuovi conservanti dovrebbero avere un ampio spettro d'azione, essere non tossici, essere attivi a bassa concentrazione, non influenzare le proprietà organolettiche degli alimenti e avere un buon rapporto costo-efficacia. Le piante e i loro estratti, e i peptidi antimicrobici (AMPs) sono sempre più attenzionati dal mondo scientifico grazie alla loro naturalezza, elevata biodisponibilità e innumerevoli proprietà antimicrobiche.

Nel **capitolo 1** è stata valutata l'attività antimicrobica di *Loranthus europaeus*, nota e importante pianta medicinale. A tal scopo, sono stati eseguiti diversi protocolli al fine di estrarre selettivamente composti proteici dai frutti gialli di *L. europaeus*. L'attività antimicrobica degli estratti è stata valutata verso quattro funghi fitopatogeni (*Aspergillus niger*, *Alternaria* spp., *Penicillium* spp., *Botrytis cinerea*) e diversi batteri patogeni di origine alimentare (*Listeria monocytogenes*, ceppi di *Staphylococcus aureus*, *Salmonella* Typhimurium ed *Escherichia coli*). I risultati non hanno evidenziato attività antimicotica; tuttavia, un estratto di proteina grezza ha mostrato un elevato potere battericida verso due batteri patogeni di origine alimentare, registrando valori di concentrazione minima inibente (MIC) e concentrazione minima battericida (MBC) tra 0.2 e 0.5 mg/mL. I ceppi di *S. aureus* si sono rivelati, tra i batteri testati, i più sensibili. Nello specifico, è stata osservata una forte attività battericida di due frazioni proteiche, parzialmente purificate di circa 600 e 60 kDa di massa molecolare in condizioni native, verso *S. aureus* M7. Sulla base dei risultati ottenuti, gli estratti di proteine vegetali hanno mostrato proprietà antimicrobiche tali da classificarli come potenziali agenti antimicrobici naturali utilizzabili nel settore agroalimentare per controllare la crescita di batteri patogeni negli alimenti.

Il **capitolo 2** mirava a valutare l'attività antimicrobica e antibiofilm del peptide 1018-K6, appartenete al sistema immunitario innato, contro la Salmonella enterica. In questo studio sono stati inclusi un totale di 42 ceppi, appartenenti a 3 diverse sottospecie e 32 sierotipi. Sono stati inoltre studiati i profili di resistenza agli antibiotici di tutti i ceppi e gli effetti citotossici di 1018-K6 sulle cellule di fibroblasti di mammifero. I risultati hanno rivelato che i valori di MIC e MBC rientravano rispettivamente nei range di 8-64 μg/mL e 16-128 μg/mL. Tuttavia, la maggior parte dei ceppi (97%) ha mostrato valori di MIC tra 16 e 32 mg/mL. Inoltre, le concentrazioni sub-inibitorie di 1018-K6 hanno fortemente ridotto la formazione di biofilm in diversi ceppi di *S. enterica*, indipendentemente dalla concentrazione dell'inoculo iniziale. I nostri risultati hanno dimostrato che 1018-K6 è in grado di controllare e gestire la crescita di *S. enterica* con un grande potenziale per applicazioni nei campi dell'active packaging e dei disinfettanti per impianti industriali.

A tal proposito, l'obiettivo del **capitolo 3** è stato studiare l'effetto antimicrobico di una superficie di polietilene tereftalato (PET) funzionalizzata con il peptide antimicrobico MTP1, precedentemente caratterizzato. Ne è stata, quindi, valutata la capacità di aumentare la *shelf-life* di diversi tipi di alimenti freschi come ricotta e carne di bufala. I polimeri modificati sono stati caratterizzati per quanto riguarda la procedura di attivazione al plasma mediante misurazioni dell'angolo di contatto con l'acqua e misurazioni della spettroscopia infrarossa di trasformata di Fourier totale attenuato del riflesso (ATR-FTIR). I risultati hanno dimostrato un forte effetto antimicrobico di MTP1-PET verso i microrganismi alteranti ed assenza di citotossicità verso una linea cellulare umana. Inoltre, i polimeri attivati hanno mostrato un'elevata stabilità allo stoccaggio e una buona riutilizzabilità.

Il **Capitolo 4** mirava a valutare l'efficacia del confezionamento in polietilene tereftalato (PET) funzionalizzato con il peptide antimicrobico 1018-K6 nel prolungare la durata di conservazione degli hamburger di pesce di lampuga (*Coryphaena hippurus*) A 1, 3, 5 e 7 giorni, sono state eseguite analisi microbiologiche, misurazioni del pH, a_w (attività dell'acqua) e una valutazione sensoriale.
I risultati sensoriali hanno mostrato che i campioni confezionati con imballaggi funzionalizzati sono stati considerati accettabili due giorni in più rispetto al gruppo di controllo. I dati microbiologici hanno evidenziato un'evidente attività antimicrobica della confezione attiva a 5 giorni contro coliformi, Enterobacteriaceae, *Pseudomonas* spp., *E. coli* e streptococchi fecali, con una riduzione di circa 1 Log (UFC/g) rispetto al gruppo di controllo.

Il vantaggio di utilizzare composti antimicrobici naturali è dovuto anche al loro scarso legame con la resistenza batterica, se comparati ai farmaci convenzionali. A tal fine, una migliore comprensione dei meccanismi d'azione del peptide antimicrobico è essenziale per promuoverne l'uso nell'industria alimentare.

Sebbene l'attività antimicrobica di 1018-K6 sia nei confronti dei batteri Gram-positivi che dei Gram-negativi responsabili di malattie di origine alimentare sia stata ampiamente dimostrata, il meccanismo d'azione a livello molecolare è ancora poco chiaro. Nel **capitolo 5**, sono stati condotti studi meccanicistici (saggio di saturazione del legame e metodologie di fluorescenza) utilizzando membrane modello e membrane batteriche estratte da batteri patogeni isolati in varie matrici alimentari. I risultati rivelano che l'attività antimicrobica del 1018-K6 è collegabile alla sua affinità per le membrane batteriche. Lo studio evidenzia delle differenze nell'interazione peptide-lipidi e nel partizionamento tra le membrane mimetiche dei batteri, suggerendo la necessità di interazioni più profonde tra il peptide e la membrana di batteri Gram-positivi che potrebbero essere favorite dai residui di Arg. Inoltre, la capacità del peptide di interagire con i liposomi mimetici batterici è stata confermata dalla formazione di aggregati vescicolari a rapporti lipide-peptide inferiori ad 80. In conclusione, vale la pena sottolineare la maggiore affinità del peptide 1018-K6 per le membrane batteriche rispetto a quelle del i rispettivi liposomi mimetici. Finora, le conoscenze raccolte sul meccanismo d'azione di questo peptide mostrano un'elevata specificità nei confronti dei liposomi anionici e forniscono preziose informazioni sul tempo di azione. Calcolando che il peptide necessita solo di circa 30 minuti o 2 ore di contatto per entrare rispettivamente nelle membrane dei Gram-negativi e dei Gram-positivi, la prospettiva di utilizzare il 1018-K6 nella formulazione del packaging attivo assume maggiore concretezza.

Una valida soluzione per limitare le perdite economiche e i problemi di sicurezza alimentare sopra menzionati è offerta dall'imballaggio antimicrobico, il quale agisce estendendo la durata di conservazione e migliorando la qualità e la sicurezza dei prodotti freschi.
Nel complesso, questa tesi ha fornito informazioni preziose per lo sviluppo di imballaggi antimicrobici con composti naturali. I dati raccolti ci permettono di identificarli come potenziali

soluzioni per il miglioramento della qualità microbiologica degli alimenti e il rispetto dei criteri di sicurezza alimentare.

1. Introduction

1.1 General overview

The food sector represents one of the most important economic fields in Europe, generating more than 750.000.000.000 euros per year (EU, 2020). Unfortunately, this sector suffers the burden of food losses which have been reaching very high figures in recent decades. *Food loss* is defined as "the decrease in mass (dry matter) or nutritional value (quality) of food that was originally intended for human consumption". Meanwhile, *food waste* is part of food loss and refers to "food appropriate for human consumption being discarded, whether or not after it is kept beyond its expiry date or left to spoil" (FAO, 2013). In 2011, the Food & Agriculture Organization (FAO) estimated that around 1.3 billion tons of food never reached the tables of consumers, resulting in the loss of one-third of all food produced for human consumption (FAO, 2019). In Europe, foods loss and waste achieved about 90 million tons every year (FUSIONS, 2016), corresponding to ca. 173 kg per capita, which reflected a loss of 143 billion euros each year. What is more, scientists stated that the global trend is set to grow in the near future (Lemaire and Limbourg, 2019).

Considering the numbers above, it is clear that the food industry has a huge economic impact, and microbiological contamination could be one of the major causes of food loss and food waste. It has been estimated that approximately 25% of all foods produced are lost due to microbial spoilage (Petruzzi *et al*., 2017), mainly caused by non-pathogenic spoilage microorganisms. As a result of the replication of these bacteria, undesirable alterations of sensory and nutritional characteristics occur in food matrices (Otoni *et al.*, 2016). On the other hand, the consumption of food contaminated with specific pathogenic microorganisms could severely harm public health and results in cases of foodborne diseases and/or outbreaks. In agreement with the latest data provided by the World Health Organization (WHO) in 2015, the estimated number of cases of foodborne illness and deaths annually were around 600.000.000 and 420.000, respectively (WHO, 2015). Precisely, foodborne diseases are responsible for 27.000.000 Years of Life Lost (YLL).

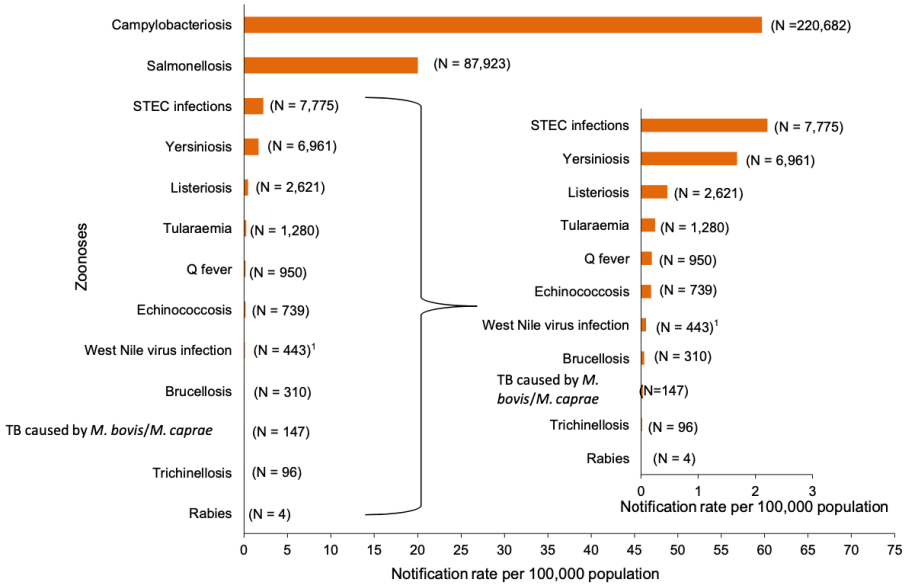
Several food categories are a suitable substrate for bacterial replication, especially huge perishable ones such as fish and fishery products, dairy products, and meat and meat products.

In fish and fishery products the deterioration processes are the result of the combined action of enzymatic autolysis, oxidation, and microbial growth (Ghaly *et al*., 2010). This latter factor is considered to be the major cause of spoilage during the storage, promoted by fish intrinsic features such as the high water content and high level of pH in the post mortem, and high amount of free amino acids (Chaillou *et al*., 2015). It is important to underline that the microbial communities responsible for spoilage are a small fraction of the flora detectable in the fish. They are known as specific spoilage organisms (SSOs), which are different among fish species and depend on the raw materials, and preservation conditions (Møretrø *et al*., 2016). The selection of spoilage bacteria is carried out in particular by manipulating the physical and chemical environmental parameters through the application of mild or strong technologies. For instance, it was reported that *Pseudomonas* spp. was the SSOs for fish species stored in aerobically conditions, while the modified atmosphere packaging select the CO_2-resistant *Photobacterium phosphoreum* (Chaillou *et al*., 2015; Murhekar *et al*., 2017). However, whichever species is responsible for deterioration, the bacterial growth causes up to 25-30 % of losses in this food sector (Hassoun *et al*., 2017).

As reported for fishery products, bacterial spoilage also represents a problem in the meat industry (Devlieghere *et al*., 2004). Meat and meat products are characterized by a nutrient content which makes them an ideal environment for the growth of a wide variety of microorganisms (Mayr *et al*., 2003), including spoilage bacteria (Ercolini *et al*., 2006). Several authors, such as Nychas *et al*., 2008, have reported that the status of contamination and the identifiable microbial populations are attributed to contaminations occurring along the whole farm-to-fork chain. Moreover, also for this type of product, the preservation parameters play a crucial role in the selection of bacterial communities, allowing to predict their presence or absence (Gram *et al*., 2002). The replication of spoilage

microorganisms reflects on the sensory appearance of products as a consequence of physical damage and activation of chemical processes that result in the production of off-flavours and off-odours. *Pseudomonas* spp., Enterobacteriaceae*, Brochothrix thermosphacta* and Lactic Acid Bacteria (LAB) are the main genera responsible for the deterioration of meat and meat products (Gram *et al*., 2002; Ercolini *et al*., 2006).

Regarding pathogenic bacteria, foodborne diseases still pose significant public health challenges despite the progress in the food safety field. The recent review "The burden of foodborne diseases in the WHO European Region" reported that the most common causes of foodborne diseases are diarrhoeal disease agents such as norovirus, with among 15 million cases, and *Campylobacter* spp., which is responsible for almost 5 million cases (WHO, 2017). However, what is worrying is that the number of confirmed cases has been underestimated because notification is not mandatory for each biological hazard responsible for illness; besides, several cases have been overshadowed as they were not diagnosed, seeming to be not connected to ingestion of unsafe food. All this happens despite in 2005 the European Union (UE) defined how to declare food poisoning events and made outbreak communication mandatory for Member States (MS). Indeed, in agreement with Directive 2003/99/EC, each MS submit data on zoonoses, antimicrobial resistance and food-borne outbreaks which were examined by the European Food Safety Authority (EFSA). In collaboration with the European Centre for Disease Control (ECDC), collected data were used to compose the annual Community Summary Report. The latest scientific Zoonoses Report of EFSA and ECDC (2021) reconfirmed campylobacteriosis and salmonellosis as the first and the second most reported zoonoses in humans with 220.682 and 87.923 confirmed cases, respectively (Figure 1.1). To follow, the Shiga toxin-producing *Escherichia coli* (STEC), with a high rate of hospitalization (50 in 2019). Moreover, even if with only 2621 confirmed invasive human cases in 2019, *Listeria monocytogenes* boasted to be one of the most serious food-borne diseases because of the high percentage of case fatality (17.6%).



Note: The total number of confirmed cases is indicated between parentheses at the end of each bar.
[1] Exception: West Nile virus infection for which the total number of cases was used.

Figure 1.1 *Notification rates and reported numbers of confirmed human cases of zoonoses in the EU, 2019 (EFSA and ECDC, 2021).*

Nowadays, the food industry attempts to control biological hazards and limit the risk of harming world public health, especially knowing that the spread of diseases is increasing by strengthening the international markets. The scenario of the last decades has some flaws that push the sector to project its aspiration on the search for new strategies of preservations. The extensive use of chemical antimicrobial compounds, in fact, had led to the development of resistant microorganisms which are

responsible for the phenomenon of antibiotic resistance. In addition, the traditional way of adding antimicrobials to food products by direct insertion had shown several limitations such as the short action time due to the rapid spread from the surface to the inner portion of the food matrix (Radusin *et al*., 2013). At the same time there is a growing consumer awareness about the nature and the origin of all ingredients that reach his table, asking for foods without additives or choosing food containing natural additives if additive-free foods are not available (Barreiro *et al*., 2014; Carocho *et al*., 2015).

1.2 Active packaging: antimicrobial packaging

It is popularly known that as a "cover" the packaging separates the food from external sources of contamination and guarantees protection against physical and chemical damages from farms to kitchens (Dehnad *et al*., 2014; Hashemi *et al*., 2018; Pilevar *et al*., 2019). Nevertheless, to meet consumers demands, researchers are working to offer innovative solutions that have the double purpose of preserving the food and providing information about its quality and sensory properties over time. Thanks to these scientists, the concepts of smart/intelligent and active packaging (AP) have been incorporated into the food industry. Briefly, the first one informs the consumers about the changes in food features by using indicators; while AP controls the growth of microorganisms thanks to the presence in its composition of specific compounds (Khaneghah *et al*., 2018; Quesada *et al*., 2016). The idea of adopting an antimicrobial packaging capable of reducing, retarding, or inhibiting microbial replication attracts many Food Business Operators (FBO). Indeed, thanks to the malleability of this device that allows it to be used for direct (interacting with the packaged food) or indirect contact (package headspace) (Otoni *et al*., 2016), the fields of applicability of this innovative strategy are innumerable.

In today' world, the plastic materials used in the packaging of food products are polyamide (PA), polystyrene (PS), polypropylene (PP), polyethylene (PE), polyvinylchloride (PVC) and polyethylene terephthalate (PET), particularly popular because of their reduced costs.

AP can be obtained from new engineering materials and nowadays several forms have been launched into the marketplace, such as devices containing volatile antimicrobials, polymer films that incorporate antimicrobial substances (extrusion, casting) and coating, adsorption or grafting of substances on the polymer surface (Radusin *et al*., 2013).

One of the most common strategies for extending the *shelf-life* of perishable foods or/and reducing the risk of foodborne disease is the use of packaging that incorporates natural antimicrobial compounds (Liang *et al*., 2019; Barbiroli *et al*., 2017). Several authors (Gogliettino *et al*., 2020) have already demonstrated the concrete opportunity of including antimicrobial substances in the food packaging material.

The list of antimicrobial agents that can be used in the development of AP is long and growing. Nevertheless, the choice of compounds is not accidental and depends on their physicochemical characteristics and spectrum of action; it is important to know the target microorganisms of the antimicrobial molecules in order to obtain the best results (Dobrucka *et al*., 2013). Furthermore, the nature and chemical composition of the food, and the methods of storage are factors that could affect the effectiveness of the systems, for example by influencing the ability of the antimicrobial molecules to reach bacterial cells.

1.3 A brief overview of natural antimicrobial agents: classification and mechanisms of action

Natural preservatives are good candidates as environmentally friendly additives to employ in the formulation of innovative food packaging. Several researchers are focusing their attention on the research and characterization of natural molecules extractable from different sources. Their goal is to satisfy the demand of the FBOs which attempt to conquer the consumers' approval by offering the so much sought-after "natural foods". Therefore, the engine of this evolution is the consumer who drives

the interest of the scientific communities and the food industry towards natural compounds in full compliance with naturalness and environmental concerns (Román *et al*., 2017; Smith *et al.*, 2017).
Plants, animals, bacteria, algae, and fungi are some of the sources of natural preservatives (Ghanbari *et al*., 2013; Hassoun *et al*., 2017). Several papers have devoted attention to specific natural compounds, pointing out their properties through studies *in vitro* which unveiled their main abilities.
Mei *et al*., 2019, classified the preservative natural compounds based on their source in:

- plant-derived preservatives (berry extract, rosemary extract, etc.);
- animal-derived preservatives (e.g., chitosan);
- microbial-derived preservatives (e.g., bacteriocin).

Furthermore, several bioactive compounds extracted from algae, mushrooms and so on can also provide a potential source of new natural preservatives in the food industry (Gyawali and Ibrahim, 2014).
However, the final goal is to investigate the applicability of each extract/fraction/molecule in the food industry and a simpler classification method provides information on their activities. To this end, natural preservatives can be divided (Barreiro *et al*., 2014; Carocho *et al*., 2015) for their:

- antioxidant properties;
- antimicrobial properties;
- antibrowning properties.

Natural antimicrobial substances are widely sought because they are able to guarantee safe food, acting against pathogenic and spoilage microorganisms, without bumping the sensibility of people who reject the chemical equivalents.
Ideally, natural preservatives should have the following properties:

- broad bactericidal and fungicidal activities;
- be non-toxic;
- be active at low concentrations;
- impart no flavour or colour to food;
- have no pharmaceutical applications;
- label-friendly;
- cost-effective (Carocho *et al*., 2015).

Concluding, natural preservatives endowed with broad antimicrobial mechanisms may concretely contribute to the preservation of freshness, appearance and safety of meat and meat products, dairy products (Gogliettino *et al*., 2020), and fish and fish products (Carlucci *et al*., 2015; Thong and Solgaard, 2017).

1.3.1 Plant-Derived Compounds

For many years the scientific community has been investigating all the possible applications of the plants. The attention of the food industry is also drawn to plant substances for their bioavailability, low costs, malleability, and abundance of active molecules, terms that classify them as perfect candidates for food preservatives (Giacometti *et al*., 2018).
The idea to take advantages of the full range of bioactive compounds dates back a long time, when the first drugs were made with plants (Antolak and Kregiel, 2017), exploiting the compounds that, normally, the plant organisms produce to defend their self from the attack of microorganisms. Although the evolution in drug discovery, the inclination of using plant extract or molecules as antimicrobial substances is very current. The phytochemicals are extracted from many portions of plants, from the green portions (leaves and stems) to the roots (Novak *et al.,* 2005; Olawore *et al*., 2005), and they are classified as essential oils or plant extracts.

1.3.1.1 Essential Oils (EOs)

EOs are complex mixtures of volatile organic compounds (VOCs), produced by a steam distillation process (Van de Braak & Leijten, 1999) and obtained from distinct parts of the plant such as flowers, buds, leaves, stem, bark and seeds (Riberio-Santos *et al*., 2017). The importance of the production method is underlined by the definition of an "essential oil" reported by the International Organization for Standardization (ISO) (ISO DIS 9235.2) which established that it is "a product made by distillation with either water or steam or by mechanical processing of citrus rinds or by dry distillation of natural materials" (Silva and Domingues, 2017).
The VOCs are secondary metabolites in plants that are responsible for their odour (Fleita *et al*., 2015), but their main role is linked to their antioxidant and antimicrobial properties (Calo *et al*., 2015).
The mechanism of action of essential oils is based on their hydrophobic nature which allows them to penetrate and overcome the lipid bilayer of the bacterial membrane which is modified in its fluidity and permeability (Figure 1.2). These events disturb the internal and external cellular environment, resulting in electrolyte imbalance, increased permeability, interference of the interactions between proteins and lipids (Vergis *et al*., 2015). Inevitably, cell lysis and the death of bacterial cells occur (Silva and Domingues, 2017).
It is important to point out that flavouring substances belonging to EOs are approved for use as flavourings by the EFSA Authority (EC, 1333/2008). In the United States, EOs and their constitutive parts are also recognized as flavouring compounds by the Food and Drug Administration and have GRAS (Generally Recognized as Safe) status (FDA, 2019).

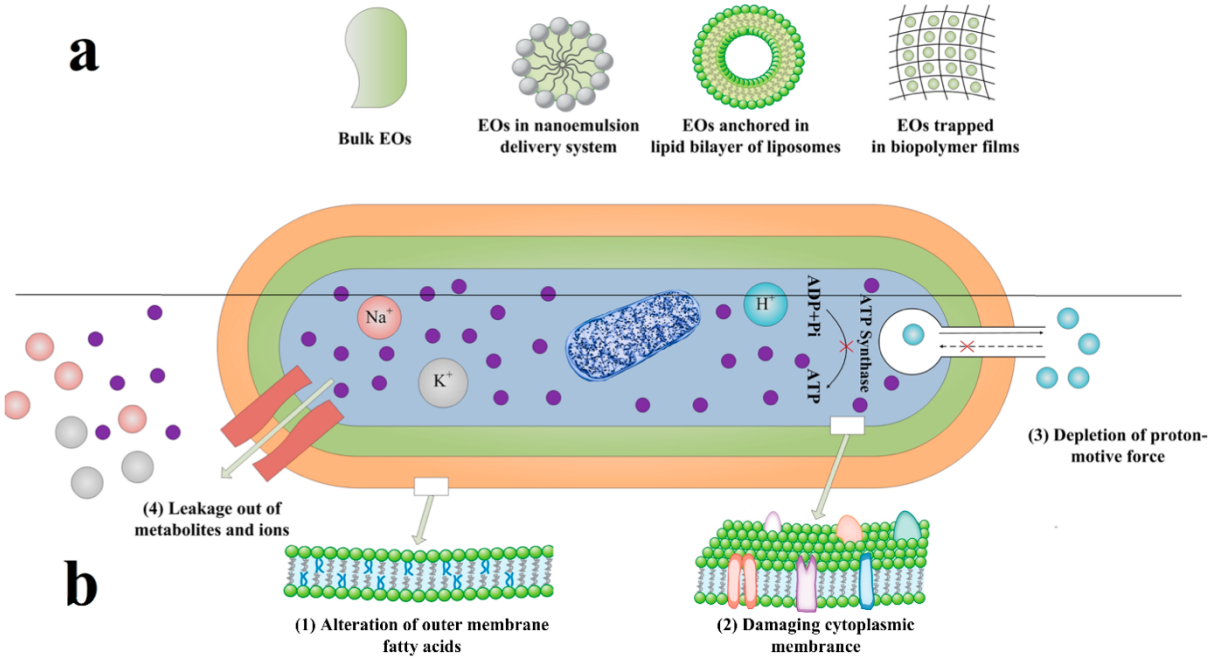


Figure 1.2 *Mechanisms of action of EOs: (**a**) Bulk EOs and several types of EO delivery systems; (**b**) common described mechanisms of action of EOs (Mei et al., 2019).*

1.3.1.2 Plant Extracts (PEs)

The plant extracts (PEs) are materials resulting from a series of steps which include a previous washing, followed by drying, grinding, and extraction, commonly with solvents (Ambrosio *et al*., 2020). Several authors have demonstrated that plant bioactive compounds are very effective against microorganisms responsible for foodborne disease and food spoilage (Ambrosio *et al*., 2020; Calo *et al*., 2015; Gyawali and Ibrahim, 2014).
PEs antimicrobial activity is mainly due to their content of polyphenols which are absorbed into the bacterial membrane and causes membrane disruption and generation of hydroperoxides. Cell death is a consequence of all these damage events (Akter *et al*., 2019; Bouarab Chibane *et al*., 2019).

1.3.2 Animal-Derived Compounds

At present, many antimicrobial compounds derived from animals or animal products have been used as food preservatives. Not all have been recognized and investigated in the same way. In the years, researchers have tested molecules as lactoperoxidase, lysozymes and lactoferrin, derived from eggs and milk, respectively; however, the chitosan is the compound that more than the other has attracted attention (Zheng, 2014). It is worth noting that the use of these molecules is limited because they are capable of causing allergies (Ford *et al*., 2010).

1.3.2.1 Chitosan

The chitosan is derived from the deacetylation process of the chitin (α-1,4-N-acetylglucosamine), a linear polymer that widely spreads in nature. It is well-known that is the constitutive element of the exoskeletons of arthropods and crustaceans (Tikhonov *et al*., 2006), however it is also found in the wall of fungi, yeasts and protozoa (Alishahi and Aïder, 2012; Muzzarelli, 2011).
The spectrum of action of chitosan has been extensively studied and characterized in the last years. It is known that it is able to act against various microorganisms including fungi and bacteria, and its effectiveness is influenced by many factors, intrinsic or not to the molecule. This latter condition has generated a wide variability of the results (Divya *et al*., 2018; Dutta *et al*., 2009) when the chitosan antimicrobial efficacy was tested.
Diverse hypotheses were formulated on the mechanism of action of the chitosan. Many authors have supported the idea that this compost is capable of penetrating in the cytoplasm, where could interact directly with the bacterial DNA or inhibit the mRNA and proteins synthesis (Sudarshan *et al*., 1992). However, the most accredited theory is founded on the binding of its positively charged amino groups (NH_3^+) with the negative carboxyl groups (COO^-) of the outer membrane or the cell wall of bacteria and fungi. This interaction may modify the membrane permeability or interfere with the exchange systems of the lipidic bilayer, blocking the transition of nutrients and oxygens (Baptista *et al*., 2020).

1.3.3 Microbial-Derived Compounds

Lactic acid bacteria (LAB) are gram-positive bacteria capable to produce several substances widely used in the food industry. Their main product is lactic acid, as a result of the fermentation of glucose (Axelsson, 1993). Nevertheless, specific strains produce inhibitory compounds with an antibacterial activity also against pathogenic microorganisms (Gálvez *et al*., 2010). Among the metabolites, bacteriocins have been considered as ideal agents for food preservation, thanks to their broad spectrum of action (Sarika *et al*., 2012).

1.3.3.1 Bacteriocins

The term “bacteriocin” is commonly used to indicate very small antimicrobial proteins/peptides (AMP) which are synthesized at the ribosomal level of different bacteria (Cotter *et al*., 2005) to inhibit the growth of adjacent bacteria (Hugas, 1998). They are natural antimicrobial compounds with positively charged elements that can interact electrostatically with the phosphate groups on the microbial membranes negatively charged, resulting in the generation of pores (Mousavi Khaneghah *et al*, 2018). It is worth pointing out that there is an extensive variety of these polypeptides, which are biochemically, genetically, structurally, and functionally different (Galvez *et al*., 2008). The importance of bacteriocins derived from LABs is due to their GRAS status.
Bacteriocins are generally categorized into four classes (Klaenhammer, 1993; Balciunas *et al*., 2013):

I. lantibiotics, low molecular weight (<5 kDa) thermostable peptides, characterized by the presence of non-proteogenic thioether amino acids lanthionine (Lan) or methyllanthionine (MeLan) (McAuliffe *et al*., 2001; Lee and Kim, 2011);

II. small thermostable peptides (<10 kDa) composed of 30 to 60 amino acids and do not contain Lan or MeLan; they are subdivided into three subclasses:

IIa: pediocin-like or *Listeria-active*, composed of 37 to 48 amino acids and with one or two α-helix (Lee and Kim, 2011),

IIb (lactocin G),

IIc (lactocin B);

III. high molecular weight (>30 kDa) thermolabile peptides, represented by helveticin J;

IV. large peptides complexed with lipids or carbohydrates.

Each class of bacteriocins has not only structural differences but also different target bacteria and mechanisms of action. The first class, for instance, is not capable to reduce or inhibit Gram-negative bacterial growth. The spectrum of action of lantibiotics is close and it includes only the Gram-positive microorganisms (Lee and Kim, 2011; Wiedemann *et al*., 2001).

Regarding the mechanism of action, the electrostatic charge of bacteriocins plays a crucial role allowing the formation of bonds between specific bacterial receptors of membrane and the antimicrobial polypeptide that result in the formation of pores (Souza *et al*., 2005; Hernández-Dominguez *et al*., 2008). This phenomenon, as in the case of other molecules already discussed, induces the increase in membrane permeability and loss of cellular nutrients and ions (Ariyapitipun *et al*., 2000). If the formation of pores is an effect common to all bacteriocins, the pore stability and conductivity differ from one class to another (Herranz *et al*., 2001).

The concrete applicability of these molecules in the food industry is impeded by many factors. Baptista *et al.* (2020) have listed the adverse conditions as reported below:

a) interaction with food macromolecules as proteins and lipids, which can be interposed between the receptors and ligands;
b) presence of proteases, which are able to act bacteriocins (Schillinger *et al*., 1996);
c) food transformation and storage could affect the polypeptide stability by modifying parameters such as pH and temperature;
d) the high initial level of contamination of the food, that could slow down the bacteriocin efficacy to prevent bacterial replication (Rilla *et al*., 2004);
e) composition of the microbiota, as some bacteriocins are not capable of inhibiting the growth of gram-negative bacteria.

In agreement with the Codex Alimentarius Commission 283–1978 and the Directive 95/2/EC of the European Union (Codex, 1978; EU, 2011), the only bacteriocin approved for direct incorporation into food as a food additive (E 234) is nisin (EC, 1333/2008). Nisin is a polypeptide of 34 amino acids belonging to the first class of bacteriocins. It was discovered in 1928 as a product of *L. lactis*. It has been introduced in the food industry as a GRAS food preservative (Gharsallaoui *et al*., 2016, Chaves *et al*., 2017), with the purpose to prevent spore germination and reduce the growth of Gram-positive pathogenic bacteria, such as *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Clostridium perfringes* and *Streptococcus* spp. (Matamoros *et al*., 2009; Solomakos *et al*., 2008).

1.3.3.2 Organic acids

Organic acids are organic compounds with one or more carboxyl groups (-COOH). They are known to possess antimicrobial activities and their potential application in food application have been extensively investigated. They originate as metabolites of the fermentative metabolism of several microorganisms. Citric and lactic acid, for instance, are produced by *Aspergillus niger*, and *Streptococcus lactis*, respectively (Davidson and Branen, 1993; Kandler, 1983).

Categorized as GRAS (Vázquez *et al*., 2005), organic acids are commonly used in the food industry as acidulants, chelating agents, flavourings, and preservatives. They are, in fact, potent antimicrobials

especially in undissociated form, when provided with hydrophobic features. In this state, organic acids overcome passively the lipid bilayer reaching the cytoplasm. The high pH facilitates the dissociation of the acid, and the accumulation of ions results in bacterial death (polar ions are responsible for inhibiting metabolic enzymes; accumulation of protons induced the acidification of the cytoplasm; accumulation of ions contributes to increasing the osmotic) (Denyer and Stewart, 1998; Cherrington *et al*., 1991).
Several organic acids, their salts or anhydrides are listed as food preservatives in the EU database (Annex II of Regulation (EC) No 1333/2008).

1.4 Antimicrobial Peptide (AMP) Structure and Mechanism of Action

1.4.1 History and role of Antimicrobial Peptides

Antimicrobial Peptides are small protein molecules used by different organisms as a weapon to defend themselves against microbial infections (Zasloff, 2002a; Zasloff, 2002b). The first AMP dates back to 1939 (Dubos and Hotchkiss, 1941). It was isolated from bacteria of the *Bacillus* genus and was utilized in mise as a preventive therapy against pneumococcal infection. Only after years, the molecule was called Gramicidin (Dubos, 1939). Since the first discovery, new peptides were unearthed from different sources. The years 1941 and 1956 were marked by two memorable events: for the first time a plant organism and an animal organism were used as a substrate for isolation and identification of antimicrobial peptides. In fact, the purothionin was isolated from the *Triticum aestivum* (Phoenix *et al*., 2013), while the phagocytin was from rabbit leukocytes (Hirsch, 1956).
The discovery of the existence of small natural compounds endowed with antimicrobial properties has attracted attention and many studies have focused on their role. Gradually, the investigators came to the conclusion that AMPs act as constituents of the immune system (Phoenix *et al*., 2013) of both prokaryotes (e.g., bacteria) and eukaryotes (e.g., fungi, plants, and animals) (Peters *et al*., 2010). Especially in mammals, AMPs constitute the first immune barrier and promote the immune system via specific events (such as the activation of T cells; prompt phagocytosis; activation of dendritic cells and chemoattraction of neutrophils) (Matejuk *et al*., 2010).
One of the most important properties of the AMPs is the variety and their diversity results in a wide spectrum of action against various pathogens. However, they bonded over some structural characteristics. For instance, most of the AMPs have a positive charge (from +1 to +9) and, therefore, are cationic (CAMPs) (Farkas *et al*., 2017; Lee *et al*., 2013). Moreover, they have an amphipathic structure with separate hydrophobic and hydrophilic domains.
A large multitude of antimicrobial peptides exist, and this allows them to be used against a so large number of microorganisms. It is true that the simplest method to classify these molecules is based on the pathogen targets, which classifies them as antibacterials, antifungals, antivirals, and antiparasitics. However, because some of them may exhibit activity versus more than one target, the adjective “antimicrobial” was generally adapted to refer to these peptides.

1.4.2 Physicochemical parameters that affect antimicrobial activities

For many years the scientific community has been aware of the role of numerous physicochemical features of AMPs in determining their general activities. These parameters are tied down with those of membrane lipids of target microorganisms (Yeaman and Yount, 2003). Nevertheless, to this day the exact relationship among all the factors is not well understood. Molecule charge, length and amino acid composition of the sequence, hydrophobicity and amphipathicity are some of the main influencing specifications (Auvynet and Rosenstein, 2009; Guilhelmelli *et al.,* 2013) of the antimicrobial peptides.
It is well known that the first interaction between the peptide and the outer membrane is due to the positive charge of the antimicrobial molecule (electrostatic interaction). Moreover, a close directly

proportional relationship exists between the positive charge and the antimicrobial activity (Dathe *et al*., 1997; Bonucci *et al*., 2014; Fillion *et al.,* 2015). However, for binding to occur, the peptide must interact with a lipid membrane characterized by specific charge density. When negatively charged lipids (such as phosphatidylglycerol (PG) and cardiolipin (CL)) are included in the membrane composition, the CAMPs partition is generally strong. On the contrary, the same peptides could very probably record weak partition into zwitterionic lipids.
For this to work, the peptide hydrophobicity is essential because it allows the partition of the molecule into the membrane. According to Lee *et al.* (2013), almost all the AMPs are characterized by a hydrophobic residues content of approximately 50%. Furthermore, the same relationship described above for the charge also exists between the antimicrobial activity and this parameter (Chen *et al*., 2007), but it is important that its optimal percentage is not exceeded. Indeed, Chou *et al*. (2008) showed that by increasing hydrophobicity the peptide not only loses its activities, but it acquires toxicity towards mammalian cells.
Regarding the peptide structure, the AMPs are classified into 4 structural groups: α-helical peptides, β-strand/sheet peptides, mixed helical/sheet peptides and extended non-helical/sheet peptides. The peptide conformation has a strong impact on its first interactions with membranes and subsequent binding. Generally, most AMPs have an unstructured flexible conformation in a solution that stiffens upon lipid interaction.
Overall, the analysis of charge, structure and hydrophobicity singularly highlighted the crucial role of each parameter. However, they never act individually and the result of the unanimous work of these three factors counts (Fillion *et al*., 2015; Shai, 1999; Zhu *et al*., 2015). It is customary, in fact, to refer to the amphipathic structure which identifies the positions and the relative proportions of hydrophilic and hydrophobic domains within the peptide.

1.4.3 Mechanisms of action

The mechanism of action of antimicrobial peptides is a hot topic that has been amply investigated in the last decades. Scientists agree that the first stage of membrane interaction is common to all AMPs, as though the events cascade of all the main mechanistic steps that occur from the first contact with the membrane to the cell death. What differs is how the peptide causes the death of the microorganism and, to this purpose, the effect of the interaction AMP-lipids can be classified as membrane disruptive/permeabilizing and non-membrane disruptive/permeabilizing (Wang *et al*., 2015). The formation of pores in the membrane is only one of the possible mechanisms of action (Lee *et al*., 2013), and more models are proposed to explicate how AMPs induce cell death.
The events cascade mentioned above consists of five moments (Koprivnjak and Peschel, 2011):

1. initial attraction;
2. interaction peptide-lipids and peptide structural adjustment;
3. peptide concentration and accumulation up to an active stoichiometric level (Moravej *et al*., 2018);
4. membrane destabilization;
5. achievement of the final targets of the membrane or inside the cell.

Briefly, the cationic residues of CAMPs are attracted via electrostatic forces from negatively charged lipids of the bacterial membrane target (Bessalle *et al*., 1992; Matsuzaki *et al*., 1997; Melo *et al*., 2009). Once nearby to lipids, peptides change their conformations, commonly thought a coil-helix transition (Bello *et al*., 1982; Dathe *et al*., 1999). However, this is not true for β-sheet AMPs which have a more rigid structure already in solution.
Following the creation of the bond, a critical concentration of peptides is necessary to induce the molecule self-association and penetrate the lipid bilayer. Once located in the membrane core, AMPs can explicate their action via a variety of mechanisms (Huang, 2006; Hall *et al*., 2012). The self-association and the multimerization are key phenomena in the achievement of the critical peptide

concentration (Holthuis and Menon, 2014). The propensity to create complex structures through peptide-peptide and peptide-lipids interactions depends on the amino acid sequence of the peptides.
Some AMPs can overcome bacterial membranes and target nucleic acids or/and proteins. The main mechanisms consist of the inhibition of nucleic acid and protein synthesis that affect, inhibition of cell wall syntheses and induction of cell death (Guilhelmelli *et al.,* 2013).
To describe the AMPs action on the membrane, authors have used different models (Lee *et al*., 2013), however, two of them have been more frequently associated with the peptides studied so far: the barrel-stave/toroidal pore models and carpet-like mechanisms.

1.4.3.1 The barrel-stave/toroidal pore models

The main two models of pore formation are the "barrel stave" mechanism and the "toroidal wormhole" mechanism. The first one was the first mechanism to be proposed (Baumann and Mueller, 1974) and foresees the formation of large structures of peptides that penetrate deeply in the bilayer forming a ring like a "barrel" pore (Figure 1.3). The term "stave" is connected to the individual spokes within the barrel. The pore structure is characterized by the position of hydrophobic domains of the AMPs facing toward the lipid face, while hydrophilic ones facing toward the aqueous pore (Breukink and de Kruijff, 1999; Ehrenstein and Lecar, 1977).
The "toroidal pore" or "wormhole" mechanism does not diverge from the previous one. After the peptide interaction with the membrane target and the conformational changes, the helices get in the horizontal position with their hydrophobic domains facing towards the phosphorous heads of lipids. As result, a breach and a positive curvature in the membrane are produced with a final thinning and destabilization of the membrane (Yeaman and Yount, 2003). On the whole, this model differs from the barrel stave pore as the peptides still interact with the lipid head groups and are not situated within the membrane hydrophobic core; however, it is less stable and reversible. This instability could be positively read as a desired condition, since authors (Wang *et al*., 2015) have described the AMPs translocation to the luminal of the model membranes through the pores. Therefore, the antimicrobial peptides may use this system to reach targets located inside the bacterial cells.

1.4.3.2 Carpet-like mechanisms

As reported above, AMPs adopt several mechanistic models which do not all result in the formation of pores. The carpet-like mechanisms are so called because of the peptides disposition on the membrane. They cover the outer surface, inserting or not into the membrane core (Taubes, 2008; Shai, 1999; Shai and Oren, 2001); then the achievement of the critical concentration, the coating of peptides induces the membrane destabilization by operating on its charge. The final result is the membrane damage with its fragmentation in micelles. So much so that many authors have considered this model as the previous step of the toroidal pore formation.

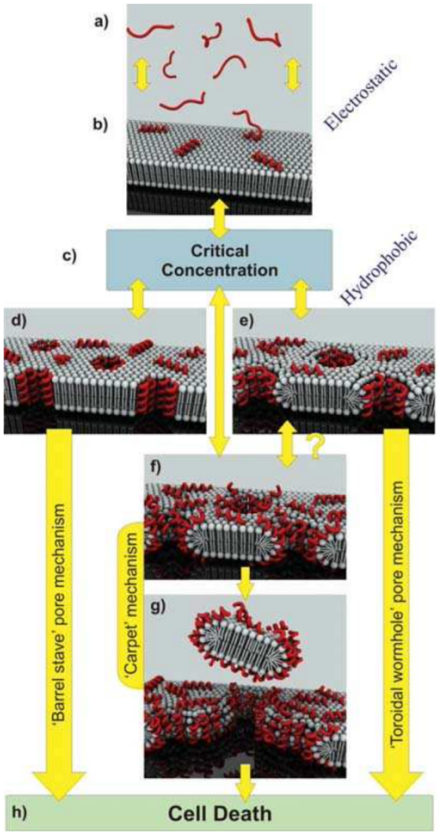


Figure 1.3 *Graphical representation of the main reported mechanisms of action of AMPs (Lee et al., 2016).*

1.5 References

Akter S, Netzel ME, Tinggi U, Osborne SA, Fletcher MT, Sultanbawa Y, 2019. Antioxidant rich extracts of *Terminalia ferdinandiana* inhibit the growth of foodborne bacteria. Foods 8, 281.

Alak G, 2012. The effect of chitosan prepared in different solvents on the quality parameters of brown trout fillets (Salmo trutta fario). Food Nutr Sci 3, 1303–1306.

Ambrosio RL, Gratino L, Mirino S, Cocca E, Pollio A, Anastasio A, Palmieri G, Balestrieri M, Genovese A, Gogliettino M, 2020. The bactericidal activity of protein extracts from Loranthus europaeus berries: a natural resource of bioactive compounds. Antibiotics 9(2), 47.

Antolak H, Kregiel D, 2017. Food preservatives from plants. In D. N. Karunaratne, & G. Pamunuwa (Eds.). Food additives (1st ed.). IntechOpen.

Ariyapitipun T, Mustapha A, Clarke AD, 2000. Survival of Listeria monocytogenes scott a on vacuum-packaged raw beef treated with polylactic acid, lactic acid, and nisin. J Food Protect 63(1), 131–136.

Auvynet C, Rosenstein Y, 2009. Multifunctional host defense peptides: antimicrobial peptides, the small yet big players in innate and adaptive immunity. FEBS J 276:6497–6508.

Axelsson LT, 1993. Lactic acid bacteria: Classification and physiology. In S. Salminen, & A. Von Wright (Eds.). Lactic acid bacteria (1 nd) (pp. 1–64). New York, NY, USA: Marcel Deker.

Balciunas EM, Martinez FA, Todorov SD, de Melo Franco BD, Converti A, de Souza Oliveira RP, 2013. Novel biotechnological applications of bacteriocins: A review. Food Control 32, 134–142.

Baptista RC, Horita CN, Sant'Ana AS, 2020. Natural products with preservative properties for enhancing the microbiological safety and extending the shelf-life of seafood: A review. Food res int 127, 108762.

Barbiroli A, Musatti A, Capretti G, Iametti S, Rollini M, Sakacin, 2017. An antimicrobial packaging for decreasing Listeria contamination in thin-cut meat: Preliminary assessment. J Sci Food Agric 97, 1042–1047.

Barreiro MF, Morales P, Ferreira ICFR, 2014. Adding molecules to food, pros and cons: A review on synthetic and natural food additives. Compr rev in food sci f 13(4), 377-399.
Baumann G, Mueller P, 1974. A molecular model of membrane excitability. J Supramol Struct 2, 538-557.
Bello J, Bello HR, Granados E, 1982. Conformation and aggregation of melittin: dependence on pH and concentration. Biochemistry 21, 461-465.
Bessalle R, Haas H, Goria A, Shalit I, Fridkin M, 1992. Augmentation of the antibacterial activity of magainin by positive-charge chain extension. Antimicrob Agents Chemother 36, 313- 317.
Bonucci A, Balducci E, Martinelli M, Pogni R, 2014. Human neutrophil peptide 1 variants bearing arginine modified cationic side chains: effects on membrane partitioning. Biophys Chem 190-191, 32-40.
Bouarab Chibane L, Degraeve P, Ferhout H, Bouajila J, Oulahal N, 2019. Plant antimicrobial polyphenols as potential natural food preservatives. J Sci Food Agric 99(4):1457-1474.
Breukink E, de Kruijff B, 1999. The lantibiotic nisin, a special case or not? Biochim Biophys Acta 1462, 223-234.
Calo JR, Crandall PG, O'Bryan CA, Ricke SC, 2015. Essential oils as antimicrobials in food systems—A review. Food Control 54, 111–119.
Carlucci D, Nocella G, De Devitiis B, Viscecchia R, Bimbo F, Nardone G, 2015. Consumer purchasing behaviour towards fish and seafood products. Patterns and insights from a sample of international studies. Appetite 84, 212-227.
Carocho M, Morales P, Ferreira ICFR, 2015. Natural food additives: Quo vadis? Trends Food Sci Technol 45(2), 284-295.
Chaillou S, Chaulot-Talmon A, Caekebeke H, Cardinal M, Christieans S, Denis C, Desmonts MH, Dousset X, Feurer C, Hamon E, 2015. Origin and ecological selection of core and food-specific bacterial communities associated with meat and seafood spoilage. ISME J 9, 1105–1118.
Chaves, C.L.; Serio, A.; Montalvo, C.; Ramirez, C.; Peréz, J.Á.; Paparella, A.; Mastrocola, D.; Martuscelli, M, 2017. Effect of nisin on biogenic amines and shelf life of vacuum packaged rainbow trout (*Oncorhynchus mykiss*) fillets. J Food Sci Technol 54(10):3268-3277.
Chen Y, Guarnieri MT, Vasil AI, Vasil ML, Mant CT, Hodges RS, 2007. Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides. Antimicrob. Agents Chemother 51, 1398-1406.
Cherrington CA, Hinton M, Mead GC, Chopra I, 1991. Advances in Microbial Physiology, Vol. 32, 87–108.
Chou HT, Kuo TY, Chiang JC, Pei MJ, Yang WT, Yu HC, Lin SB, Chen WJ, 2008. Design and synthesis of cationic antimicrobial peptides with improved activity and selectivity against Vibrio spp. Int J Antimicrob Agents 32, 130-138.
Codex (1978). Commission codex alimentarius. General Standard for Cheese. Codex Standard 283-1978. Retrieved from www.fao.org/input/download/standards/175/ CXS_283s.pdf.
Cotter PD, Hill C, Ross RP, 2005. Bacteriocins: Developing innate immunity for food. Nat Rev Microbiol 3(10), 777–788.
Dathe M, Wieprecht T, 1999. Structural features of helical antimicrobial peptides: their potential to modulate activity on model mem- branes and biological cells. Biochim. Biophys. Acta, 1462, 71-87.
Dathe M, Wieprecht T, Nikolenko H, Handel L, Maloy WL, MacDonald DL, Beyermann M, Bienert M, 1997. Hydrophobicity, hydrophobic moment and angle subtended by charged residues modulate antibacterial and haemolytic activity of amphipathic helical peptides. FEBS Lett., 403, 208-212.
Davidson PM, Branen A, 1993. Produção de ácido cítrico por Aspergillus Niger: avaliação de diferentes fontes de nitrogênio e de concentração de sacarose. In Antimicrobials in food 647.
Dehnad D, Emam-Djomeh Z, Mirzaei H, Jafari SM, Dadashi S, 2014. Optimization of physical and mechanical properties for chitosan- nanocellulose biocomposites. Carbohydr Polym 105:222–8.

Denyer SP, Stewart GSAB, 1998. Mechanisms of action of disinfectants. Int Biodeter Biodegr 41(3–4), 261–268.
Devlieghere F, Vermeiren L, Debevere J, 2004. New preservation technologies: Possibilities and limitations. Int Dairy J 14(4), 273-285.
Divya K, Smitha V, Jisha M S, 2018. Antifungal, antioxidant and cytotoxic activities of chitosan nanoparticles and its use as an edible coating on vegetables. Int J Biol Macromol 114, 572–577.
Dobrucka R, 2015. Antimicrobial packaging with natural compunds - a review. Logforum, 12, 193–202.
Dubos RJ, 1939. Studies on a bactericidal agent extracted from a soil bacillus: II. Protective effect of the bactericidal agent against experimental pneumococcus infections in mice. J Exp Med 70:11–17.
Dubos RJ, Hotchkiss RD, 1941. The production of bactericidal substances by aerobic sporulating bacilli. J Exp Med 73:629–640.
Dutta PK, Shipra T, Mehrotra GK, Joydeep D, 2009. Perspectives for chitosan based antimicrobial films in food applications. Food Chem 114, 1173–1182.
Ehrenstein G, Lecar H, 1977. Electrically gated ionic channels in lipid bilayers. Q Rev Biophys 10, 1-34.
Ercolini D, Russo F, Torrieri E, Masi P, Villani F, 2006. Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. Appl Environ Microbiol 72(7), 4663-4671.
EU, C. E. (2011). COMMISSION REGULATION (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. Retrieved from https:// www.fsai.ie/uploadedFiles/Reg1129_2011.pdf.
European Commission Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on Food Additives. *O*ff. *J. Eur. Union.* 2008, *L 354*, 16–33. Available online: https://eur-lex.europa.eu/eli/reg/2008/1333/2012-12-03 (accessed on 20 January 2020).
European Food Safety Authority, & European Centre for Disease Prevention and Control. (2021). The European Union One Health 2019 Zoonoses Report. *EFSA Journal*, *19*(2), e06406.
European Union Food Safety to Fork: Safe and Healthy Food for Everyone The EU Explained, Agriculture. Available online: http://europa.eu/pol/index_en.htm (accessed on 1 March 2020).
FAO. 2013. *Food wastage footprint; impacts on natural resources.* Summary Report. Rome. (www. fao.org/docrep/018/i3347e/i3347e.pdf).
Farkas A, Maróti G, Kereszt A, Kondorosi É, 2017. Comparative Analysis of the Bacterial Membrane Disruption Effect of Two Natural Plant Antimicrobial Peptides. Front Microbiol 23;8:51.
Fillion M, Valois-Paillard G, Lorin A, Noel M, Voyer N, Auger M, 2015. Membrane interactions of synthetic peptides with antimicrobial potential: effect of electrostatic interactions and amphiphilicity. Probiotics Antimicro 7, 66-74.
Fleita D; El-Sayed M; Rifaat D, 2015. Evaluation of the antioxidant activity of enzymatically-hydrolyzed sulfated polysaccharides extracted from red algae; Pterocladia capillacea. LWT-Food Sci Technol 63, 1236–1244.
Food and Drug Administration, U.S.D. of H. and H.S. CFR - Code of Federal Regulations Title 21. Available online: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=182.20 (accessed on 27 November 2019).
Ford LS, Taylor SL, Pacenza R, Niemann LM, Lambrecht DM, Sicherer SH, 2010. Food allergen advisory labeling and product contamination with egg, milk, and peanut. J Allergy Clin Immun 126, 384–385.
FUSIONS (Food Use for Social Innovation by Optimizing Waste Prevention Strategies), 2016. Estimates of European Food Waste Levels, Stockholm.

Galvez A, Lopez RL, Abriouel H, Valdivia E, Omar N Ben, 2008. Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. Crit Rev in Biotechnology 28(2), 125–152.
Gálvez A, Abriouel H, Benomar N, Lucas R, 2010. Microbial antagonists to food-borne pathogens and biocontrol. Curr Opin Biotechnol 21(2):142-8.
Ghaly AE, Dave D, Budge S, Brooks MS, 2010. Fish spoilage mechanisms and preservation techniques: Review. Am J Appl Sci 7, 859–877.
Ghanbari M, Jami M, Domig KJ, Kneifel W, 2013. Seafood biopreservation by lactic acid bacteria—A review. LWT-Food Sci Technol 54, 315–324.
Gharsallaoui A, Oulahal N, Joly C, Degraeve, P, 2016. Nisin as a food preservative: Part 1: Physicochemical Properties, Antimicrobial Activity, and Main Uses. Crit Rev Food Sci Nutr 56(8):1262-74.
Giacometti J, Bursać Kovačević D, Putnik P, Gabrić D, Bilušić T, Krešić G, Stulić V, Barba FJ, Chemat F, Barbosa-Cánovas G, Režek Jambrak A, 2018. Extraction of bioactive compounds and essential oils from mediterranean herbs by conventional and green innovative techniques: A review. Food Res Int 113:245-262.
Gogliettino M, Balestrieri M, Ambrosio RL, Anastasio A, Smaldone G, Proroga YTR, Moretta R, Rea I, De Stefano L, Agrillo B, Palmieri G, 2020. Extending the Shelf-Life of Meat and Dairy Products via PET-Modified Packaging Activated With the Antimicrobial Peptide MTP1. Front Microbiol 10:2963.
Gram L, Ravn L, Rasch M, Bruhn JB, Christensen AB, Givskov M, 2002. Food spoilage-interactions between food spoilage bacteria. Int J Food Microbiol 78 (1-2), 79-97.
Guilhelmelli F, Vilela N, Albuquerque P, Derengowski L, Silva-Pereira I, Kyaw C, 2013. Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance Front Microbiol 4, 353.
Gyawali R, Ibrahim SA, 2014. Natural products as antimicrobial agents. Food Control 46, 412–429.
Hall K, Lee TH, Daly NL, Craik DJ, Aguilar MI, 2012. Gly6 of kalata B1 is critical for the selective binding to phosphatidylethanolamine membranes. Biochim Biophys Acta 1818, 2354-2361.
Hashemi Tabatabaei R, Jafari SM, Mirzaei H, Mohammadi Nafchi A, Dehnad D, 2018. Preparation and characterization of nano-SiO2 reinforced gelatin-k-carrageenan biocomposites. Int J Biol Macromol 111:1091–9.
Hassoun A, Çoban ÖE, 2017. Essential oils for antimicrobial and antioxidant applications in fish and other seafood products. Trends Food Sci Technol 68, 26–36.
Hernández-Dominguez C, Hernández-Anguiano A, Cháidez-Quiroz C, Rendón-Sanchez G, Suslow T, 2008. Detección de *Salmonella* y coliformes fecales en agua de uso agrícola para la producción de melón "Cantaloupe". Agric Técnica en México 34(1), 75-84.
Herranz C, Casaus P, Mukhopadhyay S, Martínez JM, Rodríguez JM, Nes IF, Hernández PE, Cintas LM, 2001. Enterococcus faecium P21: A strain occurring naturally in dry- fermented sausages producing the class II bacteriocins enterocin A and enterocin B. FoodMicrobiology, 18, 115–131.
Hirsch JG, 1956. Phagocytin: a bactericidal substance from polymorphonuclear leucocytes. J Exp Med 103:589–611.
Holthuis JC, Menon AK, 2014. Lipid landscapes and pipelines in membrane homeostasis. Nature, 510, 48-57.
Huang HW, 2006. Molecular mechanism of antimicrobial peptides: the origin of cooperativity. Biochim Biophys Acta 1758, 1292- 1302.
Hugas M, 1998. Bacteriocinogenic lactic acid bacteria for the biopreservation of meat and meat products. Meat Sci 49(98), S139-S150.
ISO/DIS 9235.2: 1997. Aromatic Natural Raw Materials-Vocabulary. Geneva: International Standard Organization.
Kandler O, 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie van Leeuwenhoek, 49(3), 209–224.

Key Facts on Food Loss and Waste You Should Know! | SAVE FOOD: Global Initiative on Food Loss and Waste Reduction | Food and Agriculture Organization of the United Nations. Available online: http://www.fao.org/save-food/resources/keyfindings/en/ (accessed on 11 November 2019).

Khaneghah AM, Hashemi SMB, Limbo S, 2018. Antimicrobial agents and packaging systems in antimicrobial active food packaging: an overview of approaches and inter- actions. Food Bioprod Process 111:1–19.

Klaenhammer TR, 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12, 39–85.

Koprivnjak T, Peschel A, 2011. Bacterial resistance mechanisms against host defense peptides. Cell. Mol Life Sci 68:2243–2254.

Lee H, Kim HY, 2011. Lantibiotics, class I bacteriocins from the genus *Bacillus*. J Microbiol Biotechnol 21 (3), 229-235.

Lee TH, N Hall K, Aguilar MI, 2016. Antimicrobial peptide structure and mechanism of action: a focus on the role of membrane structure. Curr top med chem 16(1), 25-39.

Lemaire A, Limbourg S, 2019. How can food loss and waste management achieve sustainable development goals? J Clean Prod 234, 1221–1234.

Liang X, Feng S, Ahmed S, Qin W, Liu Y, 2019. Effect of potassium sorbate and ultrasonic treatment on the properties of fish scale collagen/polyvinyl alcohol composite film. Molecules 24, 2363.

Matamoros S, Leroi F, Cardinal M, Gigout F, Kasbi Chadli F, Cornet J, Prévost H, Pilett MF, 2009. Psychrotrophic Lactic Acid bacteria used to improve the safety and quality of vacuum-packaged cooked and peeled tropical shrimp and cold-smoked salmon. J Food Protect 72(2), 365–374.

Matejuk A, Leng Q, Begum MD, Woodle MC, Scaria P, Chou ST, Mixson AJ, 2010. Peptide-based Antifungal Therapies against Emerging Infections. Drugs Future 35(3):197.

Matsuzaki K, Nakamura A, Murase O, Sugishita K, Fujii N, Miyajima K, 1997. Modulation of magainin 2-lipid bilayer interactions by peptide charge. Biochemistry 36, 2104-2111.

Mayr D, Margesin R, Klingsbichel E, Hartungen E, Jenewein D, Schinner F, Märk TD, 2003. Rapid detection of meat spoilage by measuring volatile organic compounds by using proton transfer reaction mass spectrometry. Appl Environ Microbiol 69(8), 4697-705.

McAuliffe O, Ross P, Hill C, 2001. Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol Rev 25(3), 285-308.

Mei J, Ma X, Xie J, 2019. Review on natural preservatives for extending fish shelf life. Foods 8(10), 490.

Melo MN, Ferre R, Castanho MA, 2009. Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations. Nat Rev Microbiol *7,* 245-250.

Moravej H, Moravej Z, Yazdanparast M, Heiat M, Mirhosseini A, Moosazadeh Moghaddam M, Mirnejad R, 2018. Antimicrobial peptides: features, action, and their resistance mechanisms in bacteria. Microb Drug Resist 24(6), 747-767.

Møretrø T, Moen B, Heir E, Hansen AÅ, Langsrud S, 2016. Contamination of salmon fillets and processing plants with spoilage bacteria. Int J Food Microbiol 237, 98–108.

Mousavi Khaneghah A, Hashemi SMB, Limbo S, 2018. Antimicrobial agents and packaging systems in antimicrobial active food packaging: An overview of approaches and interactions. Food Bioprod Process 111, 1–19.

Murhekar S, Wright MH, Greene AC, Brownlie JC, Cock IE, 2017. Inhibition of *Shewanella* spp. growth by *Syzygium australe* and *Syzygium luehmannii* extracts: Natural methods for the prevention of fish spoilage. J Food Sci Technol 54, 3314–3326.

Muzzarelli RAA, 2011. Formation and diagenesis. In Chitin nanostructures in living organisms (1st ed., pp. 1–34). Dordrecht, The Netherlands: Gupta, N., Ed.; Springe.

Novak J, Draxler L, Göhler I, Franz CM, 2005. Essential oil composition of Vitex agnus-castus — comparison of accessions and different plant organs. Flavour Frag J 20, 186–192.

Nychas GJE, Skandamis PN, Tassou CC, Koutsoumanis KP, 2008. Meat spoilage during distribution. Meat Sci 78(1-2), 77-89.
Olawore NO, Ogunwande IA, Ekundayo O, Adeleke KA, 2005. Chemical composition of the leaf and fruit essential oils of Murraya paniculata (L.) Jack. (Syn. Murraya exotica Linn.). Flavour Frag J 20, 54–56.
Otoni CG, Espitia PJP, Avena-Bustillos RJ, McHugh TH, 2016. Trends in antimicrobial food packaging systems: Emitting sachets and absorbent pads. Food Res Int 83, 60–73.
Peters BM, Shirtliff ME, Jabra-Rizk MA, 2010. Antimicrobial peptides: primeval molecules or future drugs? PLoS Pathog 6:e1001067.
Petruzzi L, Corbo MR, Sinigaglia M, Bevilacqua A, 2017. Microbial Spoilage of Foods. In The Microbiological Quality of Food, 1–21.
Phoenix DA, Dennison SR, Harris F, 2013. Antimicrobial peptides: their history, evolution, and functional promiscuity. In Antimicrobial Peptides, pp. 1–37.
Pilevar Z, Bahrami A, Beikzadeh S, Hosseini H, Jafari SM, 2019. Migration of styrene monomer from polystyrene packaging materials into foods: characterization and safety evaluation. Trends Food Sci Technol 91:248–61.
Quesada J, Sendra E, Navarro C, Sayas-Barberá E, 2016. Antimicrobial active packaging including chitosan films with Thymus vulgaris L. essential oil for ready-to-eat meat. Foods 5:57.
Radusin T, Škrinjar MM, Cabarkapa I, Pilic B, Novakovic´ AR, Hromiš NM, 2013. Actual and future trends in antimicrobial food packaging. Agro Food Ind Hi Tech 24, 44–48.
Ribeiro-Santos R, Andrade M, de Melo NR, Sanches-Silva A, 2017. Use of essential oils in active food packaging: Recent advances and future trends. Trends Food Sci Technol 61, 132–140.
Rilla N, Marti B, Rodri ANA, 2004. Inhibition of a methicillin-resistant Staphylococcus aureus strain in Afuega ' l Pitu cheese by the nisin Z– Producing strain Lactococcus lactis subsp. lactis IPLA 729. J Food Protect 67(5), 928–933.
Román S, Sánchez-Siles LM, Siegrist M, 2017. The importance of food naturalness for consumers: Results of a systematic review. Trends Food Sci Technol 67, 44-57.
Sarika AR, Lipton AP, Aishwarya MS, Dhivya RS, 2012. Isolation of a bacteriocin-producing Lactococcus lactis and application of its bacteriocin to manage spoilage bacteria in high-value marine fish under different storage temperatures. Appl Biochem Biotech 167, 1280–1289.
Schillinger U, Geisen R, Holzapfel WH, 1996. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends Food Sci Tech 7, 58–64.
Shai Y, 1999. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta 1462*,* 55-70.
Shai Y, Oren Z, 2001. From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. Peptides 22, 1629-1641.
Silva F, Domingues FC, 2017. Antimicrobial activity of coriander oil and its effectiveness as food preservative. Crit Rev Food Sci Nutr 2017, *57*, 35–47.
Smith S, Varble S, Secchi S, 2017. Fish consumers: Environmental attitudes and purchasing behavior. J Food Prod Mark 23(3), 267-282.
Solomakos N, Govaris A, Koidis P, Botsoglou N, 2008. The antimicrobial effect of thyme essential oil, nisin, and their combination against Listeria monocytogenes in minced beef during refrigerated storage. Food Microbiol 25, 120–127.
Souza EL, Silva CA, Sousa CP, 2005. Bacteriocins: Molecules of fundamental impact on the microbial ecology and potential food biopreservatives. Brazilian Arch Biol Technol 48(4), 559-566.
Sudarshan NR, Hoover DG, Knorr D, 1992. Antibacterial action of chitosan. Food Biotechnol 6, 257–272.
Taubes G, 2008. The bacteria fight back. Science 321, 356-361.

Thong NT, Solgaard HS, 2017. Consumer's food motives and seafood consumption. Food Qual Prefer 56:181-188.

Tikhonov VE, Stepnova EA, Babak VG, Yamskov IA, Palma-Guerrero J, Jansson HB, Lopez-Llorca LV, Salinas J, Gerasimenko DV, Avdienko ID, 2006. Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2(3)-(dodec-2-enyl)succinoyl/-derivatives. Carbohyd Polym 64, 66–72.

Van de Braak SAAJ, Leijten GCJJ, 1999. Essential oils and oleoresins: A survey in the Netherlands and other major markets in the European Union. Rotterdam: CBI, Centre for the Promotion of Imports from Developing Countries.

Vázquez JA, González MP, Murado MA, 2005. Effects of lactic acid bacteria cultures on pathogenic microbiota from fish. Aquaculture 245, 149–161.

Vergis J, Gokulakrishnan P, Agarwal RK, Kumar A, 2015. Essential Oils as Natural Food Antimicrobial Agents: A Review. Crit Rev Food Sci Nutr 55, 1320–1323.

Wang G, 2015. Improved methods for classification, prediction, and design of antimicrobial peptides. Methods Mol Biol 1268, 43-66.

Wiedemann I, Breukink E, Van Kraaij C, Kuipers OP, Bierbaum G, De Kruijff B, Sahl HG, 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J Biol Chem 276(3), 1772-1779.

World Health Organization, 2017. *The burden of foodborne diseases in the WHO European region*. WHO Regional Office for Europe.

World Health Organization. *WHO Estimates of the Global Burden of Foodborne Diseases: Foodborne Disease Burden Epidemiology Reference Group 2007–2015*; World Health Organization (WHO): Geneva, Switzerland, 2015; pp. 1–15.

Yeaman MR, Yount NY, 2003. Mechanisms of antimicrobial pep- tide action and resistance. Pharmacol Rev 55, 27-55.

Zasloff M, 2002a. Antimicrobial peptides in health and disease. N Engl J Med 347, 1199-1200.

Zasloff M, 2002b. Antimicrobial peptides of multicellular organisms. Nature 415, 389-395.

Zheng Z, 2014. Ingredient technology for food preservation. Ind Biot 10, 28–33.

Zhu X, Zhang L, Wang J, Ma Z, Xu W, Li J, Shan A, 2015. Characterization of antimicrobial activity and mechanisms of low amphipathic peptides with different alpha-helical propensity. Acta Biomater 18, 155-167.

2. AIM

Food spoilage and foodborne diseases have a bearing on the environmental, economic, and social aspects and could seriously harm the public health. For all these reasons, the research in food wastage and food pathogenic bacteria field is expanding. Scientists and Food Business Operators (FBOs) has the communal objective to discover molecules whose nature could satisfy main requirements such as naturalness, safety, bioavailability, and cheapness.

In this regard, the general aim of this thesis was to develop an "end-product" with antimicrobial activity ready to be put on the food market. Therefore, each chapter focused on specific step of the experimental plan, which included the discovery and characterization of natural compounds, the evaluation of their potential role in the formulation of novel active packaging, and the study of their mechanisms of action. In particular, the antimicrobial activity of a crude protein extract from the European medicinal plant *Loranthus europaeus* against the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes* was evaluated (**Chapter 1**). Moreover, the bactericidal and antibiofilm activity of the peptide 1018-K6 against a large panel of serotypes of *Salmonella enterica* was investigated. The resistance profiles of all the strains included in this study were also determined (**Chapter 2**). Once the class of molecules that best suited to achieving the goal was identified, the next objective was to develop a new class of packaging materials, functionalized with the bactericidal peptides MTP1 (**Chapter 3**) and 1010-K6 (**Chapter 4**), to evaluate both the usefulness and effectiveness of the active coatings on the microbial quality and safety of fresh perishable products and the potential extension of their shelf-life. The ultimate goal was the characterization of the binding affinity of peptide 1018-K6 toward mimetic and biological bacterial membranes, and the discovery of the mechanistic method used to kill bacterial cells (**Chapter 5**).

3. Chapter 1

The Bactericidal Activity of Protein Extracts from *Loranthus europaeus* Berries: A Natural Resource of Bioactive Compounds

Abstract: *Loranthus europaeus* is a well-known and important medicinal plant, with a long history of traditional medicine use. Several studies showed that it contains many bioactive compounds with a wide range of pharmacological effects. In light of these past research, *L. europaeus* were chosen to consider its potential antimicrobial action. To this aim, different protocols were performed to selectively extract protein compounds, from *L. europaeus* yellow fruits, and evaluate the antimicrobial activity against four phytopathogenic fungi (*Aspergillus niger*, *Alternaria* spp., *Penicillium* spp., *Botrytis cinerea*) and a number of foodborne bacterial pathogens (*Listeria monocytogenes*, *Staphylococcus aureus* strains, *Salmonella* Typhimurium and *Escherichia coli*) by using serial dilutions and colony formation assays. Results evidenced no antifungal activity but a notable bactericidal efficiency of a crude protein extract against two foodborne pathogens, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values between 0.2 and 0.5 mg/mL, being *S. aureus* strains the most susceptible bacteria. Moreover, a strong bactericidal activity against *S. aureus* M7 was observed by two partially purified protein fractions of about 600 and 60 kDa molecular mass in native conditions. Therefore, these plant protein extracts could be used as natural alternative preventives to control food poisoning diseases and preserve foodstuff avoiding health hazards of chemically antimicrobial applications.

Keywords: *Loranthus europaeus*; Protein extract; Antibacterial agents; Natural compounds; *Staphilococcus aureus.*

Note: Ambrosio RL, Gratino L, Mirino S, Cocca E, Pollio A, Anastasio A, Palmieri G, Balestrieri M, Genovese A, Gogliettino M, 2020. The bactericidal activity of protein extracts from Loranthus europaeus berries: a natural resource of bioactive compounds. Antibiotics 9(2), 47.

3.1 Introduction

In chapter XVI, 95 of the *Naturalis Historiae*, Pliny the Elder described the harvesting of the mistletoe growing on the oaks by Gaul druids, a cult related to the medicinal and magic properties attributed to mistletoes (Pliny, 1938). Indeed, these plants were highly reputed as a remedy for many ills, and specifically effective *contra venena* (Schurè, 1888), but the traditions about magic and curative properties of mistletoes are not confined to Central-Northern Europe. The anthropologist James G. Frazer in his land-marking "The Golden Bush" (Frazer, 1890) presented a detailed survey of the symbolic role of mistletoes in the ancient Italic cult of Diana Nemorensis, and in the 1900s growing evidence has been presented on their occurrence as sacred plants in folklore and mythology of several Indo-European cultures (Alexander, 2009).

Mistletoe is a generic term encompassing all the obligate hemiparasitic species of Angiosperms, presently placed in five phylogenetically unrelated different families, within the order Santalales. Loranthaceae and Viscaceae are the families that include most of mistletoes worldwide diffused (Watson, 2001). Archaeological findings support the use of *Viscum album* in the religious cults of druids in England, between the first and second century AD (Stead *et al*., 1986), but *V. album* is not the only mistletoe diffused in Europe. Indeed, magic and curative properties were attributed also to other mistletoe species *Loranthus europaeus*, the "true" golden bush, prevalently diffused in the Mediterranean territories of the Continent (Ramm, 2015). According to Liu *et al.* (2018), the Loranthaceae originated in Australasia during Late Cretaceous, when these two Continents were connected or contiguous; also the genus *Loranthus* has an Australasian origin, but dispersed also to North Asia and Europe. In the course of the last century, the majority of *Loranthus* species have been

transferred to different genera (Hegi, 1981), and presently the genus is represented by about ten species (Nickrent *et al*., 2010). European yellow mistletoe, *L. europaeus* Jacq., is the only species of the genus that migrated westward to Europe, and is presently diffused in Central Asia, Anatolia, South Russia, and South-Western Europe (Shahi Shavvon *et al*., 2012). *L. europaeus* (European yellow mistletoe) is a small shrub with brown bark and deciduous leaves during the winter, with yellowish-green flowers. The fruit is a spheroidal golden-yellow berry, with a sticky liquid inside. As the other hemiparasitic Santalales, *L. europaeus* actively photosynthesizes, but gains from the host plants water, inorganic nutrients and also organic compounds, such as amino acids and sugars (Hibberd and Jeschke, 2001). *L. europaeus* can establish a relation with different *Quercus* species, with *Q. pubescens* being by far the first choice host plant, although this mistletoe can also attack other trees such as chestnut (Gebauer *et al*., 2012). *L. europaeus* is presently used to treat many numerous aliments in the folk medicine of different Asiatic and European Countries (Sharquie *et al*., 2017; Vitasović Kosić, *et al*., 2017). In several regions of Central Italy, the whole plant macerated in wine or grappa was used to cure atherosclerosis and hypertension (Leporatti and Corradi, 2001), whereas in Calabria region (South Italy) the leaves were also topically applied to cure wounds (Leporatti and Pavesi, 1989). The therapeutic effects of *L. europaeus* have been attributed to the presence of a wide array of active substances: the mixture of flavonoids isolated from the plants has shown marked antioxidant properties (Harvala, 1984; Katsarou *et al*., 2012), and a stimulatory effect on lymphocyte proliferation has been attributed to flavonoids and terpenoids isolated from the leaves (Cholakova *et al*., 2002). Recently, an anti-leishmaniasis effect has been attributed to the presence of high concentrations of quercetin in the whole plant extract (Sharquie *et al*., 2017). The antimicrobial and cytotoxic activities attributed to *L. europaeus* could be also due to the presence of the so-called defense peptides, more recently known as plant defensins, largely occurring in different genera of parasitic members of Santalaceae and Loranthaceae (Larsson *et al*., 2007). Plant defensins have shown a specific activity not only against pathogenic fungi, but also to yeast models (Parisi *et al*., 2019), and for this reason are considered a possible source of therapeutic compounds also against human fungal infections (Sathoff *et al*., 2019).
The insights gained from this work demonstrated, for the first time, the antimicrobial activity of a crude protein extract from the European medicinal plant *L. europaeus* against the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes*. The bactericidal effect of two partially purified protein compounds isolated from the yellow berries was also examined against the methicillin-resistant *S. aureus* M7, whose growth was completely inhibited already at a concentration of 0.01 $mg \cdot mL^{-1}$. It should finally be noted that this study started thanks to the correlation of the anthropological and biogeographic data made by the coauthor of the manuscript Angelo Genovese.

3.2 Materials and Methods

3.2.1 Collection of Plant Material

Shoots of berries of European mistletoe parasitic plant *Loranthus europaeus* were collected between December 2017 and March 2018 from infected oaks (*Quercus pubescens*) in the forest of Valle del Torrente, Savucchia, Carpanzano (Cs), Calabria, South Italy, Italy (39.14453° N–16.31390° E) at about 3–4 meters from the ground level. All the picked samples were yellow-berried leafless aerial shoots. The berries were rounded with a diameter of 0.5–1 cm and were stored at −80 °C until protein extraction. The yellow berries were gently collected by the Prof. Angelo Genovese of University of Naples “Federico II”.

3.2.2 Preparation of Crude Extracts

Total protein extraction from yellow berries was carried out using three protocols. *L. europaeus* frozen berries were pitted and finely powdered in liquid nitrogen using a mortar and pestle and the

pulverized mixtures were used for all the extraction protocols. Specifically, protein extracts were obtained by adding to the powdered materials a defined volume of each extraction buffer: 100 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM PMSF (extract 1); 100 mM Sodium Acetate pH 5.0, 1 mM DTT, 1 mM PMSF (extract 2); 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mM DTT, 5% SDS (extract 3). The extracts 1 and 2 were shaken on a rotatory shaker for 16 h at 4 °C and then centrifuged at 16,000× *g* for 40 min at 4 °C. The extract 3 was shaken on a rotatory shaker for 3 h at 4 °C and then centrifuged at the same conditions described above. All the resulting supernatants were collected and extensively dialyzed in bags with 10 kDa MWCO (Molecular weight cut-off) at 4 °C against 50 mM sodium acetate pH 5.0 for extract 2 and 50 mM Tris-HCl pH 8.0 for extract 1 and 3. The extracted proteins Bradford's (Bradford, 1976) method using bovine serum albumin as standard.

3.2.3 Antifungal Activity Assays

The antifungal activity of the three extracts was evaluated against four phytophatogenic fungi (*Aspergillus niger*, *Botrytis cinerea*, *Penicillium* spp., and *Alternaria* spp.) as described in Agrillo *et al.* (2019). Before the antifungal testing, the protein extracts 1, 2 and 3 were sterilized by filtration through 0.22 µm sterile filters (Millex GV). Tests were performed pouring the extracts (300 µL) in wells (0.5 cm in diameter) aseptically punched on the PCA plates, previously scraped with fungi spores (2×10^4 conidia/mL) and by incubating the plates for 48 h at 28 °C. The antifungal activity was evaluated measuring the diameter of the inhibition zone on PCA plates.

3.2.4 Bacterial Culture and Inoculum Preparation

Methicillin-resistant *Staphylococcus aureus* (MRSA, M7), *Staphylococcus aureus* (MSSA, SA4), *Listeria monocytogenes* (92), no pathogenic *E. coli* (O157) and *Salmonella* Typhimurium isolated from different foods, were used in the microbiological assays. Bacterial cultures were stored at −80 °C. Before the experiments, the frozen stocks of each strain were plated on selective agar and incubated at 37 °C for 16 h to obtain single colonies. Working cultures were produced daily by transferring a loopful of culture to Tryptic Soy Broth (TSB, Biotec, Grosseto, Italy) and incubating for 16 h at 37 °C. To obtain the bacterial suspension, the density of the cell was assessed spectrophotometrically (OD_{600}) and the solution was adjusted to 0.1. Enumeration of the inoculum was completed by diluting to approximately 3.0 Log $CFU \cdot mL^{-1}$ and spread-plating 100 µL on selective plate agar. Plates were aerobically incubated at 37 °C for 48 h.

3.2.5 Antibacterial Activity Assay of Plant Fruit Extracts

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were carried out on berry extract 1 and 2 (after dialysis against sodium acetate at 50 mM) according to Clinical and Laboratory Standards Institute (CLSI, 2015), with some modifications (Owuama, 2017). The stock solution (0.50 $mg \cdot mL^{-1}$) of the two berry extracts was diluted at different concentrations (from 0.41 to 0.01 $mg \cdot mL^{-1}$) in Tryptic Soy Broth (TSB; Biotec, Grosseto, Italy) to a total volume of 1 mL for each tube. 10 µL of each strain, 1.0×10^4 $CFU \cdot mL^{-1}$, was inoculated. At the same time, equal volumes of sterile Tryptic Soy broth were inoculated as a control. The tubes were incubated for 20 ± 2 h at 37 °C and thereafter observed for turbidity. MIC is defined as the lowest concentration of the extract at which no bacterial growth was detected. MBC is defined as the lowest concentration of peptide at which more than 99.9% of the bacterial cells are killed. To determine the MBC, 100 µL of the bacterial cell suspension was taken based on the MICs, cultivated on agar plate and then incubated for 24–48 h at 37 °C. At least six technical replicates were included for each group, and all experiments were performed in triplicate.

3.2.6 Antibacterial Activity Assay of Partially Purified Samples

The antimicrobial efficacy of the partially purified samples (pellets 50% and 90% and gel filtration fractions) was determined according to Palmieri *et al*. (2018). The pellets were tested at concentration of 0.15 $mg \cdot mL^{-1}$ *versus Staphylococcus aureus* (MRSA, M7). Gel filtration fractions were assayed at concentrations ranging from 0.01 $mg \cdot mL^{-1}$ to 0.04 $mg \cdot mL^{-1}$. Under all the experimental conditions explored, the plate counting method was used to estimate the activities.

3.2.7 Partial Purification of the Active Components

Precipitation by ammonium sulphate, a method of protein purification, was performed on total protein extracts 2 followed by gel filtration chromatography to isolate the antibacterial compounds. Powdered ammonium sulphate was added in small portions under constant stirring at 4 °C to 50% and 90% saturation levels. Specifically, the precipitate by ammonium sulphate at 50% saturation was collected by centrifugation at 15,000× *g* for 30 at 4 °C, dissolved in 50 mM sodium acetate pH 5.0 and extensively dialyzed in bags with 10 kDa MWCO (Molecular weight cut-off) against the same buffer to completely remove the salt. The supernatant resulting from the precipitation by 50% was precipitated at 90% $(NH_4)_2SO_4$ saturation at 4 °C. After centrifugation at 15,000× *g* for 30 at 4 °C, the resulting pellet was re-suspended in 50 mM sodium acetate pH 5.0 and extensively dialyzed against the same buffer in bags with 10 kDa MWCO (Molecular weight cut-off). All fractions obtained following ammonium sulphate precipitations were tested for antibacterial activity as described above. The sample obtained from salt precipitation which resulted active in the antibacterial tests were loaded on a gel filtration column Yarra 3µm Sec-4000 column (Pharmacia Biotech, Milan, Italy) connected to an HPLC system (Shimadzu, Milan, Italy) and pre-equilibrated in 50 mM sodium acetate containing 50 mM NaCl, pH 5.0. The samples were eluted at a flow rate of 0.5 $mL \cdot min^{-1}$. Fractions were pooled, concentrated and tested for the antibacterial activity as previously described.

3.2.8 SDS-PAGE Analysis

In order to monitor purity, electrophoretic analyses were performed on 10% polyacrylamide gel under denaturing conditions (SDS-PAGE) according to the procedure described by Laemmli (1970). Standard proteins (Page rule Unstained ladder) were purchased from Thermo Scientific (Massachusetts, USA).

3.2.9 Spectroscopic Analyses

The fluorescence of plant fruit pigments was determined using a Jasco FP-8200 spectrofluorometer. The extraction of the main pigments was performed on the plant extract 2 before and after the dialysis using ethyl acetate as solvent. The extract obtained was centrifuged at 16,000× *g* for about 10 minutes. The supernatant was collected, and the fluorescence emission spectra were collected at 25 °C in a 1 cm path length quartz cuvette using excitation and emission slit widths of 2.5 nm. The samples were excited at different λ_{exc} and the emission ranges used were: 500–800 nm (480- nm λ_{exc}), 600–800 nm (425-nm λ_{exc}), and 600–800 nm (470-nm λ_{exc}).

3.2.10 Statistical Analysis

All experiments were performed at least five times. Statistical analysis was carried out using the software GraphPad Prism®, version 6 (GraphPad, San Diego, California, USA). Statistical analysis of microbiological data was performed by using Student's t-test ($p < 0.05$) and the results were presented as mean ±standard deviation (s.d.).

3.3 Results and discussion

3.3.1 Samples Collection

In Italy, *Loranthus europaeus* is prevalently diffused in oak forests of Apennines, extending from Central to South Italy. For this study, the forest of Carpanzano (Calabria), located at an altitude of 610 m, was selected. Carpanzano is a continental territory of Calabria, far from the sea, with cold winters and higher precipitations during spring and fall. Visible tufts of *L. europaeus* were scattered on numerous *Q. pubescens* trees and samples were collected during winter, when mistletoe twigs are leafless, and fruits acquire a bright yellow color (Figure 3.1).

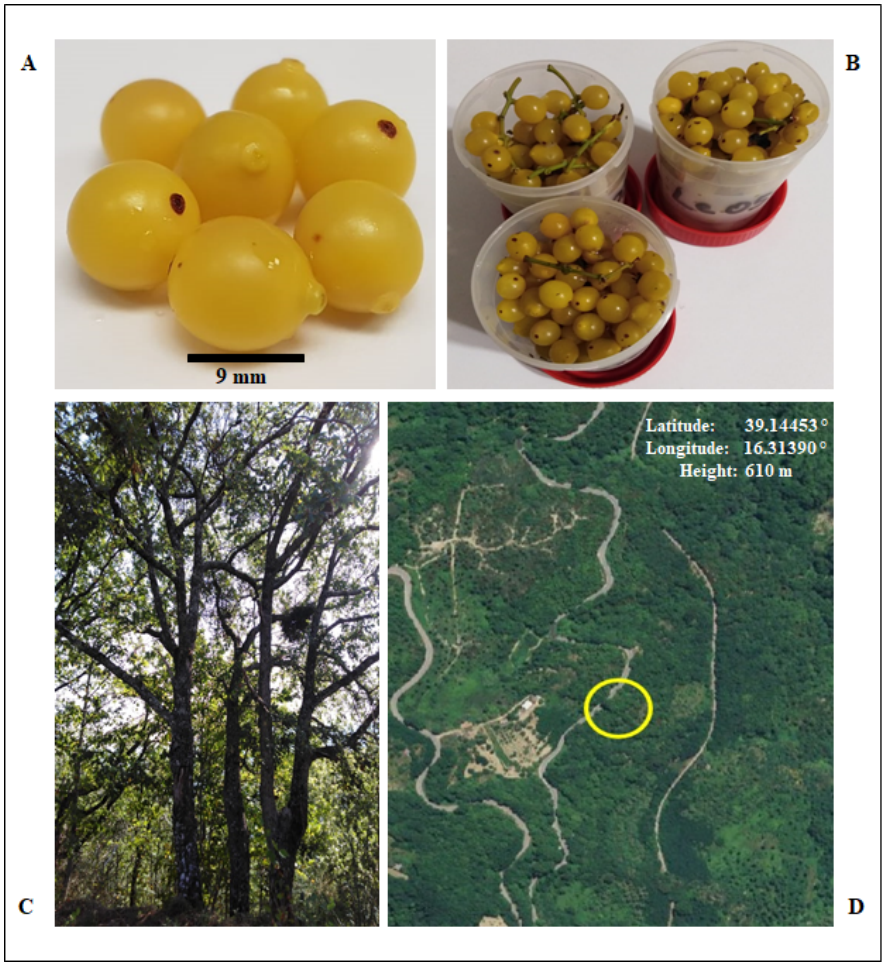


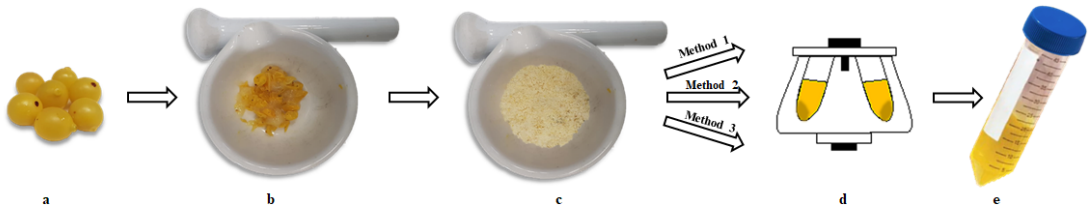
Figure 3.1 *(**A-B**) Ripen berries of L. europaeus; (**C**) oak tree hosting L. europaeus twigs; (**D**) the sampling site (Carpanzano forest, Calabria, Italy).*

3.3.2 Preparation of protein extracts from Loranthus europaeus berries and plant extracts yield

With the aim to recover and isolate new plant protein agents with antimicrobial activity, that can possibly be used as natural preservatives in the food and pharmaceutical industries, an efficient protein extraction method from *Loranthus europaeus* berries was developed. Indeed, the extraction efficiency is strongly affected by several factors such as the starting plant material, the buffer composition and the method used as well as the presence of interfering substances (Tiwari *et al.*, 2011). It is worth noting that at present there are relatively few reports on the extraction protocols of antibacterial proteins from plant berries respect to those on the wide variety of smaller molecules, obtained usually through ethanol or methanol extraction (Cowan, 1999).

In this study, three of simply, fast and common extraction protocols used for proteins were carried out for berries with some modifications (Tattersall *et al*., 1997; Jammer *et al*., 2015; Palaniyandi and Muthuswamy, 2015; Wang *et al*., 2008), taking into account both the pH and the presence of strong anionic detergents such as SDS (Figure 3.2A). As far as the protein recovery is concerned, the quantitative comparison among the different extracts showed that the highest yield was obtained with method 3, followed by method 2 and method 1 that gave the lowest protein yield (Figure 3.2B). However, it is worth noting that the protein quantitation assay on extracts from protocol 3 was affected by the presence of SDS-containing buffer that persisted even after extensive dialysis of the sample, thus interfering with the protein yield results (Friedenauer and Berlet, 1989).

A



B

Method	Extraction buffer	Protein yield (mg/g berry)
1	**Extract 1:** Tris-HCl 100 mM, pH 8.0; DTT 1 mM; PMSF 1 mM	1.46 ± 0.10
2	**Extract 2:** Sodium Acetate 100 mM, pH 5.0; DTT 1 mM; PMSF 1 mM	3.26 ± 0.15
3	**Extract 3:** Tris-HCl 500 mM, pH 8.0; DTT 10 mM ; EDTA 10 mM ; SDS 5%	3.89 ± 0.17

Figure 3.2 *(**A**) Representative scheme of the different steps applied for the preparation of crude protein extracts from the yellow berries of L. europaeus. **a**: yellow berries; **b**: pitted berries; **c**: powdered berries in liquid nitrogen using a mortar and pestle; **d**: centrifugation of the mixture; e: crude protein extract. Finely ground powder of plant fruit was used as starting material in all three protocols. (**B**) Table reporting protein yields from berries of L. europaeus using three extraction protocols. Data are presented as means ± standard deviation (s.d.) of three different samples analyzed in triplicate.*

The protein pattern of the three extracts was assessed by SDS-PAGE analysis and a representative Coomassie-stained gel is reported in Figure 3.3. The protein extracts 1 and 2 showed a similar electrophoretic profile in contrast to that associated with the extract 3, possibly resulting from the use of SDS in the extraction buffer, which is known to be extremely effective in the solubilization of membrane proteins (Arachea *et al*., 2012).

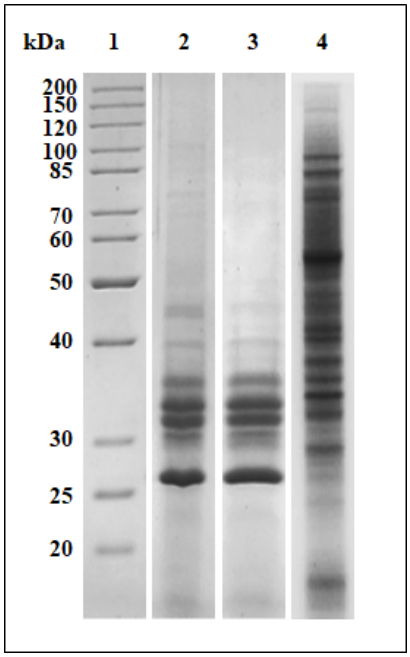


Figure 3.3 *SDS-PAGE (10%) analysis of the total protein extracts from L. europaeus berries using the three methods. Lane 1: molecular weight markers (Thermo Scientific); crude protein extracts obtained by: method 1 (Lane 2); method 2 (Lane 3); and method 3 (Lane 4). Protein bands were detected by Coomassie blue staining. Equal amounts of proteins were loaded for each Lane. The gel is representative of three independent experiments on three different protein preparations.*

3.3.3 Antifungal Activity

An initial *in vitro* screening was done to evaluate the antifungal activity of all the plant extracts against four of the most common phytopathogenic fungi. As depicted in Figure 3.4, none of the two extracts at pH 8.0 and 5.0 (Method 1 and 2) showed antifungal activity against any of the tested microorganisms, even at the highest amount investigated. In addition, the two protein samples seemed to promote the sporulation of *Aspergillus niger* (colony diameter of 3.5 ± 0.3 cm and 2.0 ± 0.8 cm for extract 2 and extract 1, respectively after 48 h of incubation*)*, possibly due to the presence in the plant extracts of some additional nutrients which could further stimulate the fungal growth (Figure 3.4C). As far as the SDS-extract (Method 3) is concerned, it is worth noting that even after extensive dialysis of the samples a residual amount of detergent persisted in the protein mixtures, resulting in a strong interference with the antifungal and antibacterial activity assays, which require a complete removal of this detergent. For these reasons, the SDS-extracts were not further considered for our investigations.

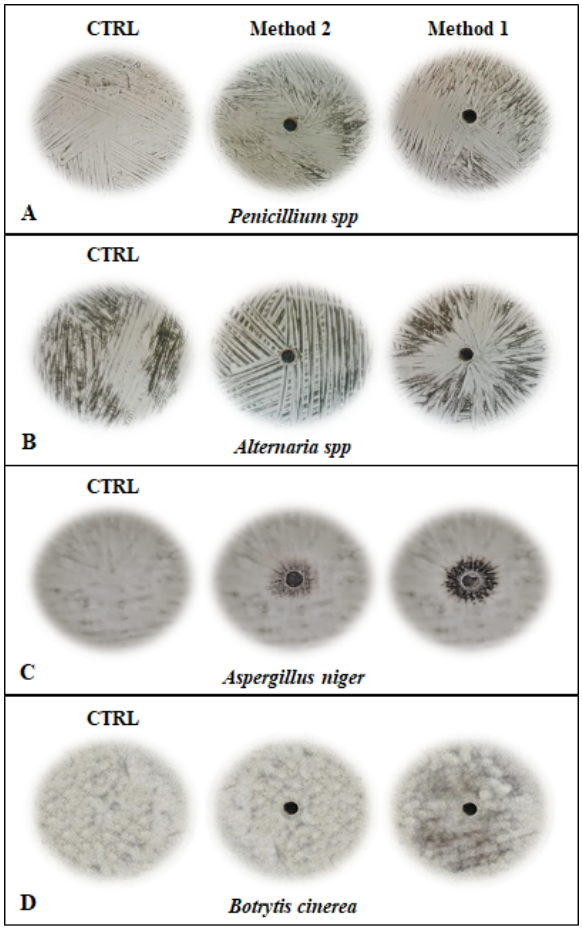


Figure 3.4 *Antifungal activity assay of plant extract 1 (Method 1) and plant extract 2 (Method 2) against different phytopathogenic fungi: (**A**) Penicillium spp; (**B**) Alternaria spp; (**C**) Aspergillus niger; (**D**) Botrytis cinerea. CTRL: each tested fungus without treatment. The plates were incubated at 28 °C for 48 h. The pictures are representative of three independent experiments on three different protein preparations.*

3.3.4 Antibacterial Activity

In order to explore the potential use of the protein samples as antimicrobial agents, the antibacterial activity of the extracts 1 and 2 was evaluated against a panel of bacteria, including 3 strains of Gram-positive *(L. monocytogenes*, *S. aureus* MSSA, and MRSA) and 2 strains of Gram- negative bacteria (*Salmonella* Typhimurium and *E. coli*), commonly associated with infectious diseases. Specifically, to compare the effect of the two extracts on the growth of the microorganisms under investigation, the MIC and MBC values were determined by using the serial dilution assay. It is known that sodium acetate can affect the bacterial growth (Smith, 2016; Cabezas-Pizarro *et al*., 2018), therefore preliminary experiments were performed in order to assess the effects of different concentrations of acetate on the growth of the foodborne pathogens, considering that the extract 2 was obtained using acetate as extractant. The obtained results evidenced a linear decrease of bacterial growth rate with the increase in acetate concentration starting from 60 mM (data not shown). For this reason, all the subsequent experiments with extract 2 were performed only after dialysis of the sample in order to have a final concentration of 50 mM acetate that did not interfere with the antimicrobial assays. Interestingly, the tested microorganisms revealed a different sensitivity to the two types of extracts. Overall, the results demonstrated that the extract 1 was less effective in suppressing the microbial growth of all pathogens tested, exhibiting MIC values 2-fold higher than those observed for the acetate-extract. It can be hypothesized that the variation in MIC values between the two plant-fruit samples arose from a diverse nature of the proteins extracted by using the acetate respect to the Tris buffer. Hence, the extract 1 was not considered for any further study based on its weak antibacterial activity. As far as plant fruit extract 2 is concerned (Figure 3.5A), it exhibited an efficient and

significant antimicrobial activity against *L. monocytogenes, S. aureus* MRSA, and *S.* Typhimurium, with MIC values ranging from 0.16 to 0.50 mg·mL^{-1}, being *S. aureus* MRSA the most sensitive bacterial species. Indeed, the protein sample was found to be ineffective against *E. coli* and *S. aureus* MSSA SA4 even at the highest amount (0.50 mg·mL^{-1}) assayed. To investigate further the antimicrobial effects of the extract 2, the MBC was evaluated revealing that it displayed a strong bactericidal activity against *L. monocytogenes* and S. aureus MRSA, with MBC values of 0.38 and 0.20 mg·mL^{-1}, respectively. These results clearly indicated that this protein extract was bacteriostatic at concentrations lower than those required to explain bactericidal activity against *L. monocytogenes*, being MBC value higher than the corresponding MICs. Instead, the MBC determined against *S. aureus* MRSA was on a par with the corresponding MIC (both at about 0.2 mg·mL^{-1}), thus demonstrating that the tested sample should be considered to have a strong bactericidal mode of action. On the other hand, *S.* Typhimurium needed protein concentrations higher than 1 mg·mL^{-1} to be killed, indicating that the active substances were only bacteriostatic towards this strain. Therefore, according to the results obtained, the Gram-positive bacteria were more sensitive to the plant extract 2 than the Gram-negative microorganisms, presumably as consequence of the different bacterial membrane structures. Specifically, lipopolysaccharides layer and periplasmic space of Gram-negative bacteria could be the reasons of the relative resistance of this class of bacteria to the plant extract 2 treatment. However, this explanation represents a simplification as other mechanisms could play a role in this process. Interestingly, in relation to the antibacterial spectrum of the crude extract (Figure 3.5A), it is important to emphasize the strong growth inhibition of methicillin-resistance *S. aureus* M7 strain (Figure 3.5 B,C), which is one of the most pathogenic bacterium resistant to multiple drugs, having acquired resistance to a variety of them.

A

Bacteria	Plant fruit extract 2	
	MIC (mg mL^{-1})	MBC (mg mL^{-1})
L. monocytogenes 92	0.28±0.05	0.38±0.02
S. aureus MRSA M7	0.16±0.04	0.20±0.01
S. Typhimurium	0.50±0.03	ND
S. aureus MSSA SA4	NI	NI
E. coli O157	NI	NI

ND = not determined
NI = not inhibited

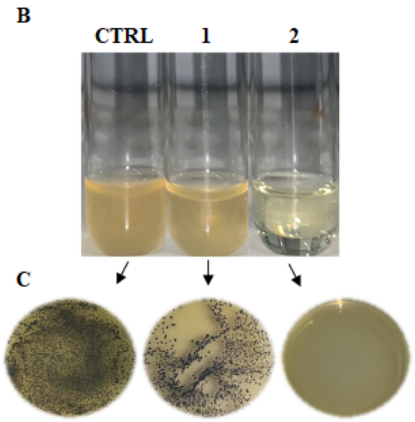


Figure 3.5 *(**A**) Table of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of plant fruit extract 2 against different foodborne pathogens. (**B**) Antimicrobial test in vitro of plant fruit extract 2 against S. aureus MRSA M7. **CTRL**: S. aureus MRSA M7 control; (**1**) protein extract 2 at 0.08 mg·mL^{-1} concentration; (**2**) protein extract 2 at 0.16 mg·mL^{-1} concentration (MIC value). (**C**) MBC value (0.2 mg·mL^{-1}) determined by the standard plate count. Data are presented as means ± standard deviation (s.d.) of three different samples analyzed in triplicate. * Significant difference ($p < 0.05$) between the treated and the control samples.*

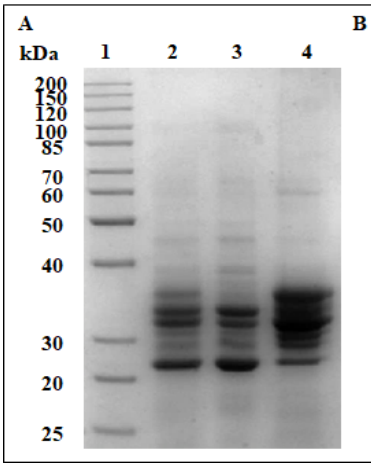
Antibacterial studies were also performed against a no foodborne Gram-negative pathogen *Pseudomonas protegens* N, a widespread plant-protecting bacterium isolated from water samples of an irrigation well located in the region of Djebira in Bejaia, northern Algeria (Agrillo *et al.*, 2019; Tabli *et al.*, 2018). The obtained results clearly demonstrated that all the plant extracts under investigation were ineffective to inhibit the growth of the soil microorganism, confirming that the *L. europeaus* antibacterial proteins appeared to be less potent both versus pathogenic and not pathogenic Gram-negative bacteria. In accordance with the reported findings concerning the screening of antimicrobial potentiality and taking into account the sensitivity of the tested bacteria, extract 2 and *S. aureus* MRSA M7 were chosen to perform the further analyses.

3.3.5 Spectroscopic Analysis

Many of the colors associated with higher plants are due to the presence of pigment molecules, such as chlorophylls and the carotenoids, which confer them a natural fluorescence. Therefore, the intense color of these pigments makes them ideal candidates for absorption spectroscopy studies, having a unique visible spectrum, which can provide a positive identification (Hee-Ock *et al*., 2012). In this context, the pigment content in terms of chlorophyll a, chlorophyll b and carotenoids present in the plant extract 2 was determined by spectrofluorometric analysis, performing their extraction using ethyl acetate as solvent that is considered the best extractant for this class of molecules (Kumar *et al*., 2010). As shown in Figure 3.S1, the photosynthetic fluorescence emission spectra obtained from the organic extracts evidenced the presence of three main bands: one of chlorophyll a at 650–684 nm, the second at 642–670 nm, characteristic to chlorophyll b, and the last one at 500–600 nm probably due to carotenoids. The same experiment was performed on the plant extract 2 after dialysis in bags with 10 kDa MWCO (Molecular weight cut-off), revealing that it was completely abolished the fluorescence emission peaks corresponding to the three pigment molecules, which were lost during dialysis. Therefore, it is reasonable to assess that the strong antibacterial activity measured in the extract 2, whose preparation includes dialysis, can be attributed to compounds with a molecular mass higher than 10 kDa.

3.3.6 Partial Purification of the Active Compounds

With the aim to gain insight into the protein component/s responsible for the antibacterial activity of the extract 2, a partially purification procedure was performed by a combination of ammonium sulphate fractionation and gel filtration chromatography. In the first step, precipitation experiments were conducted subjecting the extract 2 to precipitation using two sequential salt saturation levels (50% and 90%). The pellets resulting from the two precipitation steps were dissolved in 50 mM sodium acetate buffer pH 5.0, extensively dialyzed to remove the ammonium sulphate, tested for antibacterial activity and analyzed by SDS-PAGE (Figure 3.6A).



B

	S. aureus MRSA M7		
	CTRL (Log CFU mL^{-1})	Treated bacterial sample (Log CFU mL^{-1})	Change in Log CFU mL^{-1} between CTRL and treated bacterial sample
Pellet 50% (0.15 mg mL^{-1})	9	5	-4
Pellet 90% (0.15 mg mL^{-1})	9	5	-4

negative sign = extent of bacterial reductions as indicated by the Log (CFU mL^{-1}).

Figure 3.6 *(**A**) SDS-PAGE analysis of protein fractions. Lane 1: molecular weight markers; Lane 2: plant extract 2; Lane 3: protein sample obtained by 50% ammonium sulphate precipitation; Lane 4: protein sample obtained by 90% ammonium sulphate precipitation. Equal amounts of total proteins were loaded for each lane. The gel is representative of three independent experiments on three different protein preparations. (**B**) Antibacterial effect of pellet 50%, pellet 90% and plant extract 2 samples against S. aureus MRSA M7 reported in terms of change in the Log CFU·mL^{-1} of viable colonies observed between control and treated bacteria at 24 h. Data are representative of three independent experiments on three different protein preparations.*

In vitro antibacterial assessment of the two precipitates (named pellet 50% and pellet 90%) was carried out at the MIC value (0.15 mg·mL^{-1}) determined with the total extract 2 against *S. aureus*

MRSA (Figure 3.5A) and the results were reported in terms of the change in the Log CFU·mL^{-1} of viable colonies. The bactericidal activity was defined as being equal to 3 Log CFU·mL^{-1} or greater reduction in the viable colony count relative to the initial inoculum (Scheetz *et al*., 2007). As shown in Figure 3.6B, a rapid reduction in the Log of the viable cells counted (−4 Log CFU·mL^{-1}), was detected with both samples. This acknowledged the fact that the bactericidal activity measured for the extract 2 resulted from the contribution of different protein components. However, given that the total protein yield in the 90% pellet was 5-fold lower than that obtained in 50% sample and taking into account the large amount of the starting material required to allow more detailed investigations, we firstly decided to proceed to the purification of 50% pellet. An important aspect to underline is the complete recovery of the proteins responsible of the antibacterial activity in the plant crude extract after precipitation by ammonium sulphate.

Additional purification step was conducted through the gel filtration chromatography on an SEC-4000 column. The elution profile (Figure 3.7) obtained from 50% pellet, showed five main protein fractions, which were assayed for the antibacterial activity against the *S aureus* MRSA (Figure 3.8A). A strong killing activity was exhibited by both protein fractions Fr 1 and Fr 2, with MIC values of 0.01 mg·mL^{-1} and 0.04 mg·mL^{-1}, respectively, which coincided with the MBCs. In contrast, no activity was observed with the remaining protein fractions Fr 3, Fr 4, and Fr 5. Based on the calibration curve of the gel filtration column, Fr 1 and Fr 2 displayed a molecular mass of approximately 600 kDa and 60 kDa, respectively.

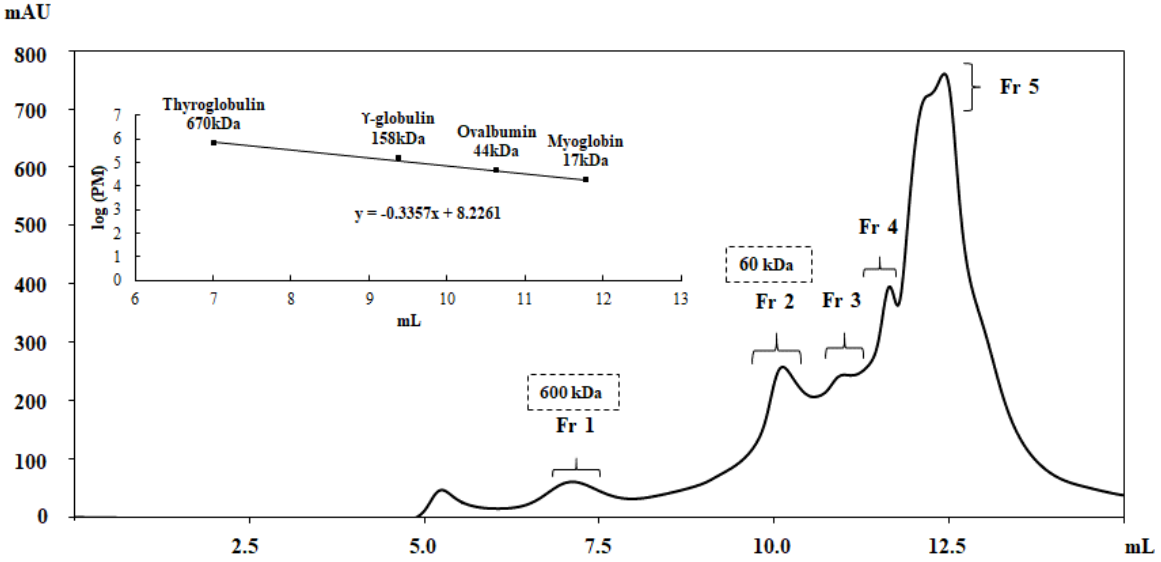


Figure 3.7 *Elution profile of pellet 50% sample obtained by gel filtration chromatography performed on YARRATM SEC-4000 column in 50 mM sodium acetate buffer pH 5.0 containing 50 mM NaCl. Insert: Calibration curve of the gel filtration YARRATM SEC-4000 column using protein standards of known molecular masses. The collected fractions are indicated*.

On the other hand, the SDS-PAGE analysis of all the gel filtration fractions revealed not only an enrichment of the active compounds (Fr 1 and Fr 2) (Figure 3.8B) but also a possible oligomeric nature of the antibacterial proteins considering the molecular mass determined under native conditions (Figure 3.7).

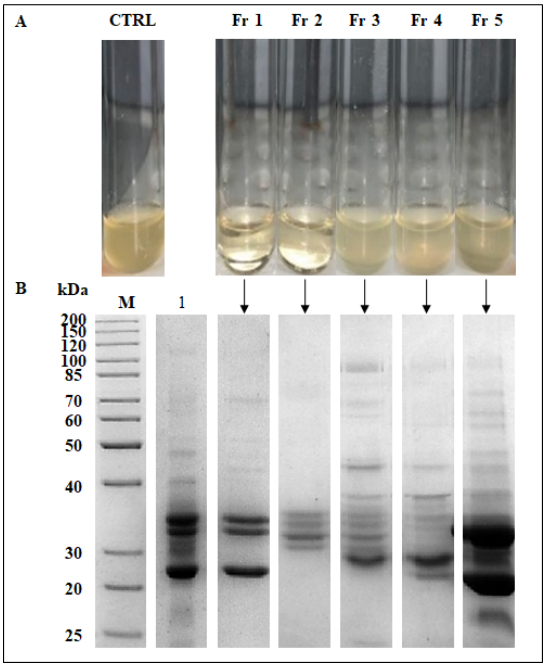


Figure 3.8 *(**A**) Antimicrobial screening assay of gel filtration fractions against S. aureus MRSA M7.* ***CTRL****: S. aureus MRSA M7 control;* ***Fr 1, Fr 2, Fr 3, Fr 4 and Fr 5****: fractions obtained after gel filtration chromatography of the pellet 50% sample. (**B**) SDS-PAGE analysis of the protein fractions. M: molecular weight markers; Lane 1: pellet 50% sample. Equal amounts of total proteins were loaded for each Lane.*

However, it cannot be excluded that more than one active protein compound could cooperate and contribute to the intrinsic antibacterial activity of the *L. europaeus* plant fruits.

3.4 References

Agrillo B, Mirino S, Tatè R, Gratino L, Gogliettino M, Cocca E, Tabli N, Nabti E, Palmieri G, 2019. An alternative biocontrol agent of soil-borne phytopathogens: A new antifungal compound produced by a plant growth promoting bacterium isolated from North Algeria. Microbiol Res 221, 60–69.

Alexander N, 2009. The Germanic word for 'sword' and delocatival derivation in Proto-IndoEuropean. JIES 37, 462–488.

Arachea BT, Sun Z, Potente N, Malik R, Isailovic D, Viola RE, 2012. Detergent selection for enhanced extraction of membrane proteins. Protein Expr Purif 86, 12–20.

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–254.

Cabezas-Pizarro J, Redondo-Solano M, Umaña-Gamboa C, Arias-Echandi ML, 2018. Antimicrobial activity of different sodium and potassium salts of carboxylic acid against some common foodborne pathogens and spoilage-associated bacteria. Rev Argent Microbiol 50, 56–61.

Cholakova M, Christov V, Dimitrova D, Evstatieva L, Alexandrova R, Nikolova E, 2002. Flavonoid and terpenoid isolated from Loranthus europaeus with stimulatory effect on lymphocyte proliferation. Experimental Pathology and Parasitology 5/9, 45-48.

Cowan MM, 1999. Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev. 12, 564–582.

Frazer JG, 1890. *The Golden Bough: A Study in Magic and Religion*. Boringhieri, Ed; Macmillan and Co., Ltd.: London, UK, 407–409.

Friedenauer S, Berlet HH, 1989. Sensitivity and variability of the Bradford protein assay in the presence of detergents. Anal Biochem 178, 263–268.

Gebauer R, Volarik D, Urban J, 2012. Quercus pubescens and its hemiparasite Loranthus europaeus: Nutrient dynamics of leaves and twigs. Acta Physiol Plant 34, 1801–1809.

Harvala E, 1984. Flavonoids of Loranthus europaeus. J Nat Prod 47, 1054–1055.

Hee-Ock B, Sung-Jin H, Chun-Sik B, Su-Hyun P, Buk-Gu H, Gorinstein S, 2012. Extraction and characterization of some natural plant pigments. Ind Crops Prod 40, 129–135.

Hegi G, 1981. Angiospermae, dicotyledones 1. In *Illustrierte Flora von Mitteleuropa, Band III, Teil 2*; Parey, P., Ed.; Verlag Paul Parey: Berlin, Germany.

Hibberd JM, Jeschke WD, 2001. Solute flux into parasitic plants. J Exp Bot 52, 2043–2049.

Jammer A, Gasperl A, Luschin-Ebengreuth N, Heyneke E, Chu H, Cantero-Navarro E, Großkinsky DK, Albacete AA, Stabentheiner E, Franzaring J, Fangmeier A, van der Graaff E, Roitsch T, 2015. Simple and robust determination of the activity signature of key carbohydrate metabolism enzymes for physiological phenotyping in model and crop plants. J Exp Bot 66, 5531–5542.

Katsarou A, Rhizopoulou S, Kefalas P, 2012. Antioxidant Potential of the Aerial Tissues of the Mistletoe Loranthus europaeus Jacq. Rec Nat Prod 6, 394–397.

Kumar P, Ramakritinan CM, Kumaraguru AK, 2010. Solvent Extraction and Spectrophotometric Determination of Pigments of Some Algal Species from the Shore of Puthumadam, Southeast Coast of India. IJOO 4, 29–34.

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Larsson S, 2007. Mistletoes and Thionins as Selection Models in Natural Products Drug Discovery. Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy 49, 65.

Leporatti ML, Corradi L, 2001. Ethnopharmacobotanical remarks on the Province of Chieti town (Abruzzo, Central Italy*).* J Ethnopharmacol 74, 17–40.

Leporatti ML, Pavesi A, 1989. Usi nuovi, rari o interessanti di piante officinali di alcune zone della Calabria. Webbia 43, 269–289.

Liu B, Le CT, Barrett RL, Nickrent DL, Chen Z, Lu L, Vidal-Russell R, 2018. Historical biogeography of Loranthaceae (Santalales): Diversification agrees with emergence of tropical forests and radiation of songbirds. Mol Phylogenet Evol 124, 199–212.

Nickrent DL, Malécot V, Vidal-Russell R, Der JP, 2010. A revised classification of Santalales. TAXON 59, 538–558.

Owuama C, 2017. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a novel dilution tube method. Afr J Microbiol Res 11, 977–980.

Palaniyandi U, Muthuswamy A, 2015. An efficient protein extraction method for proteomic analysis of black pepper (‘Piper nigrum’ L.) and generation of protein map using nano LC-LTQ Orbitrap mass spectrometry. Plant Omics 8, 500–507.

Palmieri G, Tatè R, Gogliettino M, Balestrieri M, Rea I, Terracciano M, Proroga YT, Capuano F, Anastasio A, De Stefano L, 2018. Small Synthetic Peptides Bioconjugated to Hybrid Gold Nanoparticles Destroy Potentially Deadly Bacteria at Submicromolar Concentrations. Bioconjug Chem 29, 3877– 3885.

Parisi K, Shafee TMA, Quimbar P, van der Weerden NL, Bleackley MR, Anderson MA, 2019. The evolution, function and mechanisms of action for plant defensins. Semin Cell Dev Biol 88, 107–118.

Pliny, 1938. Loeb Classical Library 330. In *Natural History*; Rackham, H., Ed.; Harvard University Press: Cambridge, MA, USA; Volume I: Books 1–2.

Ramm H, 2015. Mistletoe through Cultural and Medical History: The All-Healing Plant Proves to Be a Cancer- Specific Remedy. In *Mistletoe: From Mythology to Evidence-Based Medicine*; Zänker KS, Kaveri SV, Eds; Transl Res Biomed: Basel, Switzerland, 2015; Volume 4, 1–10.

Sathoff AE, Velivelli S, Shah DM, Samac DA, 2019. Plant Defensin Peptides have Antifungal and Antibacterial Activity Against Human and Plant Pathogens. Phytopathology 109, 402–408.

Scheetz MH, Qi C, Warren JR, Postelnick MJ, Zembower T, Obias A, Noskin GA, 2007. In vitro activities of various antimicrobials alone and in combination with tigecycline against carbapenem-intermediate or - resistant Acinetobacter baumannii. Antimicrob. Agents Chemother 51, 1621–1626.

Schurè E, 1889. Rama (le cycle aryen). *Les Grands Initiés. Esquisse de l'histoire secrète des religions*. Librairie académique Perrin et Cie: Paris, France 3–38.
Shahi Shavvon R, Saeidi Mehrvarz S, Golmohammadi N, 2012. Evidence from micromorphology and gross morphology of the genus Loranthus (Loranthaceae) in Iran. Turk J Bot 36, 655–666.
Sharquie KE, Noaimi AA, Saleh BM, Sharara ZA, Al-Salam WS, 2017. Topical 40% Loranthus europaeus Ointment as an Alternative Medicine in the Treatment of Acute Cutaneous Leishmaniasis versus Topical 25% Podophyllin Solution. JCDSA 7, 148–163.
Smith JV, 2016. Antimicrobial activity of different sodium and potassium salts of carboxylic acid against some common foodborne pathogens and spoilage-associated bacteria. Int J GEOMATE 11, 2671–2678.
Stead IM, Bourke JB, Brothwell D, 1986. *Lindow Man: The body in the Bog*. British Museum Publications: London, UK.
Tabli N, Raia A, Bensidhoum L, Palmieri G, Gogliettino M, Cocca E, Consiglio C, Cillo F, Bubici G, Nabti E, 2018. Plant growth promoting and inducible antifungal activities of irrigation well water-bacteria. Biol Control 117, 78–86.
Tattersall DB, van Heeswijck R, Høj PB, 1997. Identification and characterization of a fruit-specific, haumatin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. Plant Physiol 114, 759–769.
Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H, 2011. Phytochemical screening and Extraction: A Review. IPS 1, 98–106.
Vitasović Kosić I, Juračak J, Łuczaj Ł, 2017. Using Ellenberg-Pignatti values to estimate habitat preferences of wild food and medicinal plants: An example from northeastern Istria (Croatia). J Ethnobiol Ethnomed 13, 31.
Wang W, Tai F, Chen S, 2008. Optimizing protein extraction from plant tissues for enhanced proteomics analysis. J Sep Sci 31, 2032–2039.
Watson DM, 2001. Mistletoe—A keystone resource in forests and woodlands worldwide. Annu Rev Ecol Syst 32, 219–249.

4. Chapter 2

A Study on the Antimicrobial and Antibiofilm Peptide 1018-K6 as Potential Alternative to Antibiotics against Food-Pathogen *Salmonella enterica*

Abstract: Antimicrobial resistance has become one of the major global public health concerns, and it is indispensable to search for alternatives to conventional antibiotics. Recently, antimicrobial peptides have received great attention because of their broad-spectrum antimicrobial activity at relatively low concentrations, even against pathogens such as *Salmonella enterica*, which is responsible for most food- borne illnesses. This work aimed at evaluating the antimicrobial and antibiofilm activity of the innate defense peptide, named 1018-K6, against *S. enterica*. A total of 42 strains, belonging to three different subspecies and 32 serotypes, were included in this study. The antibiotic resistance profile of all the strains and the cytotoxic effects of 1018-K6 on mammalian fibroblast cells were also investigated. Results revealed that MIC (minimum inhibitory concentrations) and MBC (minimum bactericidal concentrations) values were in the ranges of 8–64 μg/mL and 16–128 μg/mL, respectively, although most strains (97%) showed MICs between 16 and 32 μg/mL. Moreover, sub-inhibitory concentrations of 1018-K6 strongly reduced the biofilm formation in several *S. enterica* strains, whatever the initial inoculum size. Our results demonstrated that 1018-K6 is able to control and manage *S. enterica* growth with a large potential for applications in the fields of active packaging and water disinfectants.

Keywords: antimicrobial peptide; *Salmonella*; food-borne pathogen; biofilm; 1018-K6; food preservatives

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4.1 Introduction

Salmonellosis is considered the second most reported gastrointestinal infection in humans in the European Union (EU) (EFSA and ECDC, 2021). This is due to the ability of the *Salmonella* genus serotypes to spread among a variety of species (both humans and animals act as host organisms). In 2019, 87923 confirmed human salmonellosis cases were reported in the EU (EFSA and ECDC, 2021). Indeed, the main sources of human infection in *Salmonella* outbreaks (17.9% of the total number of outbreaks) are contaminated foods, especially eggs and egg-derived products. Over the last few years, the annual number of strong-evidence food-borne outbreaks (FBOs) by eggs rose significantly, accounting for 37.0% (EFSA and ECDC, 2021a).

Human infections caused by *Salmonella* are divided into typhoid forms, due to *S*. Typhi and *S.* Paratyphi, and non-typhoid forms, especially due to *S.* Typhimurium and *S*. Enteritidis, which are responsible for over 50% of total gastrointestinal infections (EFSA and ECDC, 2021). Typically, non- typhoidal *Salmonella* causes self-limiting disease, although immunocompromised elderly or young individuals may manifest severe infections that need medical treatments over a long time with extended-spectrum antibiotics (Parry and Threlfall, 2008). However, the widespread and imprudent use of antibiotics may cause the development of multidrug-resistant strains of *Salmonella* (Anjum *et al*., 2011), which could be transferred through food-producing animals to humans (Hur *et al*., 2012). In 2019, a high proportion of *Salmonella* isolates from human patients in the EU were revealed to be resistant to sulfonamides, tetracyclines and ampicillin (EFSA and ECDC, 2021b). The increasing resistance to the "critically important antimicrobials" (CIA) of highest priority, as defined by the World Health Organization, is alarming, especially in the case of ciprofloxacin, the third-generation cephalosporins (cefotaxime and ceftazidime) and/or the carbapenems whose inefficacy is mainly due

to the extended-spectrum beta-lactamase (ESBL)/AmpC/carbapenemase-producing bacteria among *Salmonella* spp. (EFSA and ECDC, 2021b). As a result, *Salmonella* infections are becoming increasingly more difficult to treat, even with last-resort antibiotics. Therefore, in the era of novel technologies for innovative biomedical approaches, one of the main scientific challenges is the exploration of alternative solutions to the current inefficient treatments of infections with traditional antibiotics (Pateiro *et al*., 2021).

Moreover, recent studies revealed the social aspects of the life of bacteria, such as biofilm formation, which is mostly affected by environmental conditions. Biofilms play a crucial role in antimicrobial resistance phenomena, decreasing bacterial susceptibility to antibiotic action (Costerton *et al*., 1999). Indeed, the first case reported of food-borne bacterial biofilm infection was associated with *Salmonella* spp., whose biofilm-production has been largely studied and described (Duguid *et al*., 1966). The switch from a free-living state to the biofilm mode of growth allows bacterial cells to survive under adverse conditions, which may be recreated in chicken slaughterhouses and poultry farms (Wang *et al*., 2013). Previous works described *S*. Enteritidis as one of the strongest biofilm-producer serotypes (Schonewille *et al*., 2012) and identified wood as the surface that allows the best bacterial adhesion (Steenackers *et al*., 2012). As *Salmonella* biofilms show high resistance to disinfectants and antibiotics, it is necessary to develop innovative strategies to inhibit and/or prevent their formation.

In this regard, antimicrobial peptides (AMPs) represent an exciting option taken progressively into consideration. AMPs have gained increasing attention as new potential antimicrobial drugs to replace or potentiate the action of conventional antibiotics in controlling infections caused by pathogens, due to their efficient activity and unique mechanism associated with specific chemical-physical properties (Zharkova *et al*., 2019; Lei *et al*., 2019; Mika *et al*., 2011). AMPs are short amino acid sequences forming an ancient type of innate immunity found in all living organisms from bacteria to mammals, which work by providing a first line of defense against invading pathogens. Although there is a high number and wide sequence diversity of AMPs in nature, there are common structural features shared by the majority of these compounds (Maróti *et al*., 2011). These peptides are usually around 10–40 residues in length with net positive charges (from +2 to +9) and high extents of hydrophobic residues, ranging from 40 to 50%, arranged so that the folded peptide can adopt amphipathic structures (Lei *et al*., 2019; Teixeira *et al*., 2012). As a result, AMPs display a strong tendency to establish non-specific interactions with negatively charged phospholipids, such as phosphatidylglycerol, which are typically abundant in the microbial cell membranes, leading to an increased permeability, leakage of cytoplasmic components and cell death (Teixeira *et al*., 2012; Brogden, 2005). In contrast to the majority of the available antibiotics that target specific biosynthetic pathways, AMPs efficiently kill microbial pathogens in most cases by accumulation on the membrane surfaces, pore formation and damage to the cell envelope integrity (Brogden, 2005). Therefore, AMPs can be effective against a wide spectrum of organisms, such as Gram-positive and Gram-negative bacteria, as well as fungi and viruses, and their non-specific mode of action significantly prevents the induction of resistance, as it is metabolically costly for most microbes to mutate or repair membrane components (Brogden, 2005; Lazzaro *et al*., 2020; Zasloff, 2002). In addition, due to their role in the organisms as natural microbicides, AMPs are selectively cytotoxic to microorganisms, whilst they generally exhibit low or no toxic effects towards animal cells of the host organism. Indeed, the hemolytic activity and toxicity of these peptides were demonstrated to be linked to a range of physicochemical properties and to the membrane composition of the target cells. However, the cell selectivity issue needs to be seriously investigated for this class of antimicrobial compounds, as it is an important aspect that can limit their applications in vivo (Yeaman *et al*., 2003; Starr *et al*., 2020).

In a previous study, a cathelicidin-related antimicrobial peptide consisting of 12 residues and named 1018-K6 was in silico designed and characterized (Palmieri *et al*., 2018a; Palmieri *et al*., 2018b). The pep- tide displayed high structural stability as well as powerful antimicrobial and antibiofilm activities at a low-micromolar range against Gram-positive and Gram-negative bacterial pathogens, including MRSA (Palmieri *et al*., 2018a; Colagiorgi *et al*., 2020). Moreover, preliminary toxicity assays showed

that 1018-K6 did not affect the cell morphology of different human cell lines. In addition, 1018- K6 was revealed to adopt mixed α-helical/-β-sheet conformations when in contact with bacterial membrane mimics, and investigations on the antibacterial mechanisms strongly suggested that it belongs to the membrane-interacting peptide clan (Palmieri *et al*., 2018a). In light of these considerations, 1018-K6 represents a promising candidate for the development of a new generation of antibiotic molecules.
Herein, the bactericidal and antibiofilm activity of 1018-K6 against a large panel of serotypes of *Salmonella enterica* was investigated with the aim to explore the real potentials of this antimicrobial peptide further. The resistance profiles of all the strains included in this study were also determined.

4.2 Materials and Methods

4.2.1 Strain Selection

The antimicrobial activity of 1018-K6 was evaluated against 42 different *Salmonella* strains, belonging to *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae* and *S. enterica* subsp. *arizonae*. Of these, two reference strains were provided by the Spanish Type Culture Collection (CECT) and ten by the Spanish National Reference Laboratory (NRL) for salmonellosis in animals. Twenty-eight wild isolates were collected from fish (one), straw (one) and animal feces samples, of which many originated from different broiler farms. The ability of 1018-K6 to prevent biofilm formation was evaluated against a smaller panel of eleven of these species and serovars. Details about the strains investigated in this study are shown in Table 4.S1.
Wild strains were isolated from bovine and chicken feces, fish and straw according to ISO 6579-1:2017 (ISO, 2017). Briefly, samples were mixed with buffered peptone water (Merck Millipore, Germany) at 1/10 *w*/*v* and incubated for 18 h at 37°C. Then, a volume of 100 µL of the incubated sample was inoculated onto Rappaport-Vassiliadis semi-solid modified medium (Difco Laboratories, Franklin Lakes, NJ, USA) and incubated at 41°C for 48 h for selective enrichment. Then, presumptive positive plates of Salmonella were picked with an inoculating loop, spread on xylose lysine deoxycholate agar (Oxoid Ltd., Hampshire, UK) and chromID Salmonella agar (SM-ID2, bioMérieux, Marcy-l'ÉEtoile, France) and incubated at 37°C for 24 h. Presumptive Salmonella colonies from xylose lysine deoxycholate and SM-ID2 were cultured in Nutrient Agar (PanReac AppliChem, Spain) for 24 h at 37°C. Isolates were confirmed by a latex agglutination test (Microgen, London, UK) and API 20E (bioMérieux) and serotyped using the Kauffman–White typing scheme for the detection of somatic (O) and flagellar (H) antigens with standard antisera (Bio-Rad Laboratories, Hercules, CA, USA).

4.2.2 Medium and Reagents

Muller-Hinton Broth (Panreac Applichem, Barcelona, Spain) and Nutrient Agar (Panreac Applichem, Barcelona, Spain) were used to revitalize and prepare the bacterial inocula. Species confirmation was performed on selective medium XLD (Oxoid, Hamp- shire, UK) and CHROMID SM2 (Biomérieux, Marcy-l'Étoile, France). Serial dilutions were performed with 0.85% saline solution (Sodium Chloride 99.85%, Acros Organics). Crystal Violet (Panreac Applichem, Barcelona, Spain) and Methanol (EMSURE ACS, ISO, Reag. Ph. Eur., Merck, Kenilworth, NJ, USA) were used for Biofilm Inhibitory Concentration (BIC) tests, respectively, to stain the bacterial cells and solubilize dye bound in each well of the microplate. The derivative 12-mer peptide 1018-K6 was purchased from SynPeptide Co., LTD (Shanghai, China).

4.2.3 Antibacterial Activity Assay

Before each experiment, bacterial strains, frozen at 80° C, were revitalized in Muller– Hinton Broth at 37° C for 24 h and then grown on Nutrient Agar at 37° C for 24 h. Working cultures were obtained by transferring isolated colonies to 0.85% saline solution and adjusting the turbidity to 0.5 McFarland, which is equivalent to approximately 1–2 x 10^8 colony-forming units (CFU)/mL. Therefore, bacterial suspensions were diluted to obtain a concentration of 10^3 CFU/mL in Muller-Hinton Broth, confirmed by spread-plating 100 µL on selective plate agar (aerobically incubated at 37°C for 24–48 h).
The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of the peptide at which no bacterial growth is detected. Minimum Bactericidal Concentration (MBC) is defined as the lowest concentration of peptide at which more than 99.9% of the bacterial cells are killed. To evaluate MIC and MBC, the method described by Colagiorgi and coworkers was adopted (Colagiorgi *et al*., 2020). Each strain was exposed to different concentrations of peptide 1018-K6. The intermediate stock solution of the peptide was produced daily by sonicating the frozen stock and diluting it to a concentration of 256 µg/mL in Muller–Hinton Broth. Briefly, each well of the microplate was filled with 50 µL of bacterial suspension (1x10^3 CFU/mL) and 50 µL of peptide solutions at decreasing concentrations (ranging from 256 µg /mL to 0.25 µg /mL). Then, the microplate was incubated at 37°C for 20 h. Cell growth was evaluated by the unaided eyes (CLSI, 2020). After MIC determination, to evaluate the MBC values, 100 µL of bacterial suspension, in which no visible bacterial growth was observed, were seeded in Nutrient Agar plates, and the plates were then incubated for 24 h at 37°C. The same analysis was performed using two-fold increased concentrations of peptide (with respect to MIC values). The antimicrobial test was replicated at least three times for all strains.

4.2.4 Inhibition of Biofilm Formation

Inhibition of biofilm formation was determined in 96-well flat-bottom polystyrene microtiter plates by using the method described by O'Toole (2011). Culture solution and peptide intermediate stock preparations were performed daily as described above. After revitalization, bacterial cultures were serially diluted to 5 x 10^4 CFU/mL or 5 x 10^8 CFU/mL, to prove peptide antibiofilm activity in two different bacterial suspensions. Considering the MIC values, lower doses of peptide were chosen (1/2 and 1/4 of the MIC value), bearing in mind that optimal bacterial growth conditions are required to allow biofilm production. Each well of the microplate was filled with 230 µL of 1018-K6 suspension in growth medium (Muller–Hinton Broth) and 20 µL of bacterial culture. Microplates were incubated for 24 h at 37 C. At the end of incubation, the supernatant was poured off, and wells were washed three times with 300 µL of distilled water. Bacterial cells attached to the walls of the microplate were fixed by adding 250 µL of absolute methanol, left for 15 min, and then the methanol was discarded. Wells were air-dried, and the remaining bacteria were stained with 250 µL of 0.1% (*w*/*v*) crystal violet solution for 5 min. Wells were rinsed by placing the microplate under running water. Microplates were air-dried again, and the crystal violet contained within the biofilm was solubilized using 250 µL of 33% (*v*/*v*) glacial acetic acid per well. The Optical Density (OD) of each well was measured at 630 nm with a plate reader. The antibiofilm test was replicated at least three times for all strains for both inocula concentrations.

4.2.5 Antimicrobial Resistance Profile

The minimum inhibitory concentrations (MICs) of *Salmonella* strains included in this study were determined against 12 different antibiotics: ampicillin (from 1 to 128 µg/mL), chloramphenicol (from 2 to 64 µg/mL), ciprofloxacin (from 0.06 to 8 µg/mL), gentamicin (from 0.5 to 32 µg/mL), kanamycin (from 4 to 128 µg/mL), levofloxacin (from 0.5 to 32µg/mL), nalidixic acid (from 2 to 64 µg/mL), streptomycin (from 4 to 256 µg/mL), sulfamethoxazole (from 8 to1024 µg/mL), sulfisoxazole (from 8 to 1024 µg/mL), tetracycline (from 1 to 128 µg/mL) and trimethoprim (from 0.5 to 32 µg/mL). The standard antimicrobials were purchased from Sigma–Aldrich. MIC values were determined by using

the broth microdilution method as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines (2020). Briefly, for each test, antibiotic stocks of 4096 µg/mL were prepared in the diluent described in the CLSI guidelines and serially diluted in Mueller–Hinton broth (MH Broth, Panreac Applichem, Barcelona, Spain). All *Salmonella* strains were grown for 16–18 h at 37 C in Nutrient agar (Panreac Applichem, Barcelona, Spain), and a single colony from incubated plates was picked and transferred to 10 mL of 0.85% saline solution at a final concentration of 0.5 McFarland. In the next step, the saline tube was serially diluted to a final *Salmonella* concentration of 10^6 CFU/mL, and the 96-well microtiter plates were filled with 50 µL of each antibiotic dilution and 50 µL of the individual *Salmonella* strain. The microtiter plates were incubated for 24 h at 37°C and after incubation, MIC values were calculated. For each antibiotic compound and *Salmonella* strain, MIC was determined as the lowest concentration in which no visual bacterial growth was observed. *Escherichia coli* (ATCC 25922) and *Enterococcus faecalis* (ATCC 29212) were used as control strains, and the susceptibility or resistance of each isolate was determined in agreement with the 2020 CLSI recommendations (2020). Moreover, all the *Salmonella* isolates, exhibiting resistance to at least three classes of antimicrobial agents tested, were considered multi-resistant.

4.2.6 Cytotoxicity Assays on Mammalian Cells

Neutral Red Uptake (NRU) assay was used to assay the potential 1018-K6 toxicity on mammalian fibroblasts BALB 3T3 clone A31 (ATCC CCL-163), at different peptide concentrations. Cell cultures were performed in Dulbecco's Modified Eagle's Medium (DMEM) supplied with 4 mM Glutamine and 10% Newborn Calf Serum. For the experimental assay (NRU), the seeding of BALB 3T3 clone A31 (ATCC CCL-163) cells was carried out in a 96-well microtiter plate (Thermo Fisher Scientific, Milan, Italy) incubated in the humidified environment and the presence of CO_2 (5%) at 37°C for 24 h. Incubations under these specific conditions allowed cell sedimentation and the establishment of a sub-confluent monolayer before supplying 1018-K6. Mammalian cells were treated with two doses of 1018-K6 (16 µg/mL and 80 µg/mL) for 24 h at 37°C. Therefore, 150 µL of D-PBS with Ca^{2+}/Mg^{2+} were used to rinse each well before exposing cells to 50 µg/mL of Neutral Red (NR) dye solution for 3 h at 37°C. Then, after a second rinse (as described above), 150 µL of NR desorb solution (49% ddH2O, 50% ethanol, 1% acetic acid) were added to each well, and microplates were incubated in darkness under gentle agitation for 10 min. The OD of the NR extract was measured spectrophotometrically at 540 nm.
Cell viability was expressed as the percentage of BALB 3T3 clone A31 cells that survived after treatment with 1018-K6. The parameter was compared to the control sample (mammalian cells grown in DMEM with 5% NCS, 4 mM Glutamine and 0.1% DMSO) which has a cell viability of 100%:

(1) (OD treated cells OD blank)/(OD Control Cells OD blank) x 100

In order to analyze the obtained data, the ISO 10993-5:2009 was taken into account: the substance was identified as cytotoxic if the relative cell viability resulted to be <70% with respect to the control group, while the compound was classified as non-cytotoxic if the cell viability was 70% of the control sample.

4.2.7 Statistical Analysis

SPSS software version 26 (IBM Analytics, Armonk, NY, USA) was used to perform statistical analysis. Analysis of variance (generalized linear mixed model) was used to study the MBC values, the influence of the bacterial inoculum concentration on biofilm formation and the peptide effects at several doses on biofilm production for each serovar. An a posteriori contrast was performed using the Tukey test, considering a p value of <0.5 as statistically significant.

4.3 Results and Discussion

4.3.1 Antimicrobial Activity of 1018-K6 on Planktonic Salmonella enterica Cells and Salmonella Resistance Profile

Recently, a 1018-derivative antimicrobial peptide named 1018-K6, (VRLIVKVRIWRR- NH2) was designed and characterized (Palmieri *et al*., 2018a; Palmieri *et al*., 2018b; Colagiorgi *et al*., 2020). This 12-mer cationic peptide originates from the bovine host-defense peptide (HDP) bactenecins found in the neutrophil granules and belongs to the cathelicidin's family. Structural and conformational studies performed on 1018-K6 clearly revealed that it showed a propensity to assume α-helical structures in membrane-mimetic models, such as micellar solutions of SDS (Palmieri *et al*., 2018a; Palmieri *et al*., 2018b; Colagiorgi *et al*., 2020). In addition, this peptide retained its structural integrity in a wide range of pH and temperature conditions for prolonged incubation times, displaying also a significant bactericidal and antibiofilm activity against *Listeria monocytogenes* isolates from food products and contaminated environments (Palmieri *et al*., 2018a; Palmieri *et al*., 2018b; Colagiorgi *et al.,* 2020).
In this work, the efficacy of 1018-K6 on the growth of a panel of wild and reference strains of different *Salmonella* serotypes was explored for potential applications in the food manufacturing industry. Taking into account the large amount of available experimental data on *Salmonella* spp. resistance to AMPs, the study was aimed at demonstrating the efficacy of 1018-K6 against this bacterial genus. Andersson *et al*. (2016) accurately described *Salmonella* Typhimurium mechanisms of resistance (intrinsic and acquired) to cationic antimicrobial peptides. Therefore, the investigation on the peptide 1018-K6 was focused on testing its activity against a large number of strains to prove its efficiency as an antibiotic compound towards serovars, which are notoriously more resistant than others. For this reason, in order to obtain more representative results, more than 40 *Salmonella* strains across 32 different serotypes were involved in the experimental design. As reported in Table 4.1, a high variability, in terms of bacterial susceptibility to 1018-K6, was found among *Salmonella* subspecies and serovars. Nevertheless, each strain was characterized by very low minimum inhibitory concentration (MIC) values, ranging from 8 to 64 μg/mL. The highest concentration of the peptide needed to prevent visible bacterial growth was observed for *S. enterica* subspecies *salamae* 6,8: g, m, t (Table 4.1). Moreover, 1018-K6 was highly effective against 54% of the strains belonging to *S. enterica* subspecies, showing MIC values of 16 μg/mL (Figure 4.1A) towards serovars such as *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Virchow and *S.* Hadar.

Table 4.1 *MIC and MBC values of 1018-K6 against 42 Salmonella subspecies/serovars and analysis of their antibiotic resistance profiles.*

	MIC	**MBC**	**Antimicrobial Resistance Profile**
Subspecies or serovar	**μg/mL**	**μg/mL**	**Resistant to**
S. Stanleyville	8	32	-
S. Agama	16	32	-
S. Anatum	16	16	-
S. Bredeney	16	64	-
S. Cerro	16	16	-
S. Dublin	16	16	-
S. Eboko	16	16	-
S. Enteritidis	16	64	Amp
S. Hadar	16	32	Tet, sulf
S. Infantis	16	16	-
S. Jerusalem	16	16	Sulf
S. Mbandaka	16	64	-
S. Mikawasima	16	16	Amp
S. Montevideo	16	64	Sulf
S. Newport	16	16	Sulf
S. Richmond	16	16	-

S. Seftenberg	16	16	Strep
S. Typhimurium monophasic	16	16	Tet, Strep, sulf, Amp
S. Typhimurium	16	64	
S. Typhimurium 1	16	64	Tet, strep, amp
S. Typhimurium 2	16	64	Amp
S. Typhimurium 3	16	64	Amp, Strep
S. Typhimurium 4	16	64	Strep
S. Virchow	16	32	Na
S. Isangi	32	64	Sulf
S. Meleagridis	32	32	Strep, sulf, Amp
S. Barro	32	32	-
S. Dabou	32	32	-
S. Drac	32	32	-
S. Enterica 4:b	32	32	Strep
S. Enteritidis CECT 4300	32	64	-
S. Ndolo	32	32	-
S. Poona	32	32	-
S. Thompson	32	64	Amp
S. Typhimurium CECT 4594	32	64	-
S. Typhimurium 5	32	64	Strep
S. Typhimurium 6	32	64	-
S. Typhimurium 7	32	64	-
S. Typhimurium 8	32	64	-
S. arizonae 48:z4,z23	32	>128	-
S. arizonae 48:z4,z23,z32	32	128	-
S. salamae 4, 12: b-	32	64	Sulf
S. salamae 4,5,12:b	32	64	Sulf
S. salamae 6,8: g, m, t	64	64	-

Amp: ampicillin; Na Nalidixic acid; Strep: streptomycin; Sul: sulfamethoxazole; tet: tetracycline. All the experiments were performed in triplicate. The MICs and MBCs values against *Salmonella* isolates were statistically analyzed using an ANOVA test. The modes were equivalent to the medians.

A preliminary analysis of trends and correlations among the collected data from reference and wild strains (see Table 4.S1 for bacterial source) evidenced an increase in antimicrobial performances of the peptide against the environmental *Salmonella* spp., especially towards *S. enterica* subspecies Enteritidis. These results are particularly relevant if we consider that some authors (Andersson *et al*., 2016) have previously described *S.* Enteritidis and *S.* Typhimurium as the serovars more resistant than others to antimicrobial agents. Nevertheless, 1018-K6 appears to kill these pathogens efficiently, which are responsible for most of the human infection cases (EFSA and ECDC, 2021b). Moreover, the strains of *S.* Enteritidis and *S.* Typhimurium pro- vided by the Spanish Type Culture Collection (CECT) (Table 4.S1) showed higher MIC values than those determined for the same serovars isolated from chicken feces. This behavior is quite uncommon, as strains isolated from environments usually show a reduced susceptibility to antibiotics and disinfectants due to adaptation or resistance phenomena and the presence of mobile genetic elements carrying resistance genes or an altered permeability of the bacterial cell walls. However, further studies are needed to investigate the mechanisms underlying these aspects better.

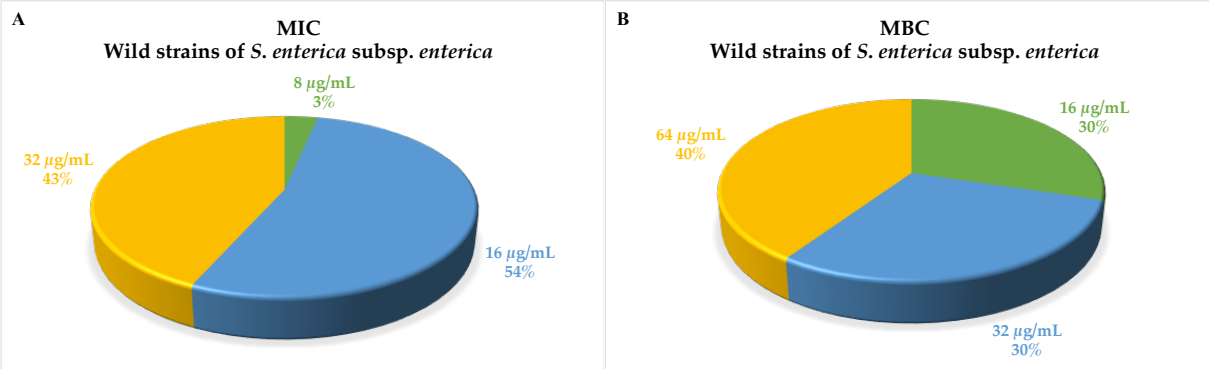


Figure 4.1 *Distributions of Minimum Inhibitory Concentration (**A**) and Minimum Bactericidal Concentration (**B**) values among strains of S. enterica subsp. enterica are shown in the pie chart as a percentage of serovars related to a specific peptide dose.*

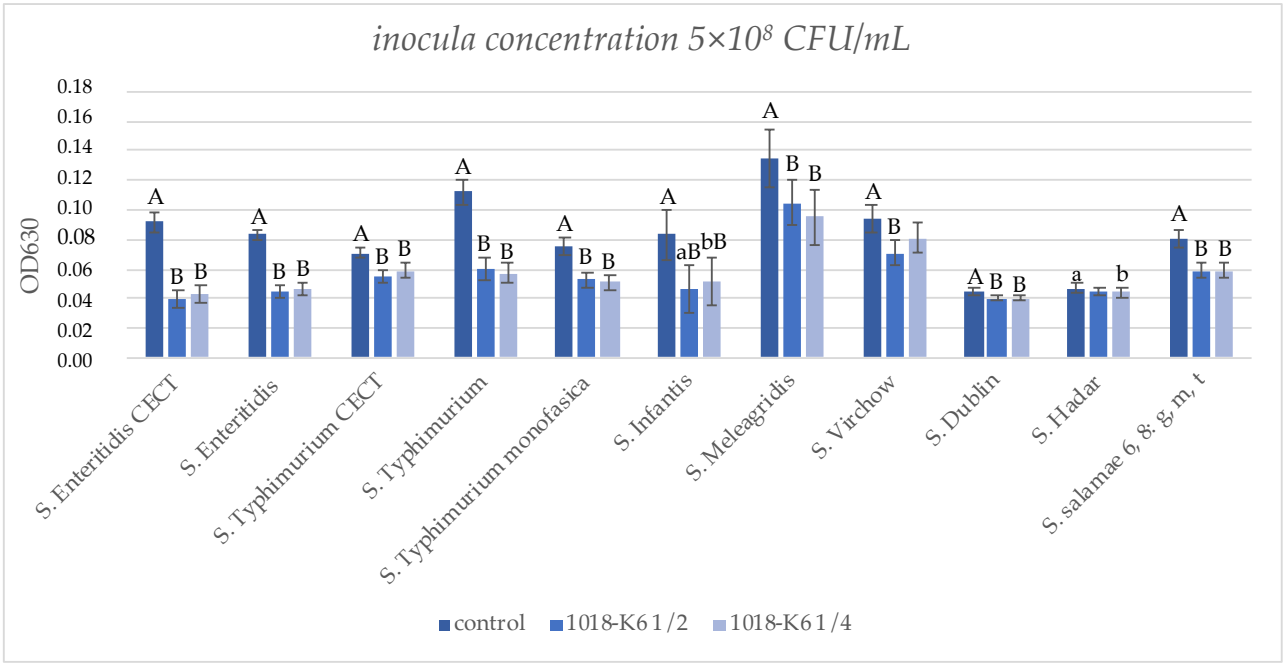
The concentration of the peptide able to kill the planktonic bacterial cells was also determined, obtaining the MBC values of 1018-K6 against the 42 *Salmonella* strains under investigation. The MBCs differed significantly between groups of strains ranging from 16 to 128 µg/mL when compared, with *S. arizonae* the most unaffected (Table 4.1).
Once again, at least 50% of bacteria belonging to the subspecies *enterica* (Figure 4.1B) were sensitive to low doses (16–34 µg/mL) of the antimicrobial peptide, while also taking into account that a concentration of 16 µg/mL was sufficient to kill all *S.* Typhimurium monophasic cells. Interestingly, the antimicrobial results in Table 4.1 suggested a generally high correlation between MBC and MIC values of 1018-K6, with some tested bacteria even having identical values. This property is surprising and quite uncommon as the MBC of antimicrobial compounds is usually much higher than the corresponding MICs values, which indicates growth inhibition and not necessarily bacterial death and cannot distinguish between bactericidal and bacteriostatic effects. Indeed, the antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC (Yazgan *et al*., 2019). As previously described, increased resistance of bacteria to commonly used antibiotics has become one of the major challenges in the current medical practice. Therefore, to gain insight into the potential of 1018-K6 as antimicrobial, the antibiotic profile of the strains included in this study was investigated. Results revealed that three strains were multi-resistant, while *S.* Typhimurium monophasic exhibited the highest resistance profile, being resistant to four different antimicrobial groups, but showed a significant sensitivity to the action of 1018-K6 with low MIC and MBC values (16 µg/mL). Therefore, the peptide was remarkably effective even against multi-resistant strains at low concentrations. On the other hand, the effects observed against *S. enterica* subsp. *arizonae* 48:z4,z23 and *S. enterica* subsp. *arizonae* 48:z4,z23,z32 were less exciting, as evidenced by increased MIC values (128 µg/mL and >128 µg/mL) and no resistance of these strains to any antimicrobial investigated. However, serotypes of this subspecies are usually characterized by low virulence as they cause diseases only in highly immunosuppressed individuals with previous pathologies (Lamas *et al*., 2018). Hence, an inverse relationship may exist between the pathogenic potential of serotypes and the resistance to the peptide 1018-K6.

4.3.2 Evaluation of the Activity of 1018-K6 against Biofilm Formation of S. enterica

Due to the propensity of *Salmonella enterica* to attach to environmental and food matrixes (Galié *et al*., 2018), 1018-K6 was tested to counteract the biofilm-forming ability of a panel of pathogenic bacteria, as the biofilms represent an important virulence factor and the main source of environmental contamination (Lamas *et al*., 2016b). The occurrence of clusters of bacteria bound to a surface and each other and embedded in a self-produced matrix is a common event in the food industry, and it has been noted that *S. enterica* under dry conditions can survive in a biofilm on stainless steel for over a year (Morita *et al*., 2011).

Biofilm inhibitory activity was evaluated against the *Salmonella* serotypes commonly recognized as a causative agent in food-borne outbreaks (*S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Virchow and *S.* Hadar) (EFSA and ECDC, 2021a), and two reference strains. Among the tested strains, some microorganisms were chosen for their well-known ability in biofilm production (i.e., *S.* Typhimurium, *S.* Infantis) (Marin *et al*., 2009). Therefore, based on optical spectroscopy, measurements of the amount of biofilm (i.e., OD_{630} nm readings following crystal violet staining) produced by each strain in the presence of sub-MIC concentrations of the peptide revealed that it led to significant reductions in biofilm growth. Interestingly, the amount of biofilm formed by *S. enterica* was not homogenous among serotypes investigated, and it did not de- pend on the inoculum concentration (Díez-García *et al*., 2012). In fact, data on biofilm formation were compared to the initial planktonic cell concentration (Table 4.S2) for each serotype, and an absence of a correlation was observed in at least 55% of strains (*S*. Typhimurium, *S*. Typhimurium monophasic, *S*. Virchow, *S*. Dublin, *S*. Hadar and *S*. *salamae*), whose variations in biofilm production were not significant, while differential responses were detected for *S*. Enteritidis CECT 4300, *S*. Enteritidis, *S*. Typhimurium CECT 4594, *S*. Infantis and *S*. Meleagridis (Table 4.S2). Therefore, our study provides evidence for the first time that the initial cell concentration may not affect biofilm formation in different *Salmonella* spp.

Histograms (Figure 4.2) were used to illustrate the relationship between biofilm forma- tion and peptide doses. The 1018-K6 differently decreased biofilm formation between species and serovars, and results obtained with the highest inoculum concentration (Figure 4.2A) indicated that it exhibited a significant inhibitory capacity versus all strains except for *S*. Hadar and *S*. Virchow. Moreover, among wild serovars, the peptide appeared to be more effective against *S*. Typhimurium and *S*. Enteritidis than the other isolates, significantly reducing biofilm mass by 49% and 44%, respectively.



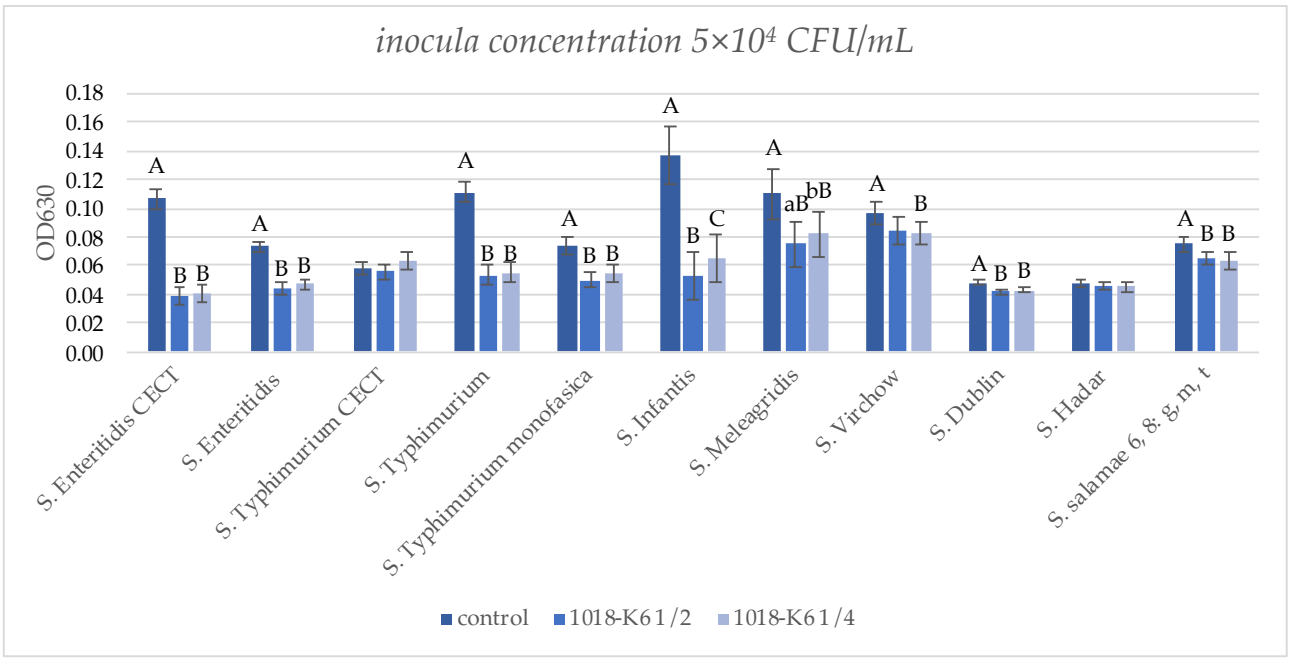


Figure 4.2 *Antibiofilm activity of 1018-K6 on 11 strains of S. enterica from inocula of 10^8 CFU/mL* ***(A)*** *and 10^4 CFU/mL* ***(B)****. Bacterial cells were grown under biofilm conditions in the absence (control) or presence of the peptide at a concentration of ½ and ¼ of the corresponding MIC values for each strain (1018-K6 ½ and 1018-K6 ¼, respectively). Statistical analysis was performed only within each strain, comparing the control with each treated bacterial sample. Average OD_{630} values are shown with error bars representing the standard error of four independent replicates. Different superscript uppercase letters indicate a significant difference within each strain at $p < 0.01$. Different superscript lowercase letters indicate a significant difference within each strain at $p < 0.05$.*

Tests using inocula of 10^4 CFU/mL confirmed the results reported above, despite the different initial concentration of planktonic cells (Figure 4.2B). Interestingly, in the case of *S.* Typhimurium CECT 4594, the lowest concentration of planktonic cells in the inoculum appeared to be better adapted to protection from the antimicrobial effects of 1018-K6, in contrast to that observed when a bacterial inoculum of 10^8 CFU/mL was used (Figure 4.2) in the biofilm assays. Overall, more studies are necessary in order to clarify key factors affecting the antibiofilm activity of 1018-K6 and the complex mechanisms of biofilm formation among serovars better, taking into account that many features can interfere in the process such as the origin of the isolates (Lamas *et al.*, 2016a).

Furthermore, bacterial biofilm reduction was found to be concentration-independent for many strains investigated (Figure 4.2), suggesting that a very low amount of 1018- K6 was necessary to inhibit the biofilm formation, and this effect appeared not to be dose-dependent. Therefore, these analyses validated the peptide efficacy on biofilm development, defining a great potentiality that might be further examined. In the last few years, several research studies explored new strategies to identify alternative antibiofilm substances able to replace the traditional compounds that often require high doses to obtain biofilm eradication/inhibition (Wang *et al*., 2019).
All these findings appeared in agreement with the previous studies (Palmieri *et al*., 2018b; Colagiorgi *et al.,* 2020) that designated 1018-K6 as an optimal antimicrobial candidate for its great activity against bacteria of the genus *Listeria* (i.e., *L. monocytogenes*) and *Staphylococcus* (i.e., *S. aureus* MRSA and *S. aureus* MSSA). Results reported in these works strongly supported the hypothesis that 1018-K6 is able to act via a cell-membrane-destabilization mode of action, which does not involve the interaction and inhibition of intracellular messengers of biofilm. However, secondary mechanisms of action of peptide could not be excluded, and further investigations are necessary to clarify these aspects better.

4.3.3 Evaluation of Cytotoxic Effects of 1018-K6 on Mammalian Fibroblast Cells

The cytotoxic potential activity of 1018-K6 on mammalian cells was mostly analyzed in vitro by the Neutral Red Uptake (NRU) assay using the mammalian BALB 3T3 clone A31 fibroblast cell line. The NRU test is a common method to quantify the cytotoxicity of different chemical compounds in cell cultures. The principle of the assay is based on the uptake of the neutral red dye which accumulates in the lysosomes of uninjured cells. The NRU in vitro analysis is usually used to investigate initial doses for in vivo acute oral systemic toxicity tests. In addition, the BALB/3T3 mouse fibroblast cells were selected in our cytotoxicity study since the BALB/3T3 system was revealed to be very sensitive to a large panel of potential carcinogens or pro-carcinogens (Dipaolo *et al*., 1972; Quarles and Tennant, 1975).
Based on these analyses, cell viability results, following the treatment with two different concentrations of 1018-K6 (16 and 80 μg/mL), were 99.9% and 97%, respectively, as determined by using the equation (1) reported in the Material and Methods section. Therefore, for Balb/3T3 cells, increasing doses of the antimicrobial peptide led to minimal inhibition of cell growth. These findings suggested that 1018-K6 did not exhibit any cytotoxic effect against the mammalian cells at the tested concentrations, even at of 80 μg/mL, which was sufficiently high to kill almost all the investigated pathogenic bacteria, except for *S. arizonae* 48:z4,z23 and *S. arizonae* 48:z4,z23,z32, which showed a MBC value of 128 μg/mL. It is noteworthy that 1018-K6 showed high selectivity towards bacteria over mammalian cells, possibly due to the different complex membrane lipids in these cells. In fact, the substantial variation in the composition of eukaryotic membranes in comparison to prokaryotic cell envelopes could explain the great selectivity of 1018-K6 for microbial cells. Indeed, it has been widely reported that the cationic antimicrobial peptides can preferentially bind to the negatively charged phospholipid bilayers of bacterial cells compared to the more neutrally charged eukaryotic cells (Teixeira *et al*., 2012; Laverty *et al*., 2011). These results further evidence the potential advantages that can be afforded by using 1018-K6 formulations in biotechnological and clinical applications.

4.4 Supplementary materials

Table 4.S1 *Source of Salmonella enterica strains and scheduled tests.*

Salmonella *subspecies and serovar*	**Source**	**MIC**	**MBC**	**BIC**
S. Enteritidis CECT 4300	CECT	✓	✓	✓
S. Typhimurium CECT 4594	CECT	✓	✓	✓
S. Agama	Spanish NRL for Salmonellosis in animals	✓	✓	

S. Dublin	Spanish NRL for Salmonellosis in animals	✓	✓	✓
S. Eboko	Spanish NRL for Salmonellosis in animals	✓	✓	
S. Hadar	Spanish NRL for Salmonellosis in animals	✓	✓	✓
S. Infantis	Spanish NRL for Salmonellosis in animals	✓	✓	✓
S. Jerusalem	Spanish NRL for Salmonellosis in animals	✓	✓	
S. Meleagridis	Spanish NRL for Salmonellosis in animals	✓	✓	✓
S. Poona	Spanish NRL for Salmonellosis in animals	✓	✓	
S. Richmond	Spanish NRL for Salmonellosis in animals	✓	✓	
S. Virchow	Spanish NRL for Salmonellosis in animals	✓	✓	✓
S. Anatum	Bovine faeces	✓	✓	
S. Bardo	Chicken faeces	✓	✓	
S. Bredeney	Chicken faeces	✓	✓	
S. Dabou	Chicken faeces	✓	✓	
S. Drac	Chicken faeces	✓	✓	
S. Enteritidis	Chicken faeces	✓	✓	✓
S. Isangi	Chicken faeces	✓	✓	
S. Mbandaka	Chicken faeces	✓	✓	
S. Mikawasima	Chicken faeces	✓	✓	
S. Montevideo	Chicken faeces	✓	✓	
S. Ndolo	Chicken faeces	✓	✓	
S. Newport	Chicken faeces	✓	✓	
S. Seftenberg	Chicken faeces	✓	✓	
S. Stanleyville	Chicken faeces	✓	✓	
S. Thompson	Chicken faeces	✓	✓	
S. Typhimurium	Chicken faeces	✓	✓	✓
S. Typhimurium 1	Chicken faeces	✓	✓	
S. Typhimurium 2	Chicken faeces	✓	✓	
S. Typhimurium 3	Chicken faeces	✓	✓	
S. Typhimurium 4	Chicken faeces	✓	✓	
S. Typhimurium 5	Chicken faeces	✓	✓	
S. Typhimurium 6	Chicken faeces	✓	✓	
S. Typhimurium 7	Chicken faeces	✓	✓	
S. Typhimurium 8	Chicken faeces	✓	✓	
S. arizonae, serotype 48: z4, z23	Chicken faeces	✓	✓	
S. arizonae, serotype 48: z4, z23, z32	Chicken faeces	✓	✓	
S. salamae, serotype 4, 12:b-	Chicken faeces	✓	✓	
S. salamae, serotype 6, 8:g, m, t	Chicken faeces	✓	✓	✓
S. Cerro	Fish	✓	✓	
S. Typhimurium monofasica	Straw (cattle feed)	✓	✓	✓

Table 4.S2 *ΔOD_{630nm} values (averaged of four independent replicates) of microbial biofilms produced by Salmonella enterica strains. Values are means ± standard error (ES).*

Salmonella *serotypes*					**Biofilm formation**		
					Inoculum 10^8 CFU/mL	Inoculum 10^4 CFU/mL	
Genus	Species	Subsp.	Serovar	Source	Average OD_{630} ± ES	Average OD_{630} ± ES	*p*
Salmonella	*enterica*	*enterica*	Enteritidis 4300	CECT	0.092 ± 0.007	0.107 ± 0.007	0.008
Salmonella	*enterica*	*enterica*	Enteritidis	wild	0.083 ± 0.004	0.073 ± 0.004	0.002
Salmonella	*enterica*	*enterica*	Typhimurium 4594	CECT	0.071 ± 0.004	0.058 ± 0.004	7.47E-08

Salmonella	*enterica*	*enterica*	Typhimurium	wild	0.112 ± 0.009	0.111 ± 0.007	0.829
Salmonella	*enterica*	*enterica*	T. monofasica	wild	0.075 ± 0.006	0.074 ± 0.006	0.722
Salmonella	*enterica*	*enterica*	Infantis	NRL	0.083 ± 0.017	0.136 ± 0.02	0.001
Salmonella	*enterica*	*enterica*	Meleagridis	NRL	0.135 ± 0.019	0.11 ± 0.017	0.028
Salmonella	*enterica*	*enterica*	Virchow	NRL	0.094 ± 0.009	0.097 ± 0.008	0.574
Salmonella	*enterica*	*enterica*	Dublin	NRL	0.045 ± 0.002	0.048 ± 0.002	0.115
Salmonella	*enterica*	*enterica*	Hadar	NRL	0.047 ± 0.003	0.048 ± 0.003	0.584
Salmonella	*enterica*	*salamae*	6, 8: g, m, t	wild	0.081 ± 0.006	0.075 ± 0.005	0.169

Statistical analysis was performed only within each strain, comparing controls of different inoculum concentration.

4.5 References

Andersson DI, Hughes D, Kubicek-Sutherland JZ, 2016. Mechanisms and consequences of bacterial resistance to anti-microbial peptides. Drug Resist Updat 26, 43–57.

Anjum MF, Choudhary S, Morrison V, Snow LC, Mafura M, Slickers P, Ehricht R, Woodward MJ, 2011. Identifying an-timicrobial resistance genes of human clinical relevance within Salmonella isolated from food animals in Great Britain. J Antimicrob Chemother 66, 550–559.

Brogden KA, 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? Nat Rev Genet 3, 238–250.

CLSI, 2020. *M100-S25: Performance Standards for Antimicrobial Susceptibility Testing*, 30th ed.; Informational Supplement; CLSI: Wayne, PA, USA.

Colagiorgi A, Festa R, Di Ciccio PA, Gogliettino M, Balestrieri M, Palmieri G, Anastasio A, Ianieri A, 2020. Rapid biofilm eradication of the antimicrobial peptide 1018-K6 against Staphylococcus aureus: A new potential tool to fight bacterial biofilms. Food Control 107, 106815.

Costerton JW, Stewart PS, Greenberg EP, 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. Science 284, 1318–1322.

Díez-García M, Capita R, Alonso-Calleja C, 2012. Influence of serotype on the growth kinetics and the ability to form biofilms of Salmonella isolates from poultry. Food Microbiol 31, 173–180.

Dipaolo JA, Takano K, Popescu NC, 1972. Quantitation of chemically induced neoplastic transformation of BALB-3T3 cloned cell lines. Cancer Res 32, 2686–2695.

Duguid JP, Anderson ES, Campbell I, 1966. Fimbriae and adhesive properties in salmonellae. J Pathol Bacteriol 92, 107–137.

European Food Safety Authority and European Centre for Disease Prevention and Control, 2021a. The European Union One Health 2019 Zoonoses Report. EFSA J 19, 6406.

European Food Safety Authority, and European Centre for Disease Prevention and Control, 2021b. The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. EFSA J. 19, e06490.

Galiè S, García-Gutiérrez C, Miguélez EM, Villar CJ, Lombó F, 2018. Biofilms in the Food Industry: Health Aspects and Control Methods. Front Microbiol 9, 898.

Hur J, Jawale C, Lee JH, 2012. Antimicrobial resistance of Salmonella isolated from food animals: A review. Food Res Int 45, 819–830.

Lamas A, Fernandez-No IC, Miranda JM, Vázquez B, Cepeda A, Franco CM, 2016a. Biofilm Formation and Morphotypes of Salmonella enterica subsp. arizonae Differs from Those of Other Salmonella enterica Subspecies in Isolates from Poultry Houses. J Food Prot 79, 1127–1134.

Lamas A, Miranda J, Vazquez B, Cepeda A, Franco C, 2016b. Biofilm formation, phenotypic production of cellulose and gene expression in Salmonella enterica decrease under anaerobic conditions. Int J Food Microbiol 238, 63–67.

Lamas A, Miranda JM, Regal P, Vazquez B, Franco CM, Cepeda A, 2018. A comprehensive review of non-enterica subspecies of Salmonella enterica. Microbiol Res 206, 60–73.

Laverty G, Gorman SP, Gilmore BF, 2011. The Potential of Antimicrobial Peptides as Biocides. Int J Mol Sci 12, 6566–6596.

Lazzaro BP, Zasloff M, Rolff J, 2020. Antimicrobial peptides: Application informed by evolution. Science 368, eaau5480.
Lei J, Sun L, Huang S, Zhu C, Li P, He J, Mackey V, Coy DH, He Q, 2019. The antimicrobial peptides and their potential clinical applications. Am J Transl Res 11, 3919–3931.
Marin C, Hernandiz A, Lainez M, 2009. Biofilm development capacity of Salmonella strains isolated in poultry risk factors and their resistance against disinfectants. Poult Sci 88, 424–431.
Maróti G, Kereszt A, Kondorosi É, Mergaert P, 2011. Natural roles of antimicrobial peptides in microbes, plants and animals. Res Microbiol 162, 363–374.
Mika JT, Moiset G, Cirac AD, Feliu L, Bardají E, Planas M, Sengupta D, Marrink S, Poolman B, 2011. Structural basis for the enhanced activity of cyclic antimicrobial peptides: The case of BPC194. Biochim Biophys Acta Biomembr 1808, 2197–2205.
Morita Y, Komoda E, Ono K, Kumagai S, 2011. Survival of biofilm-forming Salmonella on stainless steel bolt threads under dry conditions. J Food Hyg Soc Jpn 52, 299–303.
O'Toole GA, 2011. Microtiter Dish Biofilm Formation Assay. J Vis Exp 47, 2437.
Palmieri G, Balestrieri M, Capuano F, Proroga YT, Pomilio F, Centorame P, Riccio A, Marrone R, Anastasio A, 2018a. Bactericidal and antibiofilm activity of bactenecin-derivative peptides against the food-pathogen Listeria monocytogenes: New perspectives for food processing industry. Int J Food Microbiol 279, 33–42.
Palmieri G, Tatè R, Gogliettino M, Balestrieri M, Rea I, Terracciano M, Proroga YT, Capuano F, Anastasio A, De Stefano L, 2018b. Small Synthetic Peptides Bioconjugated to Hybrid Gold Nanoparticles Destroy Potentially Deadly Bacteria at Submicromolar Concentrations. Bioconj Chem 29, 3877–3885.
Parry CM, Threlfall EJ, 2008. Antimicrobial resistance in typhoidal and nontyphoidal salmonellae. Curr Opin Infect Dis 21, 531–538.
Pateiro M, Munekata PES, Sant'Ana AS, Domínguez R, Rodríguez-Lázaro D, Lorenzo JM, 2021. Application of essential oils as antimicrobial agents against spoilage and pathogenic microorganisms in meat products. Int J Food Microbiol 337, 108966.
Quarles JM, Tennant RW, 1975. Effects of nitrosocarbaryl on BALB/3T3 cells. Cancer Res 35, 2637–2645.
Schonewille E, Nesse LL, Hauck R, Windhorst D, Hafez HM, Vestby LK, 2012. Biofilm building capacity of Salmonella enterica strains from the poultry farm environment. FEMS Immunol Med Microbiol 65, 360–365.
Starr CG, Ghimire J, Guha S, Hoffmann JP, Wang Y, Sun L, Landreneau BN, Kolansky ZD, Kilanowski-Doroh IM, Sammarco MC, Morici LA, Wimley W, 2020. Synthetic molecular evolution of host cell-compatible, antimicrobial peptides effective against drug-resistant, biofilm-forming bacteria. Proc Natl Acad Sci USA117, 8437–8448.
Steenackers H, Hermans K, Vanderleyden J, De Keersmaecker SC, 2012. Salmonella biofilms: An overview on occurrence, structure, regulation and eradication. Food Res Int 45, 502–531.
Teixeira V, Feio M, Bastos M, 2012. Role of lipids in the interaction of antimicrobial peptides with membranes. Prog Lipid Res 51, 149–177.
Wang H, Ye K, Wei X, Cao J, Xu X, Zhou G, 2013. Occurrence, antimicrobial resistance and biofilm formation of Salmonella isolates from a chicken slaughter plant in China. Food Control 33, 378–384.
Wang L, Zhao X, Xia X, Zhu C, Qin W, Xu Y, Hang B, Sun Y, Chen S, Zhang H, Jiang J, Hu J, Fotina H, Zhang G, 2019. Antimicrobial Pep- tide JH-3 Effectively Kills Salmonella enterica Serovar Typhimurium Strain CVCC541 and Reduces Its Pathogenicity in Mice. Probiotics Antimicrob Proteins 11, 1379–1390.
Yazgan H, Ozogul Y, Kuley E, 2019. Antimicrobial influence of nanoemulsified lemon essential oil and pure lemon essential oil on food-borne pathogens and fish spoilage bacteria. Int J Food Microbiol 306, 108266.

Yeaman MR, Yount N, Hauger RL, Grigoriadis DE, Dallman MF, Plotsky PM, Vale WW, Dautzenberg FM, 2003. Mechanisms of Antimicrobial Peptide Action and Resistance. Pharmacol Rev 55, 27–55.

Zasloff M, 2002. Antimicrobial peptides of multicellular organisms. Nat Cell Biol 415, 389–395.

Zharkova MS, Orlov DS, Golubeva OY, Chakchir OB, Eliseev IE, Grinchuk TM, Shamova OV, 2019. Application of an- timicrobial peptides of the innate immune system in combination with conventional antibiotics—A novel way to combat antibiotic resistance? Front Cell Infect Microbiol 9, 128.

5. Chapter 3

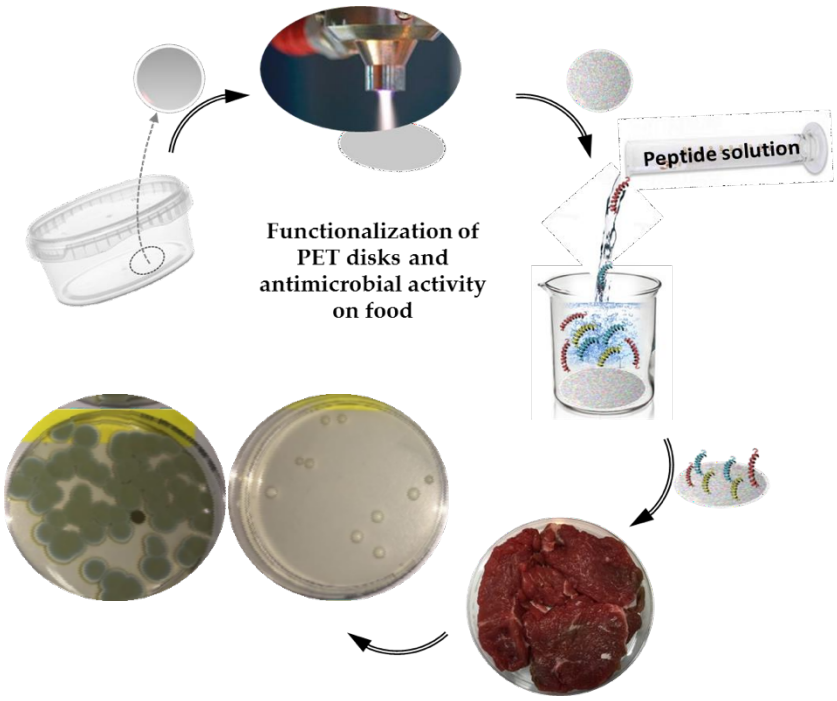
Extending the Shelf-Life of Meat and Dairy Products via PET-Modified Packaging Activated With the Antimicrobial Peptide MTP1

Abstract

Fresh products are characterized by reduced shelf-life because they are an excellent growth medium for a lot of microorganisms. Therefore, the microbial spoilage causing significant food supply losses has become an enormous economic and ethical problem worldwide. The antimicrobial packaging is offering a viable solution to tackle this economic and safety issue by extending the shelf-life and improving the quality and safety of fresh products. The goal of this study was to investigate the effects of a food contact surface of polyethylene terephthalate (PET) functionalized with the previously characterized antimicrobial peptide mitochondrial-targeted peptide 1 (MTP1), in reducing the microbial population related to spoilage and in providing the shelf-life stability of different types of fresh foods such as ricotta cheese and buffalo meat. Modified polymers were characterized concerning the procedure of plasma-activation by water contact angle measurements and Fourier transform infrared spectroscopy measurements in attenuated total reflection mode (ATR-FTIR). Results showed that the MTP1-PETs provided a strong antimicrobial effect for spoilage microorganisms with no cytotoxicity on a human colon cancer cell line. Finally, the activated polymers revealed high storage stability and good reusability. This study provided valuable information to develop alternative antimicrobial packaging for enhancing and extending the microbial quality and safety of perishable foods during storage.

Keywords: antimicrobial packaging, antimicrobial peptide, shelf-life, spoilage microorganism, cytotoxicity

Note: Gogliettino M, Balestrieri M, Ambrosio RL, Anastasio A, Smaldone G, Proroga YTR, Moretta R, Rea I, De Stefano L, Agrillo B, Palmieri G, 2020. Extending the Shelf-Life of Meat and Dairy Products via PET-Modified Packaging Activated With the Antimicrobial Peptide MTP1. Front Microbiol 10:2963.



Graphical Abstract. Scheme of the experimental procedure for the pre-activation and functionalization of PET disks and for the antimicrobial effects of MTP1-PETs against meat products.

5.1 Introduction

Short shelf-life of fresh foods represents one of the main limitations for the commercialization of this class of products, mainly due to their high content in nutrients and superficial moisture which lead to the fast growth of spoilage and pathogenic microorganisms (Aymerich *et al*., 2008; Patsias *et al*., 2008; Zhou *et al*., 2010). Indeed, it is well known that microbial growth on the surface of a product is often responsible for the undesirable changes in flavor, aroma, and other organoleptic characteristics of fresh foods, which lower their quality and shorten their commercial life (Mead, 2004; Samelis, 2006; Petrou *et al*., 2012). Although the exact figure of the total economic loss due to food spoilage is hardly to estimate, it is clear that it constitutes an enormous financial burden (Blackburn, 2006) accounting for 1.3 billion tons per year by FAO (Cichello, 2015; Bondi *et al*., 2017). Therefore, even a reduction in food waste of 20–25% could save between $120 and $300 billion per year according to a recent report by the UK Waste & Resources Action Programme (WRAP). As a preservation technique, the refrigeration is necessary to maintain the microbial quality of fresh products, but it does not guarantee by itself a long shelf-life, which in the case of some foods amounts to a time period of about 4–5 days. Therefore, demand for safe fresh products presents major challenges to the food industry to develop innovative strategies for improving the preservation process and prolonging the storage time maintaining both the natural appearance and safety of foods by reducing or eliminating spoilage bacteria. A significant support in this field derives from the use of packages, which not only act as a barrier against moisture, water vapor, and gases, but they may also serve as a carrier of active substances in the “active packaging,” thus increasing the shelf- life and assuring the safety and/or quality of food products (Suppakul *et al*., 2003). Active packaging is the most relevant innovative idea applied for consumer satisfaction. It can be defined as a mode of packaging in which product, package, and the environment interact in a positive way to extend shelf-life of products and/or to enhance safety or sensory properties while maintaining the quality of the foods (Suppakul *et al*., 2003). Among the active packaging technologies, antimicrobial packaging is

considered one of the most promising. These systems are based on the immobilization of antimicrobial agents on the surface of polymers, whose usage has strongly increased due to their large variety and the different compositions available, which make possible to adopt the most convenient packaging solutions, focusing on the specific needs of each product. One of the most common support that has found increasing applications within the packaging field is the polyethylene terephthalate (PET), a simple long-chain polymer, whose chemical inertness and physical properties have made it particularly suitable for different food applications. However, the chemical inertness of PET makes necessary to activate and functionalize its surface with specific treatments as the cold plasma before proceeding with the subsequent immobilization of bioactive compounds such as essential oils, plant extracts, bacteriocins, or enzymes (Jordá-Vilaplana *et al*., 2014; Malhotra *et al*., 2015). Some antimicrobial packages use immobilized antimicrobial peptides (AMP) to suppress the growth of microbes (Malhotra *et al*., 2015). AMPs are part of the innate immune system of all multicellular organisms (Andreu and Rivas, 1998; De Smet and Contreras, 2005; Guaní-Guerra *et al*., 2010) and include a chemically and structurally heterogeneous family, whose members have been isolated from a wide variety of animals, plants, bacteria, fungi, and viruses (Andreu and Rivas, 1998; Reddy *et al*., 2004). Nevertheless, three main characteristics that are shared by almost all known AMPs, can be distinguished: small size, highly cationic character, and tendency to adopt amphipathic structures (Nakatsuji and Gallo, 2012). These physicochemical properties make AMPs able to interact with the negatively charged microbial membranes. However, to serve as effective coating agents, the AMPs must meet several prerequisites, which include the retention of broad spectrum antimicrobial activity once bound to packaging materials. As many naturally occurring peptides lack the ability to retain these properties, there is a need to develop new and more effective AMPs, with the aim to increase safety and shelf-life of food products. Recently, starting from the human source sequence of CPT-1a (McGarry and Brown, 1997), a new AMP, named mitochondrial-targeted peptide 1 (MTP1), was designed as already stated in Palmieri *et al*. (2016) and characterized. Specifically, the 15-mer peptide was revealed to be highly stable in a broad range of pH (2–10) and temperature (15– 90C) for prolonged incubation times. Moreover, MTP1 assumed a-helix/b-sheet structures in mimicked cell membrane solutions, as revealed by CD analyses. Finally, the compound exhibited significant bactericidal activity against *Listeria monocytogenes*, one of the most important foodborne pathogens. The aim of the present study was to develop a new class of packaging materials, functionalized with the bactericidal peptide MTP1 and to evaluate both the usefulness and effectiveness of the afore mentioned active coatings on the microbial quality and safety of fresh perishable products and the potential extension of their shelf-life.

5. 2 Materials and Methods

5.2.1 Plasma Treatment

For plasma treatment and further peptide immobilization, the PET films were cut into disk-shaped pieces. Etching of PET disks was carried out using the PlasmaLab 80 Plus Reactive Ion Etching (RIE) system (Oxford Instruments, Abingdon, Oxfordshire, United Kingdom). The following parameters were modified to identify the best operative conditions: exposure time (*T*) (10–20–30–50–100–300 s); molecular oxygen concentration (O_2) (10–50–100 sccm); partial gas pressure (*P*) (0.1–0.5 atm); and power of radiofrequency generator (RF) (50–100–300 W).

5.2.2 Water Contact Angle Measurements

Water contact angles (WCA) were measured under static conditions by sessile drop method using an OCA 15EC system (DataPhysics Instruments GmbH, Filderstadt, Germany) coupled with a drop shape analysis software (SCA 20, DataPhysics Instruments GmbH, Filderstadt, Germany). A 1-µL drop was placed on the functionalized polymer surfaces, recording the images after 10 s. The WCA

values are expressed as mean ± standard deviation (s.d.) of at least three measurements on the same sample in two independent experiments (i.e., at least six measurements for each result).

5.2.3 Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy measurements in attenuated total reflection mode (ATR-FTIR) were carried out in the 4000–650 cm^{-1} spectral range with a resolution of 4 cm^{-1}, using a Thermo-Nicolet Continuum XL spectrometer (Thermo Scientific, United States). The FITR measurements were performed under inert (N_2) atmosphere. Spectra have been automatically corrected from the background using Ominc software (Thermo Scientific, United States).

5.2.4 MTP1 Immobilization Procedure

After oxygen plasma exposure, the pre-activated PET films were incubated into a MTP1 solution of 50 μM concentration prepared in sodium phosphate buffer (PB; 10 mM), pH = 7.0 for 24 h at 25C. After incubation, the liquid solution of unbound peptide was removed manually, and the functionalized PETs were extensively rinsed in water and DMSO in order to remove the traces of non-covalently bound peptide before performing all the surface characterizations. The PET containers used in all the analyses were kindly provided by the dairy "Mini Caseificio Costanzo s.r.l." located in Lusciano (Caserta, Italy).

5.2.5 Immobilization Yield Analysis of PET Polymers

Immobilization yield analysis of MTP1 on pre-activated PETs was performed by using a reverse-phase high-performance liquid chromatography (RP-HPLC) system (Shimadzu, Milan, Italy). Once immobilization was completed, the supernatant solutions were recovered after 24 h incubation and chromatographically analyzed to indirectly estimate the amount of the peptide attached to the polymeric surfaces. For these analyses, 200 μL of the samples was injected over a μBondapak C18 reverse- phase column (3.9 mm x 300 mm, Waters Corp., Milford, MA, United States) connected to an HPLC system, using a linear gradient of 0.1% TFA in acetonitrile from 5 to 95%. A reference solution was prepared with the initial peptide concentration used for the functionalization procedure under the same reaction conditions and run in parallel. Therefore, by knowing the added peptide (reference solution), the amount of peptide not bound to the polymers (expressed as a percentage) was determined by comparing the peak area. A calibration curve of the C18 column using different MTP1 concentrations was built. All measurements were performed in triplicate on three different preparations.

5.2.6 Release Test

The quantity of MTP1 released from the pre-activated PET disks was assayed by RP-HPLC following the same procedure previously described Peptide-immobilized PET slides were immersed in pure water or mozzarella cheese brine (1 mL) for 24 h at 4°C and then the recovered solutions were analyzed by RP-HPLC. The solutions in contact with the functionalized polymers at time $t = 0$ were used as control samples and run in parallel. All measurements were performed in triplicate on three different preparations.

5.2.7 Shelf-Life Testing on Dairy Products

A total of three random samples of buffalo ricotta cheese (200 g) were collected from a dairy factory. MTP1-PETs disks of 2.5 cm diameter (surface of 4.91 cm^2) were placed on the base of two Petri

dishes. Non-functionalized PETs were used as control. From each package, 30 g of ricotta cheese was weighted and laid aseptically on disks inside Petri dishes and storage at 4°C. Microbiological analyses were performed at *t*0 (beginning), *t*1 (4 days), and *t*2 (10 days) in contact with the MTP1-PETs. After incubation, 10 g of ricotta samples was added to 90 mL of buffered Peptone Water in sterile stomacher bag and homogenized for 3 min at 230 rpm using a peristaltic chomogenizer (BagMixer^{R} 400 P, Interscience, Saint Nom, France). Further 10-fold dilutions of the homogenates were made. Aerobic Plate Count (APC) and yeasts and molds were enumerated by spread plating on PCA incubated at 30°C for 48–72 h (ISO 4833-2:2013) and DRBC Agar plates incubated at 25 ± 1°C for 5 days for the colony count (21527-1:2008), respectively. The functionalized disks were washed three times by a sanitizing solution (Pursept-A Xpress, Schülke & Mayr GmbH, Germany) and exposed 2 h to UV radiations, before reusing.

5.2.8 Shelf-Life Testing on Buffalo Meat

Water Buffalo (n.5), slaughtered in an EU authorized slaughterhouse at 34 months and live weight of approximately 470 kg was chosen. The half-carcasses were cold-stored (0 ± 3°C) for 5 days and then the sirloin steak muscles (SSM) from both sides of the animal were removed. Subsequently, SSM were placed, for prolonged dry aging, in a forced ventilation patented cell named "Maturmeat" (ARREDO INOX S.r.l.) with an automatic extraction system set at a temperature of 0°C and at HR values ranging between 68 and 70% at microbiological lab of Department of Veterinary Medicine and Animal Productions (University of Naples Federico II). At 90 days of the aging period, three sirloin steaks (SS) were chosen. This aging time was selected due to the best palatability of the meat increasing tenderness, flavor, and/or juiciness. Aseptically 50 g from steaks was cut and placed on plastic disk functionalized with MTP1 (9 cm diameter, surface of 64 cm^2) inside Petri dishes and stored at 4°C. Analytical determinations were performed at *t*0 (beginning) and *t*1 (4 days) after contact with the peptide. APC was detected according to following procedures: 10 g of each sample and 90 mL of sterilized Peptone Water were placed in sterile stomacher bag and homogenized for 3 min at 230 rpm using a peristaltic homogenizer (BagMixer^{R} 400 P, Interscience, Saint Nom, France). Afterward, 10-fold serial dilutions of each homogenate were prepared in Peptone Water, followed by streaking in duplicate for APC performed according to ISO 4833-2:2013 on Plate Count Agar incubated at 30°C for 48–72 h and for yeasts and molds performed according to ISO 21527-1:2008 on DRBC plates incubated at 25 ± 1°C for 5 days. The functionalized disks were washed three times by a sanitizing solution (Pursept-A Xpress, Schülke & Mayr GmbH, Germany) and exposed 2 h to UV radiations, before reusing.

5.2.8.1 Physicochemical Analyses

The pH of samples was measured using a digital pH-meter (Crison-Micro TT 2022, Crison Instruments, Barcelona, Spain). The a_w (activity water) (Aqualab 4 TE – Decagon Devices Inc., United States) was determined by oven drying for 24-h at 105°C (AOAC, 1990). The 2-thiobarbituric acid (TBA) test (AOAC, 2000) was used to measure the lipid oxidation for each sample.

5.2.8.2 Rheological Analysis

On buffalo meat samples was performed: (a) Texture profile analysis (TPA), a compression test for determining the textural properties of meat pieces (Ruiz de Huidobro *et al*., 2005) by measuring the compression force developed by the texturometer (Shimadzu EZ test); (b) Colorimetric measurement using a Konica Minolta CR300 colorimeter (Minolta, Osaka, Japan). CIE *L** (lightness), *a** (redness), and *b** (yellowness) values were recorded for each sample. For all rheological analyses, the steak cores were collected in parallel to the muscle fibers, using a hand- held steel cork borer. A cylindrical 10 mm-diameter probe of ebonite was used for the TPA tests.

5.2.8.3 Sensory Evaluation

The sensory attributes of buffalo meat and ricotta buffalo cheese were estimated by a panel of five panelists (Altieri *et al*., 2005), which evaluated the following parameters: color, odor, taste, chewiness, and general appearance. The samples treated with not-functionalized PETs were used as control. Panelists scored each sample with a point scale, ranging from 0 (attributes most disliked) to 5 (attributed most liked). After storage period, the meat was cooked at 80°C for 10 min (to simulate the mode of administration in restaurant) for taste and chewiness evaluation. Two pieces of each sample, buffalo meat and ricotta cheese, were served to panelists at each sample time.

5.2.9 Cytotoxicity Assay

The cytotoxicity of the immobilized MTP1 was tested against HT-29 cells, a human colon cancer cell line used extensively to study the effects of different food products on human health (Martínez-Maqueda *et al*., 2015). HT-29 cells (kindly donated by Dr. Rosanna Palumbo CNR-IBB, Naples, Italy) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 10 mM L-glutamine. After reaching log phase, cells were transferred into 24-well plates ($1x10^5$/mL) and incubated for 24 h at 37°C. Therefore, MTP1-functionalized PET disks (surface of 80 mm^2) were added in each well and incubated for 24, 48, and 72 h at 37°C. Experiments were performed in quadruplicate. After incubation, the medium was removed and the remaining adherent cells were washed with PBS, fixated with 10% formaldehyde solution for 15 min at room temperature. Samples were subsequently washed with water and stained with 10% crystal violet solution for 30 min. Cell viability was quantified by eluting the dye from the stained cells with 10% acetic acid. Absorbance was measured spectrophotometrically at 595 nm (Multiskan FC; THERMO). Cells with the addition of not-functionalized PETs were set as negative control.

5.2.10 Statistical Analysis

All experiments were performed at least five times. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, United States). Statistical analysis of microbial counts was performed by using Student's *t*-test for independent data Sensory evaluations, TBA, a_W, and pH data were analyzed by Student's *t*-test for independent data. $p < 0.05$ was considered to be statistically significant.

5. 3 Results and Discussion

5.3.1 Activation and Functionalization of PET Polymer by Cold Plasma and MTP1
Packaging has a fundamental role in ensuring the safe delivery of goods throughout supply chains to the end consumer (Lindh *et al*., 2016). In this context, polymeric materials cover a large section of requirements in the field of the food industry and provide support surfaces for the immobilization of biologically active molecules. Specifically, PET is actually one of the most common polymers used in the food packaging, because of its physicochemical and mechanical properties and it is also relatively inexpensive to produce (Siracusa, 2012). However, surface properties of PET are usually inadequate in terms of wettability and adhesion properties, so it should be modified in order to improve its desired surface features and enhance its suitability, prior to any further processing, such as functionalization with biologically active molecules. In recent years, one of the most interesting procedures that have been employed to overcome these disadvantages is gaseous plasma treatment (Jordá-Vilaplana *et al*., 2014; Pankaj *et al*., 2014). Because of this procedure, the PET wettability is enhanced, and a more hydrophilic surface is created, rendering the polymer suitable for the preparation of new materials. Indeed, the versatility of PET is based on the development of metastable

reactive groups (-COOH*, -OH*) (Pankaj *et al*., 2014), which allow the covalent surface derivatization with the available chemical groups of biomolecules.

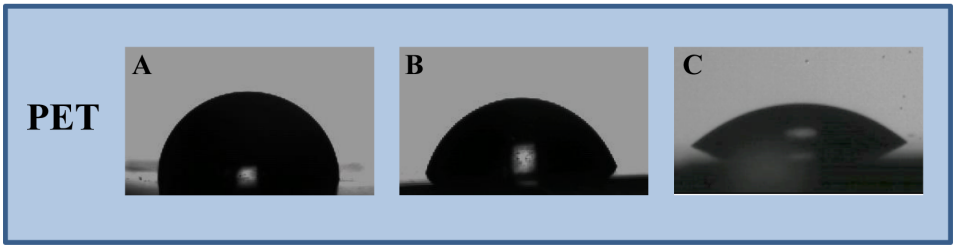


Figure 5.1 *Water contact angle measurements performed on (**A**) pristine PET, (**B**) oxygen plasma activated-PET and (**C**) MTP1-functionalized PET. The measurements were performed on five samples in duplicate.*

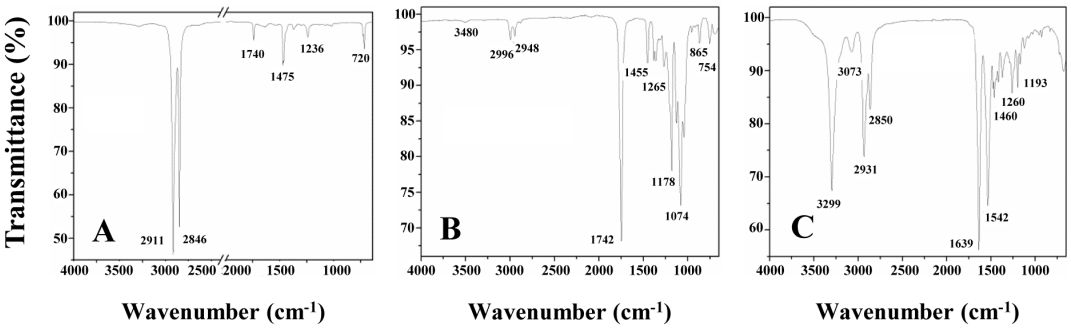
In this study, an immobilization platform on PET surfaces by using the previously characterized AMP MTP1 (Palmieri *et al*., 2016) was developed, exposing PET disks to a radiofrequency cold plasma by changing the main parameters of the process including the O_2 exposure time (T), RF power, and the concentration/the partial pressure (P) of molecular oxygen O_2. To assess the surface hydrophilicity of the PET slides after treatments, water contact angle (WCA) measurements were performed (Figure 5.1). Prior to activation, WCA was found to be 89 ± 3° (Figure 5.1A), confirming the hydrophobic nature of PET surface, whereas after plasma treatment carried out at the optimal experimental conditions (50 W and 10 s of exposure time), the contact angle was reduced to 76 ± 2°, demonstrating that the plasma process significantly increased the hydrophilicity of the surface (Figure 5.1B). It is important to note that when higher values of RF power (RF = 300 W) and long exposure times (T = 100–300 s) were applied, a macroscopic change in the roughness of the PET surface was detectable, indicative of a material degradation process. Subsequently to the plasma procedure, PET samples were incubated for 24 h in MTP1 buffer solution. As shown in Figure 5.1C, the efficiency of the coupling reaction with MTP1 was confirmed by a strong increase in the surface hydrophilicity, as indicated by the pronounced decrease of WCA value (36 ± 3°). This phenomenon was due to the reaction of the peptide free chemical groups (typically -COOH and -NH_2) with the reactive groups (-COOH* , -OH*) generated on PET surface by plasma activation (De Stefano *et al*., 2009). On the other hand, PET samples not pre-treated by radiofrequency cold plasma and incubated in same conditions in the presence of MTP1 showed the same value of WCA (75 ± 1°) of the pre-activated polymer, corresponding to negligible non-specific adsorption of the peptide on the PET surface. FTIR was also employed to confirm the success of bio-conjugation of MTP1 on PET disks. The ATR-FTIR spectrum of the not pre-activated PET (Figure 5.2A) displayed different main peaks corresponding to the C–C, C–H, and C–O bond stretching. After plasma treatment, the presence of the -OH group peaks in the FTIR spectra was detected (Figure 5.2B), consistently with the increase of the surface wettability quantified by WCA measurements (Socrates, 1994; De Stefano *et al*., 2013). The appearance of the characteristic absorption signals of the peptide, including the Amide I and Amide II bands, which arise from the peptide bonds that link the amino acids (O=C–NH) residues in the MTP1 sequence, confirmed the covalent bonding of the peptide on the PET surface (Figure 5.2C). Specifically, the Amide I band located between 1650–1560 cm^{-1} is produced mainly by the C=O stretching vibration of the peptide bond, while the absorption associated with the Amide II band at higher frequencies in the 1580–1490 cm^{-1} interval led primarily to bending vibrations of the N–H bond (Figure 5.2C). The main absorption peaks observed in ATR-FTIR spectra of PET samples are listed in the table reported in Figure 5.2D. Control samples not subjected to radiofrequency cold plasma treatment and incubated in aqueous peptide solution displayed an FTIR spectrum almost identical to that of Figures 5.2A,B (data not shown) (Edge *et al*., 1996; Silverstein and Webster, 1998; Chen *et al*., 2013).

5.3.2 Coupling Yield and Stability of MTP1-Immobilized PET Disks

In order to consider PET materials as promising candidates for applications in the food industry as antimicrobial packaging that are able to efficiently increase the food quality and safety, the AMPs used as coating agents must retain several prerequisites, including the broad activity spectrum and the biocompatibility. Nevertheless, the immobilization of AMPs is still challenged by suboptimal coating strategies leading to (i) inadequate surface concentrations and (ii) loss of antimicrobial activities with no specific binding chemistry culminating in changed orientations of the peptide molecules and/or associated cell toxicities. Therefore, it is firstly important to develop an effective surface tethering strategy that would impart the desired antimicrobial characteristics of the targeted biomaterial. This challenge strongly depends on the appropriate peptide concentration required to ensure a high efficiency of the immobilization procedure used and a great surface coating.

In this work, RP-HPLC analysis was employed to quantitatively measure the surface-immobilized yield starting from different MTP1 concentrations. Using this method, the amount of the PET-tethered peptide was indirectly evaluated by comparing the peak area (in the RP-HPLC-chromatograms) of the peptide not bound to the polymer after the bio-conjugation, with that of the initial peptide concentration used (at $t = 0$). The data obtained from these analyses demonstrated that the immobilization efficiency was concentration-dependent, reaching the highest yield (53%) using 50 μM, corresponding to a surface coverage of 11.2 nmol/cm^2 of PET. The chromatographic profile obtained for the MTP1 50 μM concentration and used to calculate the immobilization yield is reported in Figure 5.S1. The coupling yield was further confirmed by interpolation using a six-point calibration curve, which was generated utilizing known MTP1 concentrations (measured using an analytical balance) (Figure 5.S1 *insert*).

An important pre-requisite for AMP-coated packaging is the stability of the immobilized peptides. Therefore, the functionalized PET disks were incubated under different physiological conditions (i.e., immersion in pure water or in a representative liquid food matrix such as mozzarella cheese brine) up to 24 h, and the peptide-release was analyzed by RP-HPLC. As shown in the chromatograms reported in Figure 5.S2, both pure water and mozzarella brine did not cause MTP1 leakage from the PET polymers after 24 h incubation either at 4 or 25°C. In addition, no peptide-release was detectable even after prolonged incubation times (until to 72 h) in all the conditions analyzed (data not shown), thus highlighting the high stability of our system, that makes it an appropriate candidate for food applications.



D

Absorption bands (cm^{-1})	Bands
3480	OH group (hydroxyl)
3073	Symmetrical stretch of CH
2911 and 2846	C-H, Symmetrical stretching
1740	Stretching of C=O of carboxylic acid group
1639 and 1542	Amide I and II
1475 and 1455	Stretching of the C-O group deformation of the O-H group, and bending and wagging vibrational
1236 and 1178	Terephthalate Group (OOC-C6H4-COO)
1096 and 1050	Methylene group and vibrations of the ester C-O bond
865	Aromatic rings 1,2,4,5; Tetra replaced
720	Interaction of polar ester groups and benzene rings

Figure 5.2 *ATR-FTIR spectra of PET samples (**A**) before radiofrequency cold plasma treatment, (**B**) after plasma treatment and (**C**) after MTP1 bio-conjugation. (**D**) Table reporting the main absorption peaks observed in ATR-FTIR spectra of PET samples.*

5.3.3 Effect of MTP1-PETs on the Microbiological Quality of Dairy Products and Meat

Fresh food such as dairy products and meat because of their specific composition represent good support for a rapid growth of spoilage microorganisms that strongly influence the storage life of this class of products. In the case of cheese and meat, parameters such as water activity, pH, temperature, types, and viability of contaminating microorganisms are reported as some of the key factors that affect their rate of spoilage. Therefore, it is not surprising that these foods differ widely in spoilage characteristics. Among the troublesome microorganisms, yeasts and aerobic psychotropic Gram-negative bacteria can be considered the main causative agents of microbial spoilage and therefore they are recognized indicators of the hygienic quality of the foods. Indeed, psychotropic bacteria can produce large amounts of extracellular hydrolytic enzymes, and the recontamination of food products with these bacteria is determinant for their shelf-life, while yeasts are responsible of the main food degradations. These microorganisms are able to grow under a great variety of conditions and to survive in different environments resulting in unwanted physical and chemical changes, altering texture, smell, taste, or appearance of fresh products and rendering them not feasible for human consumption anymore. Therefore, extending the shelf-life of meats and dairy products represents the main challenge for food companies, and it is vital because, in the real world, these products do have fixed lifetimes after which they will perish.

In this context, the effectiveness of MTP1-PETs was evaluated through a comparison of the development of total aerobic mesophilic bacteria (APC) and yeasts on ricotta cheese and meat samples, stored under refrigeration (4°C) 1 day more than the normal shelf-life set by the company and analyzed in order to simulate a commercial storage time interval. With the aim to determine the possible influence of MTP1-PETs on the microbial growth in the products under investigation, the evolution of APC and yeast counts of samples treated with not- functionalized PETs (control) was assessed (Figure 5.3). Firstly, the initial values of the microbial counts were approximately in the

range usually found for this variety of foods (4–5 Log CFU/g). As expected, bacterial microorganisms were able to proliferate in the control samples, with APC values ranging from 4–8 Log CFU/g during the period of monitoring (Figure 5.3A). On day 4 of treatment, meat samples in contact with MTP1-PETs were characterized by a significant ($p < 0.05$) bacteria growth inhibition (2.0 ± 0.2 Log CFU/g) respect to those exposed to the control PETs. It is worth to note that the microbiological acceptability limit of 7 Log CFU/g, as defined by the International Commission on Microbiological Specifications for Food (ICMSF) (1986), was reached in the control samples in the 4 days of storage, while this microbial count was observed in the treated samples after 6 days of storage with a concomitant worsening of the sensorial characteristics of the meat. These results indicated that MTP1-PET films might be an effective coating to extend the fresh meat shelf-life, which is generally estimated to be 2–3 days beyond a sell- by date.

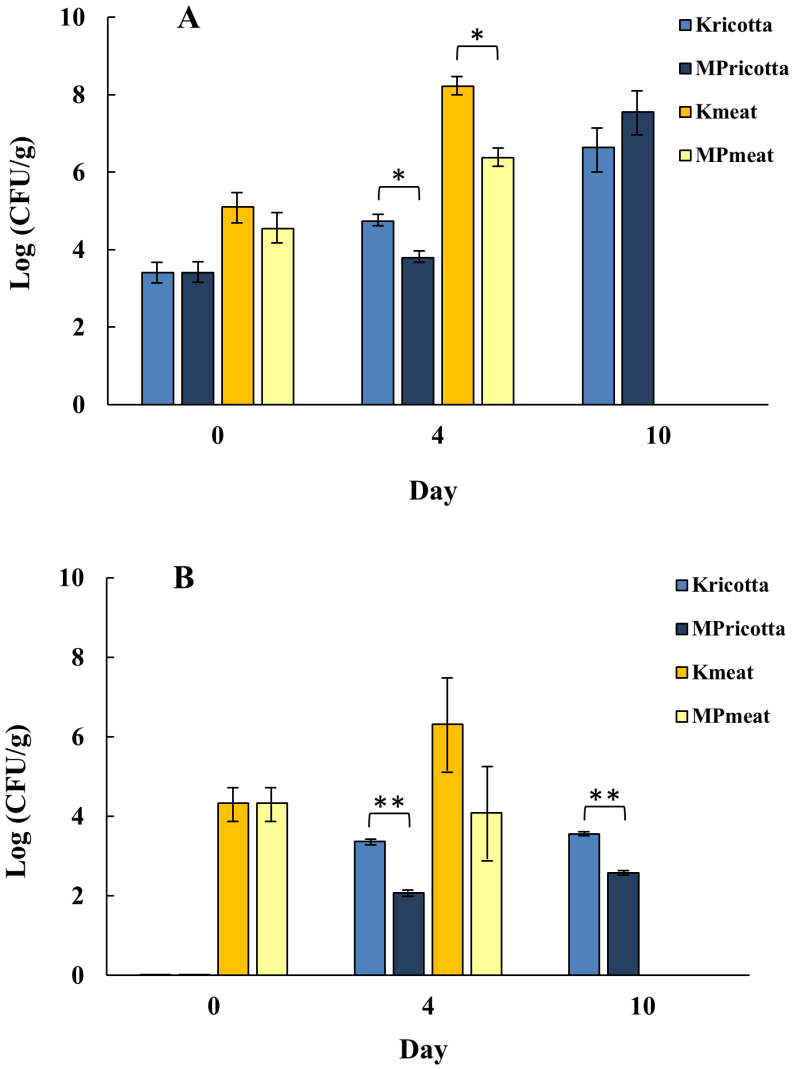


Figure 5.3 *Evolution of (**A**) Aerobic Plate Count (APC) and (**B**) yeast counts of ricotta cheese and meat, treated with MTP1-PETs (MP) during different days. Not-functionalized PETs were used as control (K). Data are presented as means ± s.d. of five different samples analysed in triplicate. * Significant difference ($p<0.05$) between the treated and the control samples; ** Significant difference ($p<0.01$) between the treated and the control samples.*

Concerning ricotta cheese samples, as reported in Figure 5.3A, a statistically significant 1 Log reduction ($p < 0.05$) of APC in the MTP1-PET samples was observed respect to control after 4 days of storage. Besides, no significant differences in APC values were detected between samples of ricotta cheese stored on the PETs activated or not at day 10 of treatment, probably indicating the limited

effect of long storage time on ricotta cheese preservation of the functionalized polymers (Figure 5.3A).
Similar results were obtained by evaluating yeasts growth (Figure 5.3B). After 4 days of storage 1 Log CFU/g increasing was revealed in meat products on control PETs in contrast to values of yeast counts equivalent to those determined at *t*0 and measured for MTP1-PET meat samples. As far as ricotta cheese is concerned, both at 4 and 10 days of treatment, it was observed the same significant ($p < 0.01$) trend of yeasts count reduction (1 Log CFU/g) confirming the possible effectiveness of the active PET to prolong the shelf-life of this kind of products. Interestingly, the results obtained for ricotta cheese samples are very notable considering that the yeasts constitute the most representative microbiological typology that strongly affects the shelf-life of this class of products, although no limits were fixed by European rules. However, Argentinian food regulations set up a maximum limit at no >3.5 Log CFU/g yeasts for cheeses with water content >55%, such as ricotta. As shown in Figure 5.3B, PET-coated ricotta samples did exceed this limit until the end of storage (10 days) while the control samples reached the limit already after 4th day.
Remarkably, the same results in terms of APC and yeast counts were determined even after three times of reuse of MTP1-PETs. Representative APC and yeast plates were reported in Figure 5.4.

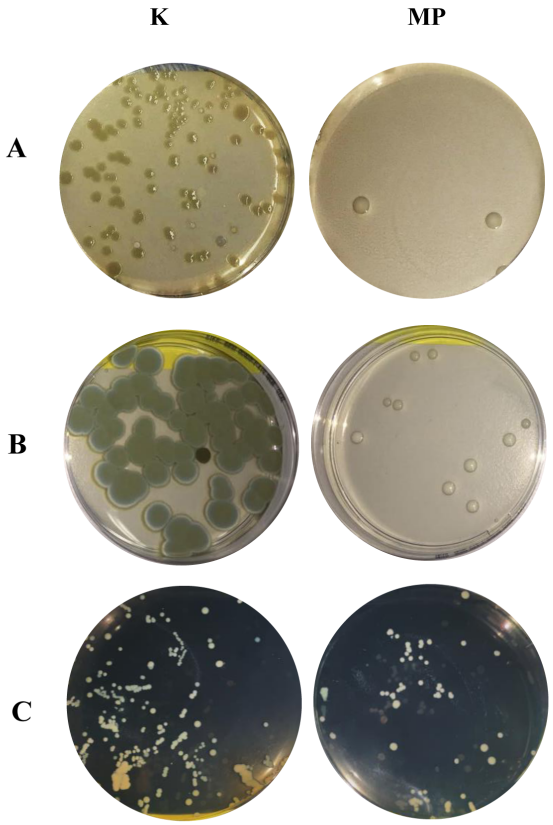


Figure 5.4 *Representative (**A**) APC and (**B**) yeast plates of meat, and (**C**) APC plates of ricotta cheese incubated in the presence of not-functionalized PETs (K) or MTP1-PETs (MP) during four days.*

5.3.4 Effect of MTP1-PETs on Physicochemical, Rheological, and Sensory Quality of Dairy Products and Meat

During storage, food products undergo physicochemical, rheological, and sensory changes that may affect their organoleptic qualities and in turn, discourage their consumption. In this context, it is essential for a packaging to preserve also the inherent qualities of a product during storage beyond the microbiological safety. Therefore, a detailed analysis concerning the afore mentioned parameters

was performed on the buffalo meat and ricotta cheese samples treated or not with MTP1-PETs at the investigation times. All data are reported in Tables 5.1 and 5.2.

Table 5.1 *Effects of MTP1-PETs on physical-chemical (pH, a_w and TBA-test), rheological parameters (color and texture) and sensory evaluation of buffalo meat samples.*

	Day 0	Day 4				
		Control		MTP1-PETs		
		M	SD	M	SD	t-test
pH	5.630	5.700	-	5.810	-	-3.024*
a_w	0.983	0.978	-	0.980	-	-2.450
TBA-test	0.673	0.768	-	0.722	-	-5.489**
Lightness	28.270	32.643	10.570	31.143	16.910	0.701
Redness	10.753	7.700	2.460	10.863	2.390	3.398*
Yellowness	5.583	3.180	9.880	5.913	3.320	-1.843
Chroma	16.337	10.880	18.460	16.777	8.450	-2.785*
Hue angle	0.479	0.392	0.090	0.499	0.010	-0.976
Adhesiveness	-35.643	-29.420	-	-22.187	0.080	-6.320**
Conhesion	39.178	31.583	1.470	32.345	0.130	0.080
Hardness	343.142	336.928	2.320	331.237	2.010	0.223
Cohesion	0.384	0.365	0.020	0.357	0.010	-0.452
Friability	121.750	128.428	1.900	127.654	0.980	-0.541
Elasticity	0.781	0.784	0.010	0.779	0.060	-0.014
Gumminess	137.002	126.784	1.410	121.350	2.010	0.418
Chewiness	104.887	99.949	1.620	101.279	6.820	-0.385
Resilience	0.215	0.196	0.010	0.192	0.020	-0.260
Color	4.200	2.800	0.800	3.400	1.200	-189.737
Taste	4.400	3.600	1.200	3.800	0.800	-0.632
Odor	3.600	2.800	0.800	3.200	0.800	-141.421
Chewiness	4.400	3.400	1.200	3.800	0.800	-126.491
Overall acceptance	4.400	2.800	0.800	3.800	0.800	-3.536*

Levels of significance of treatments: $^{**}p \leq 0.01$: $^{*}p \leq 0.05$
M: means. SD: standard deviations.

Interestingly, a significant increase ($p < 0.05$) in the overall acceptance parameter was measured in the MTP1-PETs respect to the controls at each investigation time both for buffalo meat and ricotta buffalo cheese. On the contrary, the findings showed that the meat products had little differences in terms of color, taste, odor, and chewiness along the storage, but they were not statistically meaningful ($p > 0.05$), However, panelists indicated that the odor intensity in the MTP1-PETs ricotta cheese sample was more desirable than the control ($p < 0.05$) after 4 and 10 days of storage.
As far as the physicochemical parameters is concerned, there were no substantial changes in the pH and a$_w$ values and in general no significant differences were found across all groups of meat and the dairy product following the MTP1-PET treatment during storage (Tables 5.1, 5.2). In addition, the levels of oxidative deterioration (TBA) did not change in any meaningful way between the MTP1-PETs samples and the controls but for buffalo meat samples after 4 days of contact ($p < 0.01$), suggesting that MTP1 had a slight effect on the lipid oxidation of meat products.

Table 5.2 *Effects of MTP1-PETs on physical-chemical (pH, a_w and TBA-test) and sensory evaluation of buffalo ricotta cheese samples.*

Day 0	Day 4	Day 10

		Control		MTP1-PETs			Control		MTP1-PETs		
		M	SD	M	SD	t-test	M	SD	M	SD	t-test
pH	6.770	6.900	0.010	6.860	-	0.894	6.950	-	6.950	-	-0.229
a_w	0.983	0.989	-	0.991	-	-0.695	0.989	-	0.991	-	-118.695
TBA-test	0.009	0.010	-	0.012	-	-0.133	0.015	-	0.012	-	1.270
Color	4.800	4.400	1.200	4.800	0.800	-126.491	3.000	2.000	3.600	1.200	-1.500
Taste	4.200	4.000	-	4.200	0.800	-1.000	2.600	1.200	3.000	2.000	-1.000
Odor	4.800	2.800	0.800	3.600	1.200	-2.529*	1.800	0.800	2.600	1.200	-2.529*
Chewiness	5.000	3.800	0.800	4.200	0.800	-141.421	2.200	0.800	3.200	0.800	-3.535*
Overall acceptance	4.800	3.600	1.200	4.400	1.200	-2.309*	2.400	1.200	3.400	1.200	-2.887*

Levels of significance of treatments: **p ≤ 0.01: *p ≤ 0.05
M: means. SD: standard deviations.

Color of foods is another important parameter to evaluate their quality, since the consumers associate it with freshness. As reported in Table 5.1, the not treated samples suffered a gradual lean browning during the retail period, reflecting in terms of an increase of L^* and a decrease in a^* and C^* values respect to $t0$. Conversely, lower L^* and greater redness (resulting in higher a^* and C^*, $p < 0.05$) were found in the MTP1-PET meats compared with the controls at 4 days. Finally, the rheological analysis evidenced no significant change in the textural qualities of the buffalo meat samples following the MTP1-PET treatments.

5.3.5 Cytotoxicity Testing of Immobilized MTP1

In addition to antibacterial activity, cytotoxicity is another important parameter influencing the application of any material for industrial and medical purposes. Therefore, in order to ascertain the non-toxic behavior of MTP1-modified PETs, cell viability assay was carried out on the human colon cancer cell line HT-29, that is receiving special interest in studies focused on the effects of food products on human health (Martínez-Maqueda *et al*., 2015). To this aim, cell viability was determined by exposing these cells to functionalized polymers for different time intervals, using the non-conjugated PET disks as control. As reported in Figure 5.5, the mammalian cells remained viable up to 72 h of incubation with the functionalized PET, with no significant difference in cell viability in terms of absorbance between the controls and the MTP1-PET disks. These results indicated that the amount of peptide immobilized on PETs was not toxic to mammalian cells, thus suggesting that the projected polymers could be considered safe to be applied as antimicrobial packages in the food industry.

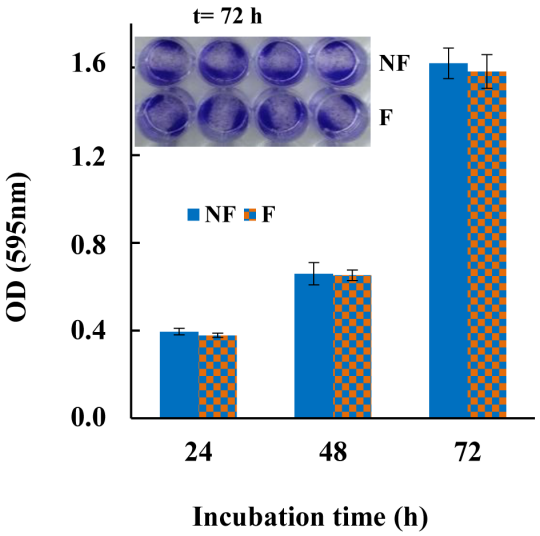


Figure 5.5 *Cytotoxicity of MTP1-PETs on HT-29 cell line measured with crystal violet at 24, 48 and 72 h incubation. The data are shown as means ± s.d. of two separate experiments performed in quadruplicate.* ***Insert****: HT-29 cell line after staining with crystal violet incubated with MTP1-PET (F) for 72 h. Not-functionalized disks were used as control (NF).*

5.4 Supplementary materials

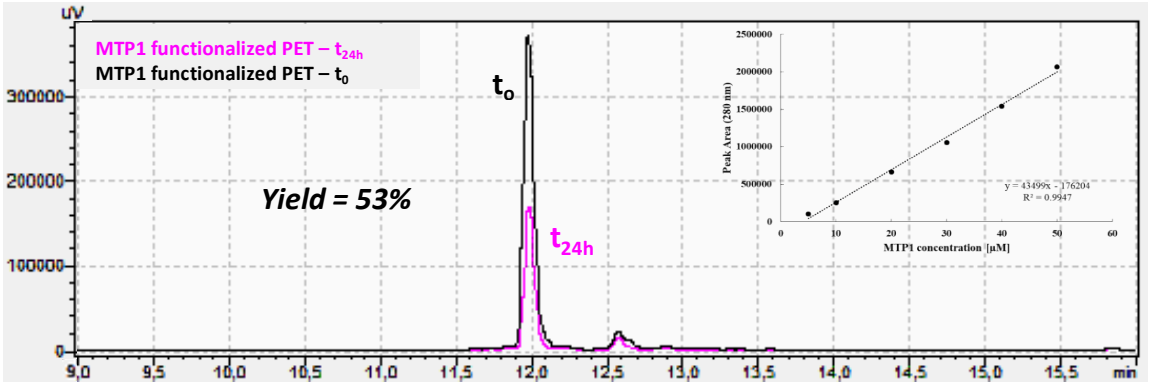


Figure 5.S1 *Immobilization yield (%) of MTP1 on PET surface determined by reverse-phase HPLC chromatography on a C18 column after the coupling reaction (24 h). Pre-activated PET surfaces by plasma were incubated for 24 h with MTP1 (50 µM) in PB pH 7.0. The solutions recovered after incubation were further analysed. The peptide solution placed in contact with the pre-activated surface at time 0 (t = 0) was used as control. The chromatograms are representative of three independent experiments.* ***Insert****: Calibration curve of the C18 column obtained using different MTP1 concentrations*

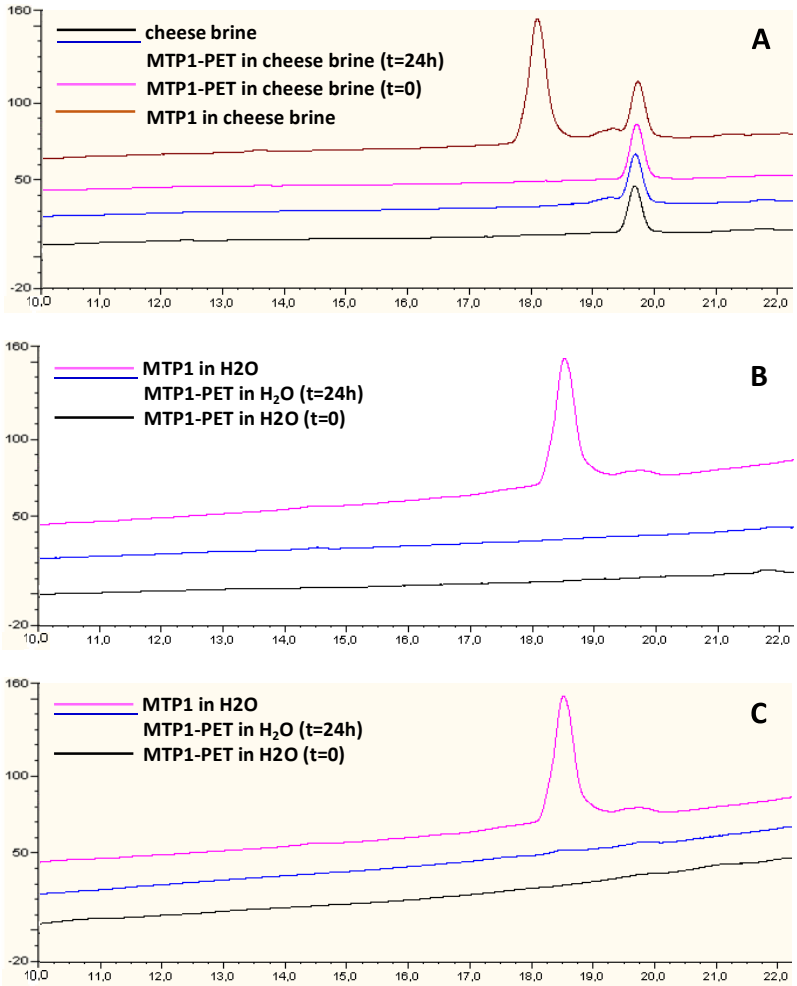


Figure 5.S2. *Release analysis of MTP1 from functionalized PET performed by reverse- phase HPLC chromatography on a C18 column. MTP1-PETs were incubated for 24 in (A) mozzarella cheese brine at 4 °C or in pure water at (B) 4 °C or (C) 25 °C. After incubation, the solutions were recovered and injected on C18. The solutions in contact with MTP1-PET at time 0 (t=0) and MTP1 peptide (50μM) were used as controls.*

5.5 References

Altieri C, Speranza B, Del Nobile MA, Sinigaglia M, 2005. Suitability of bifidobacteria and thymol as biopreservatives in extending the shelf life of fresh packed plaice fillets. J Appl Microbiol 99, 1294–1302.

Andreu D, Rivas L, 1998. Animal antimicrobial peptides: an overview. Biopolymers 47, 415–433.

AOAC, 1990. *Ocial Methods of Analysis*, 15th Edn, Method 950.46, Vol. 11, Arlington, VA: Association of Ocial Analytical Chemists.

AOAC, 2000. *Ocial Methods of Analysis*, 17th Edn, Method 976.18, Gaithersburg, MD: Association of Ocial Analytical Chemists.

Aymerich T, Picouet PA, Monfort JM, 2008. Decontamination technologies for meat products. Meat Sci 78, 114–129.

Blackburn C deW., 2006. "Introduction," in *Food Spoilage Microorganisms*, ed. C. deW Blackburn, (Cambridge: Woodhead), xvii–xxiii.

Bondi M, Lauková A, de Niederhausern S, Messi P, Papadopoulou C, 2017. Natural preservatives to improve food quality and safety. J Food Qual 2017:3

Chen Z, Hay JN, Jenkins MJ, 2013. The thermal analysis of poly(ethylene terephthalate) by FTIR spectroscopy. Thermochim Acta 552, 123–130.

Cichello SA, 2015. Oxygen absorbers in food preservation: a review. J Food Sci Technol 52, 1889–1895.

De Smet K, Contreras R, 2005. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol Lett 27, 1337–1347.

De Stefano L, Oliviero G, Amato J, Borbone N, Piccialli G, Mayol L, Rendina I, Terracciano M, Rea I, 2013. Aminosilane functionalizations of mesoporous oxidized silicon for oligonucleotide synthesis and detection. J R Soc Interface 10:20130160.

De Stefano L, Rotiroti L, De Tommasi E, Rea I, Rendina I, Canciello M, Maglio G, Palumbo R, 2009. Hybrid polymer-porous silicon photonic crystals for optical sensing. J Appl Phys 106:023109.

Edge M, Wiles R, Allen NS, McDonald WA, Mottock SV, 1996. Characterization of the species responsible for yellowing in melt degraded aromatic polyesters-I: yellowing of poly (ethylene terephthalate). Polym Degrad Stab 53, 141–151.

Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM, 2010. Antimicrobial peptides: general overview and clinical implications in human health and disease. Clin Immunol 135, 1–11.

International Comission on Microbiological Specifications for Food (ICMSF), 1986. *Microorganisms in Foods. 2. Sampling for Microbiological Analysis: Principles and Specific*. Hoboken, NJ: Blackwell Scientific Publications.

Jordá-Vilaplana A, Fombuena V, García-García D, Samper MD, Sánchez-Nácher L, 2014. Surface modification of polylactic acid (PLA) by air atmospheric plasma treatment. Eur Polym J 58, 23-33.

Lindh H, Olsson A, Williams H, 2016. Consumer perceptions of food packaging: contributing to or counteracting environmentally sustainable development? Packag Technol Sci 29, 3–23.

Malhotra B, Keshwani A, Kharkwal H, 2015. Antimicrobial food packaging: potential and pitfalls. Front Microbiol 6:611.

Martínez-Maqueda D, Miralles B, Recio I, 2015. "HT29 cell line," in *The Impact of Food Bioactives on Health:in vivo and ex vivo Models*, ed. K. Verhoeckx, (Berlin: Springer).

McGarry JD, Brown NF, 1997. The mitochondrial carnitine palmitoyltransferase system. from concept to molecular analysis. Eur J Biochem 244, 1–14.

Mead GC, 2004. "Shelf-life and spoilage of poultry meat," in *Poultry Meat Processing and Quality*, ed. G. C. Mead, (Cambridge: CRC Press), 283–303.

Nakatsuji T, Gallo RL, 2012. Antimicrobial peptides: old molecules with new ideas. J Investig Dermatol 132, 887–895.

Palmieri G, Balestrieri M, Proroga YT, Falcigno L, Facchiano A, Riccio A, Capuano F, Marrone F, Neglia G, Anastasio A, 2016. New antimicrobial peptides against foodborne pathogens: from in silico design to experimental evidence. Food Chem. 211, 546–554.

Pankaj SK, Bueno-Ferrer C, Misra NN, Milosavljevic V, O'Donnel CP, Bourke P., Keener KM, Cullen PJ, 2014. Applications of cold plasma technology in food packaging. Trends Food Sci Technol 35, 5–17.

Patsias A, Badeka AV, Savvaidis IN, Kontominas MG, 2008. Combined effect of freeze chilling and MAP on quality parameters of raw chicken fillets. Food Microbiol 25, 575–581.

Petrou S, Tsiraki M, Giatrakou V, Savvaidis IN, 2012. Chitosan dipping or oregano oil treatments, singly or combined on modified atmosphere packaged chicken breast meat. Int J Food Microbiol 156, 264–271.

Reddy KV, Yedery RD, Aranha C, 2004. Antimicrobial peptides: premises and promises. Int J Antimicrob Agents 24, 536–547.

Ruiz de Huidobro F, Miguel E, Blázquez B, Onega E, 2005. A comparison between two methods (Warner-Bratzelr and texture profile analysis) for testing either raw or cooked meat. Meat Sci 69, 527–536.

Samelis J, 2006. "Managing microbial spoilage in the meat industry," in *Food Spoilage Microorganisms*, ed. W. Blackburn, (Boca Raton, FL: CRC Press LLC).

Silverstein RM, Webster FX, 1998. *Spectrometric Identification of Organic Compounds*. New York, NY: Wiley.
Siracusa V, 2012. Food packaging permeability behaviour: a report. Int J Polym Sci 2012:11.
Socrates G, 1994. *Infrared and Raman Characteristic Group Frequencies*, 3rd Edn. New York, NY: Wiley & Sons Ltd.
Suppakul P, Miltz J, Sonneveld K, Bigger SW, 2003. Active packaging technologies with an emphasis on antimicrobial packaging and its application. J Food Sci 68, 408–420.
Zhou GH, Xu XL, Liu Y, 2010. Preservation technologies for fresh meat: a review. Meat Sci 86, 119–128.

6. Chapter 4

Effect of novel active packaging containing antimicrobial peptide on the shelf-life of fish burgers (*Coryphaena hippurus*) during refrigerated storage

Abstract

Fishery products are high perishable foods. In the last decades, the scientific community has working to achieve the ambitious goal of reducing the impact of microbial deterioration on food losses through innovative solutions, including antimicrobial packaging. This study aims to evaluate the efficacy of packaging in polyethylene terephthalate (PET) functionalized with the antimicrobial peptide 1018-K6 to extend the *shelf life* of fish burgers. 21 samples of burger (200 g) of of dolphinfish (*Coryphaena hippurus*) were divided into two experimental groups: *i)* control group (HL CTR: not functionalized PET discs); and *ii)* treated group (HL 1018-K6: PET discs functionalized with 1018-K6 peptide). At 1, 3, 5 and 7 days, Total bacterial count at 5 ° and 32 ° C, Enterobacteriaceae, Coliforms, *E. coli*, *Pseudomonas* spp., Mesophilic Lactic Acid Bacteria, Fecal streptococci and Coagulase positive staphylococci were investigated. In addition, pH, a_w (activity water) and the sensory analyses were carried out.
Sensory results showed that samples packed with functionalized packaging were considered acceptable two days longer than the control group. Microbiological data underlined an evident antimicrobial activity of the active packaging at 5 days against coliforms, Enterobacteriaceae, *Pseudomonas* spp., *E. coli* and fecal Streptococci, with a reduction of about 1 Log (CFU/g) compared to the control group.
In conclusion, antimicrobial packaging seems to offer a concrete tool capable of slowing down surface microbial replication and, therefore, extending the shelf-life and improving the health and hygiene aspect of fresh fish products.

Keywords: antimicrobial packaging, spoilage microorganisms, perishable foods.

Note: Ambrosio RL, Palmieri G, Marrone R, Balestrieri M, Gogliettino M, Anastasio A, 2021. Valutazione dell'efficacia di imballaggi funzionalizzati con peptide antimicrobico sulla "Shelf Life" di hamburger di lampuga (*Coryphaena hippurus*). XXX Convegno Nazionale AIVI.

6.1 Introduction

Fish and fishery products are considered as a rich source of high-quality proteins, n-polyunsaturated fatty acids (PUFAs), vitamins (A, B and D) and minerals (P, Mg, Se and I) (Gökoğlu and Yerlikaya, 2015), impacting their promotion as health foods. In fact, thanks to the dissemination among consumers of information on their health benefits (Thilsted *et al.,* 2016), fish consumption has doubled from 9.9 kg in 1960 to 20 kg in 2015 (FAO, 2016), up to reach 24.36 kg in 2018 (EUMOFA, 2020). However, due to the composition (Chotphruethipong and Benjakul, 2019, Abbas *et al*., 2009), seafood is very susceptible to deterioration phenomena which are the main cause of rejection by consumers. This happens because, as a result of decay processes, secondary products and microbiological metabolites formed can lead to the formation of off-odor and off-flavor, texture and color changes. Although *Pseudomonas putrefaciens* and *Pseudomonas fluorescens* are recognized as the major spoilage microorganisms in seafood (Ghaly *et al*., 2010), the total microbial count has a great connection with the fish spoilage because related to the management of critical phases along the food chain (*i.e.* handling, storage and method of packaging). Over the years, synthetic antimicrobials have been used in the storage of fish products; however, consumers have associated these compounds with toxicity, increasing the interest of people toward natural bioactive compounds

(Xu *et al*., 2017) such as essential oils, plant extracts (Ambrosio *at al*., 2020; Marrone *et al*., 2021), bacteriocins, enzymes or antimicrobial peptides (AMP) (Festa *et al*., 2021).
Several agents with antimicrobial activity can be incorporated and coated onto the packaging materials, including antimicrobial peptides (Gogliettino *et al*., 2020) that are immobilized on the surface of polymers. AMPs represent a valid option to consider as innovative molecules able to replace the common antibiotics. They are short amino acid sequences that are part of the innate immune system of all multicellular organisms (Andreu and Rivas, 1998; De Smet and Contreras, 2005; Guaní-Guerra *et al*., 2010) and some of them have been isolated from a wide variety of animals, plants, bacteria, fungi, and viruses (Reddy *et al*., 2004). In a previous study, a cathelicidin-related antimicrobial peptide consisting of 12 residues and named 1018-K6 was in silico designed and characterized (Palmieri *et al*., 2018a; Palmieri *et al*., 2018b; Colagiorgi *et al*., 2020; Festa *et al*., 2021), showing high structural stability as well as powerful antimicrobial and antibiofilm activities against Gram-positive and Gram-negative food bacterial pathogens (*Listeria monocytogenes, Staphylococcus aureus* MRSA and MSSA, and *Salmonella enterica*).
Since most of the time the spoilage in fish starts from the surface growth of microorganisms, the direct surface application of antimicrobial compounds is becoming the goal of many scientists and stockholders (Gogliettino *et al*., 2020). The antimicrobial packaging represents a good solution for microbial spoilage considering that the coating not only provides a physical barrier to endogenous agents but also, through the direct application of antimicrobial compounds, acts monitoring the replication of microbial communities (Khaneghah *et al*., 2018).
The present study aimed to evaluate the antimicrobial activity of a new class of active packaging, functionalized with the bactericidal peptide 1018-K6, against bacteria responsible for the food spoilage in fish burgers of dolphinfish (*Coryphaena hippurus*), characterizing the potential extension of their shelf-life.

6.2 Materials and Methods

6.2.1 Coating formulation

One of the most common support in the food industry is polyethylene terephthalate (PET). It has been used in the production of 1018-K6 -PET or non-functionalized PET disks, according to the protocol of Gogliettino *et al.* (2020).

6.2.2 Samples preparation

Fish burgers of dolphinfish (*Coryphaena hippurus*) were purchased from a local fishery industry in Naples, Italy. A total of 21 samples (200 g) were included in the experimental trial. Specifically, 3 burgers were immediately analysed to fix the initial levels of the microbial communities; while the other samples (18) were randomly assigned into two different groups: a control group (HL CTR) and a functionalize films group (HL 1018-K6). Burgers were coated aseptically with 1018-K6 -PET or non-functionalized PET disks (5x5 cm), which were placed on the upper and lower surfaces of each sample. Subsequently, all burgers were stored in polystyrene trays cover with film paper at 4±1°C for 7 days. Microbiological, physicochemical, and sensorial analyses were performed at four sampling intervals: *day 0* (beginning of the experimental study), *day 3*, *day 5* and *day 7*.

6.2.3 pH and a_w measurements

The pH measurements were carried out with a digital pH meter (Crison-Micro TT 2022, Crison Instruments, Barcelona). Water activity (a_w) was measured with Aqualab 4 TE (Decagon Devices Inc., USA).

6.2.4 Microbiological analyses

Standard methods were used to conduct microbial analysis. Ten grams of each sample were aseptically transferred in a sterile stomacher bag with 90 mL (1:10 (w/v)) of sterilized Peptone Water (PW, Oxoid) and homogenized for three minutes at 230 rpm using a peristaltic homogenizer (BagMixer®400 P, Interscience, Saint Nom, France). Appropriate serial decimal dilutions of each homogenate were prepared for the following microorganism counts: Total Viable Count (TVC) and Psychotropic bacteria Count (PTC) were performed on Plate Count Agar (PCA, Oxoid, Madrid, Spain) incubated at 30°C for 48/72 h and 7°C for 10 days, respectively (ISO 4833-1:2013 and ISO 17410:2019); Total Coliforms on Violet Red Bile Lactose Agar (VRBL, Oxoid, Madrid, Spain) incubated at 37°C for 48 h (ISO 4831:2006); Enterobacteriaceae on Violet Red Bile Glucose Agar (VRBG, Oxoid, Madrid, Spain) incubated at 37°C for 48 h (ISO 21528-2:2017); Lactic Acid Bacteria (LAB) on MRS agar with Tween 80 (Oxoid, Madrid, Spain), incubated at 30°C for 72 h (ISO 15214:2015); *Pseudomonas* spp. on Pseudomonas Agar Base with CFC supplement (Oxoid, Madrid, Spain) incubated at 25°C for 48 h (ISO 13720:2010); *β-glucuronidase-positive Escherichia coli* (ISO 16649-1:2018) on Triptone Bile X-glucuronide Agar (TBX, Oxoid, Madrid, Spain) at 44°C for 24 h; *Enterococcus faecalis* on KAA (Kanamycin Aesculin Azide, Oxoid, Madrid, Spain) at 37°C for 48 h; coagulase positive staphylococci on Baird-Parker Agar (Oxoid, Madrid, Spain) at 37°C for 24/48 h (ISO 6888-1:1999). After counting, the data were expressed as logarithms of the number of colony-forming units (CFU/g) and means and standard error were calculated.

6.2.5 Sensory evaluation

Sensory evaluation including odor, color, and general acceptability was performed by 5 trained panelists. They were trained (2 sessions of 2 h) to fix the appropriate attributes in order to minimize individual differences and ensure results repeatability. Sensory assessments were performed under controlled humidity, light, and temperature. A Likert scale (9-point) was used to assess each attribute; in the scale, 9 corresponded to excellent, 8 to very good, 7 to good, 6 to reasonable, 5 to not good (acceptable limit), 4 to disliked, 3 to bad, 2 to very bad, and 1 to completely unacceptable (Angiolillo *et al.* 2018). Coded samples were randomly and simultaneously distributed to each panelist.

6.2.6 Statistical analyses

Microbiological and sensory data were statistically analysed using SPSS version 26 (IBM Analytics, Armonk, NY, US). Generalized linear mixed model was adopted to study parameters of dolphinfish burgers at each sampling time, including the fixed effect of packaging used at different sampling times (0, 3, 5 and 7 days).

6.3 Results and Discussion

6.3.1 pH and a_w

The pH of fish burgers increased over the whole shelf-life, from the initial value of 6.23 ± 0.01 to final values of 6.39 ± 0.02 and 6.31 ± 0.03 in the control and treated groups, respectively. The detected pH values in samples are almost neutral and in agreement with those reported by Messina *et al*. (2018) in fresh fillets of dolphinfish. The rise in pH could be linked to the protein degradation and the subsequent production of alkaline compounds which affected negatively sensorial characteristics such as odor, color and texture. Furthermore, pH values relatively high (pH > 6) could facilitate the replication of common spoilage microorganisms, creating an environment suitable for their survival (Gram and Huss, 1996). The trends highlighted a value overlapping among the two experimental

groups (data not showed), pointing out the absence of interferences between the active packaging and the phenomena underlying the modification of the pH.
Water activity influences significantly the perishability of fish and fish products, being one of the main predisposing factors for microbial replications. The a_w values related to each experimental group along the storage time did not show differences connectable to the coating typologies. Indeed, the a_w homogeneously decreased both in the control group as in treated ones, ranged from 0.973 ± 0.001 to 0.967 ± 0.003 and 0.965 ± 0.008 (HL CTR and HL 1018-K6, respectively).

6.3.2 Microbiological results

Microbiological results (Table 6.1) revealed that the application of antimicrobial films slowed down the growth trend of many bacterial classes indagated.
TVC and PTC represent important keys to investigate the contamination status of foods. In the absence of mandatory regulatory references regarding the microbiological aspect of fish and fishery products, the maximum acceptable limit has been set at 7 log cfu/g by the International Commission on Microbiological Specifications for Foods (ICMSF). As shown in Table 6.1, the initial levels of TVC and PTC in fish burgers were found to be lower than that microbiological limit. Nevertheless, the bacterial counts on day 1 are not comparable with other studies that reported values lower than 3-4 Log cfu/g (Albertos *et al*., 2019; Ehsani *et al*., 2020) in fish burgers. Clearly, the shredding process underlying the manufacture of these typologies of food could affect their overall hygiene quality, making them more sensitive to microbiological contamination (Roohinejad *et al*., 2017). Regarding the differences in concentration between TVC and PTC at the beginning of the experimental trial, similar results are reported by several authors (Hoel *et al*., 2017) in fish burgers. Total aerobic counts, both mesophilic and psychrophilic, increased over the storage and reached the highest level at 5 days of storage in both experimental groups. Between the two packaging systems, differences in TVC levels ($p < 0.01$) were found, highlighting the potential role of the active coating in suppressing the growth of this bacterial population.

Table 6.1 *Microbiological results of fish burgers.*

	Day	0	3	5	7
		M ± *sE*	*M* ± *sE*	*M* ± *sE*	*M* ± *sE*
TVC 30°C	HL CTR	5.66 ± 0.05^{A}	$6.77\pm0.06^{B,X}$	$7.04\pm0.00^{aC,X}$	$6.67\pm0.14^{BCb,x}$
	HL 1018-K6	5.66 ± 0.05^{A}	$5.81\pm0.07^{a,Y}$	$6.44\pm0.00^{B,Y}$	$6.22\pm0.14^{bB,y}$
PTC 7°C	HL CTR	4.89 ± 0.06^{A}	$6.35\pm0.09^{B,x}$	7.04 ± 0.14^{aC}	$6.67\pm0.05^{bC,X}$
	HL 1018-K6	4.89 ± 0.06^{A}	$6.03\pm0.12^{B,y}$	7.43 ± 0.13^{C}	$6.17\pm0.15^{B,Y}$
Total Coliforms	HL CTR	3.53 ± 0.13^{A}	$3.80\pm0.00^{A,x}$	$4.49\pm0.10^{B,X}$	$3.81\pm0.08^{A,X}$
	HL 1018-K6	3.53 ± 0.13^{a}	3.19 ± 0.23^{y}	$3.11\pm0.09^{b,Y}$	3.22 ± 0.14^{Y}
Enterobacteriaceae	HL CTR	3.55 ± 0.15^{A}	3.61 ± 0.19^{aA}	$4.27\pm0.13^{ABb,X}$	2.65 ± 0.03^{B}
	HL 1018-K6	3.55 ± 0.15^{aA}	3.61 ± 0.19^{aA}	$3.12\pm0.09^{bA,Y}$	2.46 ± 0.09^{B}
Pseudomonas spp.	HL CTR	3.72 ± 0.06^{A}	$5.89\pm0.03^{B,X}$	$6.54\pm0.05^{C,X}$	5.33 ± 0.14^{D}
	HL 1018-K6	3.72 ± 0.06^{A}	$4.19\pm0.23^{A,Y}$	$5.07\pm0.12^{Ba,Y}$	5.57 ± 0.16^{Bb}
E. coli	HL CTR	ni^{A}	$2.85\pm0.04^{B,X}$	$3.79\pm0.13^{C,X}$	$3.32\pm0.06^{D,X}$
	HL 1018-K6	ni^{A}	$2.22\pm0.09^{B,Y}$	$2.45\pm0.08^{ab,Y}$	$2.20\pm0.08^{bB,Y}$
Enterococcus faecalis	HL CTR	3.46 ± 0.08^{A}	$3.33\pm0.07^{Aa,x}$	$4.19\pm0.23^{B,X}$	$3.89\pm0.24^{b,X}$
	HL 1018-K6	3.46 ± 0.08^{A}	$2.52\pm0.28^{BC,y}$	$2.06\pm0.10^{B,Y}$	$2.73\pm0.04^{C,Y}$
Lactic Acid Bacteria 30°C	HL CTR	3.48 ± 0.08^{A}	$3.98\pm0.06^{B,x}$	$4.76\pm0.10^{C,X}$	4.98 ± 0.04^{C}
	HL 1018-K6	3.48 ± 0.08^{A}	$3.81\pm0.03^{B,y}$	$3.94\pm0.02^{C,Y}$	4.94 ± 0.08^{D}

Staphylococcus coagulase positive	HL CTR	4.82±0.05^{A}	4.98±0.28	5.37±0.13^{B,X}	4.63±0.11^{A,X}
	HL 1018-K6	4.82±0.05^{A}	4.36±0.29	4.21±0.14^{B,Y}	4.04±0.11^{B,Y}

**ni*: not isolated

In each sampling day, three samples by experimental group were analyzed. Statistical analysis was performed comparing experimental groups at each sampling time and within each experimental group along the ripening period. All data were presented as the mean (M) ± standard error (sE). Different superscript uppercase letters indicate a significant difference at $p < 0.01$. Different superscript lowercase letters indicate a significant difference at $p < 0.05$. $^{a–d}$ Mean values in the same row (same group in different days) with different letters presented significant differences. $^{x–y}$ Mean values in the same column (different groups on the same sampling time) with different letters presented significant differences.

It is well known that specific microbial groups are responsible for the off-flavors and the unpleasant odors typical of deteriorated fish products (Geeroms *et al*., 2008); certainly, the microorganisms belonging to the *Pseudomonas* species pull their weight among fish spoilage bacteria and are recognized as one of the most common reasons of some opportunistic and food-borne illnesses (Nowzari *et al*., 2013). The counts of these bacteria increased over the storage period, albeit differently among the two groups. The control group reached the highest value on the 5th day, followed by a plateau phase up to the 7th day. This finding is in agreement with the growth curves of TCV. In this regard, Total Bacteria Count and *Pseudomonas* spp. trends could justify the observed progressive decay of organoleptic characteristics of control fish burgers. In the groups packed with the films containing antimicrobial peptides the increase of *Pseudomonas* spoilage bacteria was slow and progressive, recording significative lower values than the control group on 3rd and 5th days ($p < 0.01$). These results highlighted the coating potential in retarding their growth.

Regarding the Enterobacteriaceae, Total Coliforms and *Enterococcus faecalis*, the growth curves were similar, with a gradual decrease subsequently occurring up to the 5th day of storage, and counts lower than the start time (significant differences were found for Enterobacteriaceae and *Enterococcus faecalis*, $p < 0.01$). It is interesting to note the differences in concentration of these bacterial communities among the two experimental groups exactly on the 5th day, when the highest values of these microorganisms were detected. The novel storage system caused a slowdown in the replication of these bacteria with a strong reduction of microbial load in the samples belonging to the treated group. The sensibility of these bacterial genera and species, recognized as an indicator of hygiene, to antimicrobial activities of the innovative packaging gives value to this system and promotes its applicability as a "controller tool" for *Escherichia coli*. Indeed, as displayed in Table 6.1, a very low proliferation of this bacterium was recorded in the samples kept in contact with the active disks. In this regard, the authors have already proved the bactericidal efficacy of the free peptide 1018-K6 against a specific genus belonging to the Enterobacteriaceae, *Salmonella* spp. (Festa *et al*. 2021). In particular, several serotyped wild strains were selected on the base of their resistance to common antibiotics and used to validate and demonstrate the peptide antimicrobial efficacy also against microorganisms which have developed defense mechanisms.

Results on the active packaging also pointed out the striking ability of the bound peptide to reduce the growth of bacteria belonging to *Staphylococcus* species. Indeed, the antimicrobial coating appears to successfully act on the survival and replicative capacity of the class of microorganisms investigated, showing a significant ($p < 0.01$) difference between the control groups and treat one on 5th and 7th days. Also this result is supported by the good results obtained during the studies *in vitro* with the free antimicrobial peptide 1018-K6 against the Gram-positive bacterium *Staphylococcus aureus* (Colagiorgi *et al*., 2020). Specifically, the concentration of AMP necessary to kill the microorganisms at a concentration of about 10^5 cfu/mL was found to be 20 μM.

6.3.3 Sensory evaluation

Figure 6.2 reports the sensory evaluation of fish burgers packaged in different types of films. As can be deduced from data, for seven days of storage, color values were acceptable in all samples with no significant differences between the two experimental groups (Figure 6.1). Regarding the odor, a

control group achieved an unacceptable mean score (less than five) on 7th day, accentuating a great difference among the two system of packaging. It is worth noting that the overall quality score of CTR samples was characterized by a gradual decrease, more pronounced after the first 5 days of storage. Specifically, the sample without active films exceeded the limit (score=5) after one week.

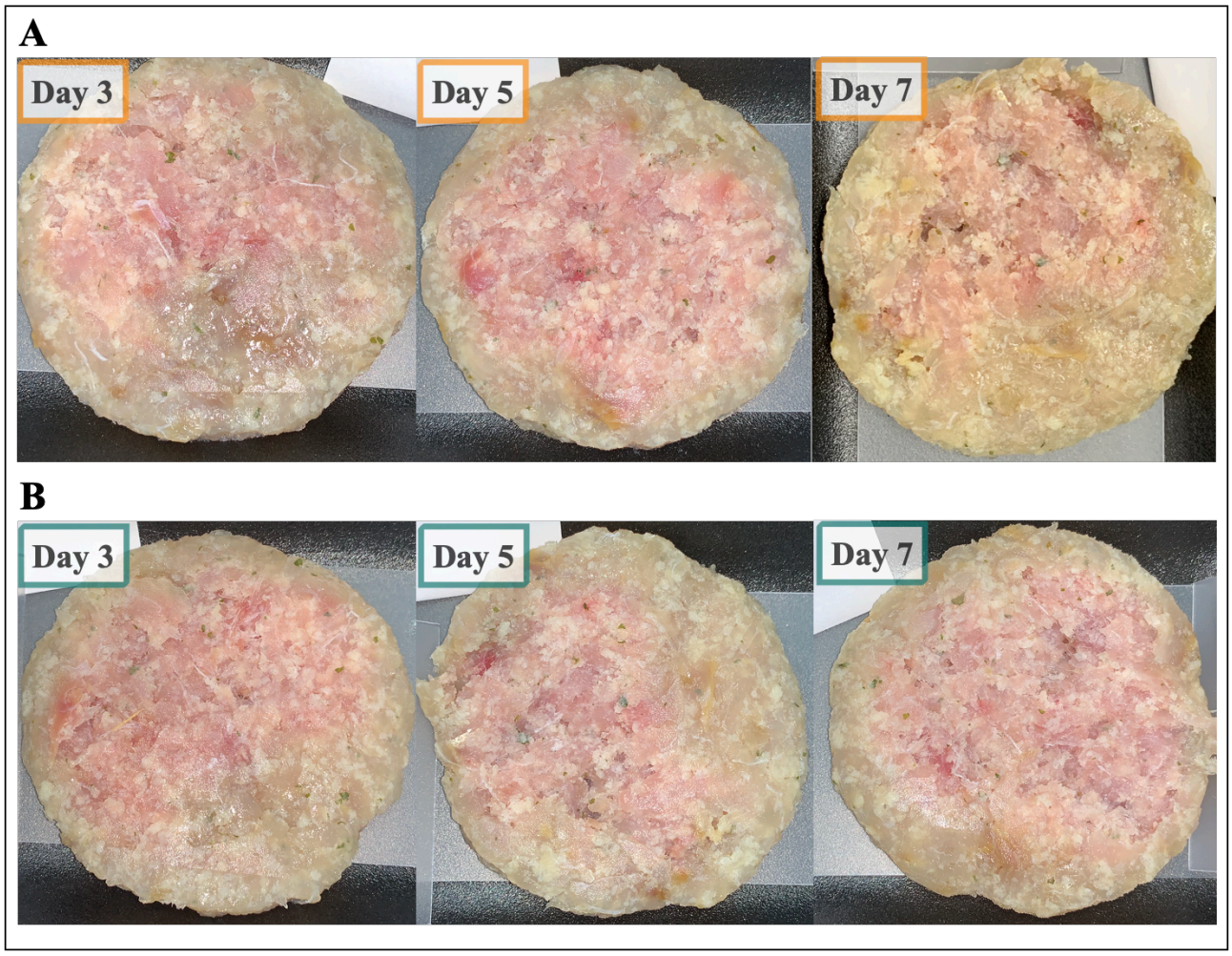


Figure 6.1 *Fish burgers coated with non-functionalized PET (**A**) or 1018-K6 -PET disks (**B**) at each sampling time.*

As can be seen, the off-odor influenced the overall acceptability of the control samples which are judged by the panelists as unpleasing food. Consumers' acceptability depends on the appearance of marine foods, affected by organoleptic values such as color, odor and overall appearance. Many authors (Wang *et al*., 2007) have attributed great weight to the odor, considering it the most critical sensory characteristic for fish products. Based on our findings and other authors (Panza *et al*., 2020; Rico *et al*., 2020), natural antimicrobial systems demonstrate to have a great potential for extending shelf-life, being able to control and reduce one of the many important factors that affect the durability of food, the microbial replication.

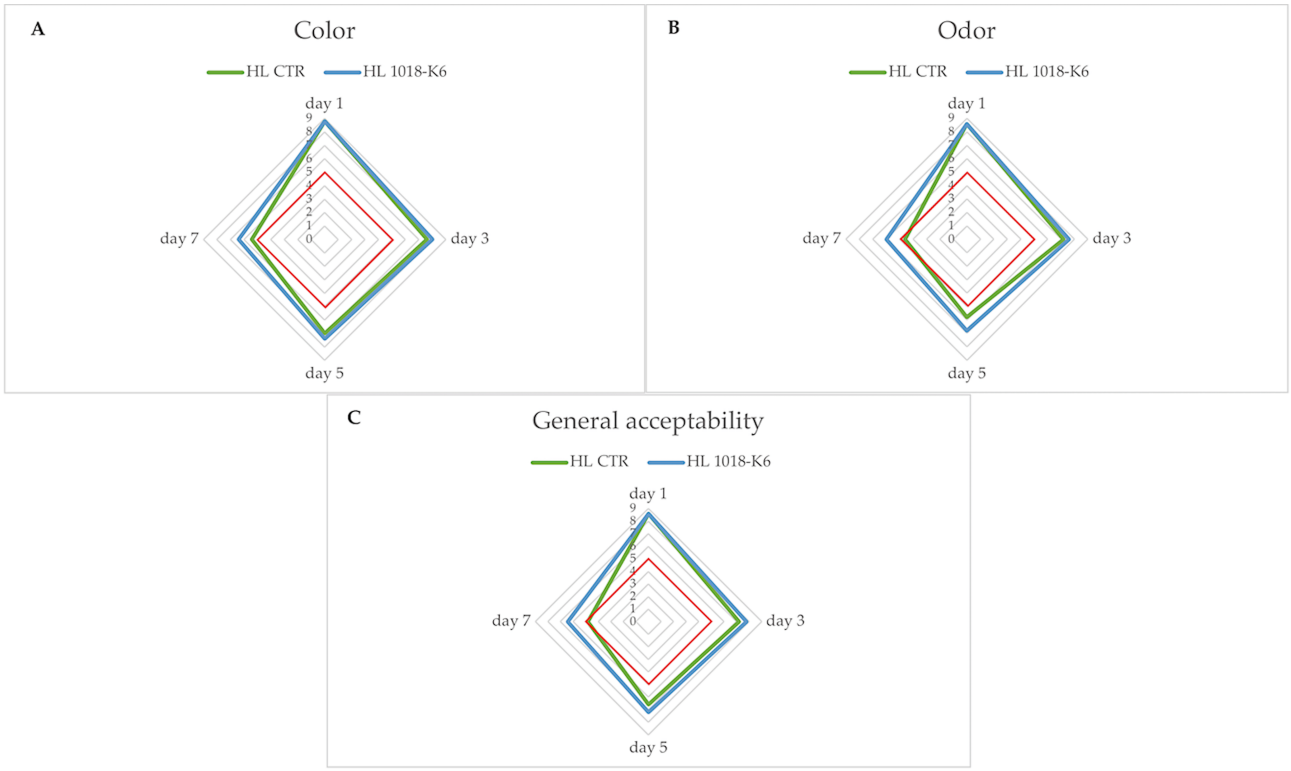


Figure 6.2 *Changes in color (**A**), odor (**B**), and overall appearance (**C**) of fish burgers during storage at refrigerator.*

6.4 References

Abbas KA, Saleh AM, Mohamed A, Lasekan O, 2009. The relationship between water activity and fish spoilage during cold storage: A review. J Food Agric Environ 7(3-4), 86–90.

Albertos I, Martin-Diana AB, Burón M, Rico D, 2019. Development of functional bio-based seaweed (*Himanthalia elongata* and *Palmaria palmata*) edible films for extending the shelflife of fresh fish burgers. Food Packag Shelf Life 22, 100382.

Ambrosio RL, Gratino L, Mirino S, Cocca E, Pollio A, Anastasio A, Palmieri G, Balestrieri M, Genovese A, Gogliettino M, 2020. The bactericidal activity of protein extracts from Loranthus europaeus berries: a natural resource of bioactive compounds. Antibiotics 9(2), 47.

Andreu D, Rivas L, 1998. Animal antimicrobial peptides: an overview. Biopolymers 47, 415–433.

Angiolillo L, Conte A, Del Nobile MA, 2018. A new method to bio-preserve sea bass fillets. Int J Food Microbiol 271, 60–66.

Chotphruethipong L, Benjakul S, 2019. Use of cashew (Anacardium occidentale L.) leaf extract for prevention of lipidoxidation in Mayonnaise enriched with fish oil. Turkish J Fish Aquat Sci 19(10), 825–836.

Colagiorgi A, Festa R, Di Ciccio PA, Gogliettino M, Balestrieri M, Palmieri G, Anastasio A, Ianieri A, 2020. Rapid biofilm eradication of the antimicrobial peptide 1018-K6 against Staphylococcus aureus: A new potential tool to fight bacterial biofilms. Food Control 107, 106815.

De Smet K, Contreras R, 2005. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol. Lett. 27, 1337–1347.

Ehsani A, Hashemi M, Afshari A, Aminzare M, Raeisi M, Zeinali T, 2020. Effect of different types of active biodegradable films containing lactoperoxidase system or sage essential oil on the shelf life of fish burger during refrigerated storage. LWT–Food Sci Technol 117, 108633.

EUMOFA, 2020. *The EU Fish Market*. Luxembourg: EUMOFA (European Market Observatory for Fisheries and Aquaculture Products).

FAO, 2016. *The state of world fisheries and aquaculture. Contributing to food security and nutrition for all*. Food and Agriculture Organization of the United Nations.

Festa R, Ambrosio RL, Lamas A, Gratino L, Palmieri G, Franco CM, Cepeda A, Anastasio A, 2021. A Study on the Antimicrobial and Antibiofilm Peptide 1018-K6 as Potential Alternative to Antibiotics against Food-Pathogen *Salmonella enterica*. Foods 10(6):1372.

Geeroms N, Verbeke W, Van Kenhove P, 2008. Consumers' health-related motive orientations and ready meal consumption behaviour. Appetite 51, 704–712.

Ghaly A, Dave D, Budge S, Brooks M, 2010. Fish spoilage mechanisms and preservation techniques: Review. Am J Appl Sci 7(7), 859–877.

Gogliettino M, Balestrieri M, Ambrosio RL, Anastasio A, Smaldone G, Proroga YTR, Moretta R, Rea I, De Stefano L, Agrillo B, Palmieri G, 2020. Extending the Shelf-Life of Meat and Dairy Products via PET-Modified Packaging Activated With the Antimicrobial Peptide MTP1. Front Microbiol 10:2963.

Gökoglu N, Yerlikaya P, 2015. Seafood chilling, refrigeration and freezing: Science and technology (1st ed.). Chichester, UK: John Wiley and Sons, Ltd.

Gram L, Huss HH, 1996. Microbiological spoilage of fish and fish products. Int J Food Microbiol 33, 121–137

Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM, 2010. Antimicrobial peptides: general overview and clinical implications in human health and disease. Clin Immunol 135, 1–11.

Hoel S, Jakobsen AN, Vadstein O, 2017. Effects of storage temperature on bacterial growth rates and community structure in fresh retail sushi. J Appl Microbiol 123(3), 698–709.

ICMSF. (1978). International Commission of Microbiological Specifications for Foods. Microorganisms in Foods. 2. Sampling for Microbial Analysis. Principles and Specific Applications.

Khaneghah AM, Hashemi SMB, Limbo S, 2018. Antimicrobial agents and packaging systems in antimicrobial active food packaging: an overview of approaches and inter- actions. Food Bioprod Process 111:1–19.

Marrone R, Smaldone G, Ambrosio RL, Festa R, Ceruso M, Chianese A, Anastasio A, 2021. Effect of beetroot (*Beta vulgaris*) extract on Black Angus burgers shelf life. Ital J Food Saf 10(1):9031.

Messina CM, Bono G, Arena R, Randazzo M, Morghese M, Manuguerra S, La Barbera L, Ozogul F, Sadok S, Santulli A, 2019. The combined impact of cold smoking and natural antioxidants on quality and shelf life of dolphinfish (Coryphaena hippurus) fillets. Nutr Food Sci 7(4), 1239-1250.

Nowzari F, Shábanpour B, Ojagh SM, 2013. Comparison of chitosan–gelatin composite and bilayer coating and film effect on the quality of refrigerated rainbow trout. Food Chem 141(3), 1667–1672.

Palmieri G, Balestrieri M, Capuano F, Proroga YT, Pomilio F, Centorame P, Riccio A, Marrone R, Anastasio A, 2018a. Bactericidal and antibiofilm activity of bactenecin-derivative peptides against the food-pathogen Listeria monocytogenes: New perspectives for food processing industry. Int J Food Microbiol 279, 33–42.

Palmieri G, Tatè R, Gogliettino M, Balestrieri M, Rea I, Terracciano M, Proroga YT, Capuano F, Anastasio A, De Stefano L, 2018b. Small Synthetic Peptides Bioconjugated to Hybrid Gold Nanoparticles Destroy Potentially Deadly Bacteria at Submicromolar Concentrations. Bioconj Chem 29, 3877–3885.

Panza O, Lacivita V, Palermo C, Conte A, Del Nobile MA, 2020. Food By-Products to Extend Shelf Life: The Case of Cod Sticks Breaded with Dried Olive Paste. Foods, 9(12), 1902.

Reddy KV, Yedery RD, Aranha C, 2004. Antimicrobial peptides: premises and promises. Int J Antimicrob Agents 24, 536–547.

Rico D, Albertos I, Martinez-Alvarez O, Lopez-Caballero ME, Martin-Diana AB, 2020. Use of Sea Fennel as a Natural Ingredient of Edible Films for Extending the Shelf Life of Fresh Fish Burgers. Molecules, 25(22), 5260.

Roohinejad S, Koubaa M, Barba FJ, Saljoughian S, Amid M, Greiner R, 2017. Application of seaweeds to develop new food products with enhanced shelf-life, quality and health-related beneficial properties. Int Food Res, 99, 1066–1083.

Thilsted SH, Thorne-Lyman A, Webb P, Bogard JR, Subasinghe R, Phillips MJ, Allison EH, 2016. Sustaining healthy diets: The role of capture fisheries and aquaculture for improving nutrition in the post-2015 era. Food Policy 61, 126–131.

Wang T, Sveinsdóttir K, Magnússon H, Martinsdóttir E, 2007. Combined Application of Modified Atmosphere Packaging and Superchilled Storage to Extend the Shelf Life of Fresh Cod (Gadus morhua) Loins. J Food Sci 73, S11–S19.

Xu DP, Li Y, Meng X, Zhou T, Zhou Y, Zheng J, Zhang J, Li HB, 2017. Natural antioxidants in foods and medicinal plants: Extraction, assessment and resources. Int J Mol Sci 18(1), 96.

7. Chapter 5

Preliminary study on the mechanism of action of 1018-K6 using model and biological membranes of food pathogenic bacteria

Abstract

The growing emergence of antibiotic resistance has increased the interest in antimicrobial peptides (AMPs), which have been associated with less common resistance than conventional drugs. To this purpose, a better understanding of their mechanisms of action is essential.
1018-K6 is a cathelicidin-related cationic peptide designed from the sequence of the innate defense regulator peptide-1018 (IDR-1018), which derived from the bovine host-defense peptide (HDP) bactenecin. Although its antimicrobial activity towards both Gram-positive and Gram-negative bacteria responsible for foodborne diseases has been extensively demonstrated, the mechanism of action at the molecular level is still unclear. Mechanistic studies (binding saturation assay, and fluorescence methodologies) were carried out using model membranes and bacterial membranes extracted from pathogenic bacteria isolated in various food matrices. Results reveal that the antimicrobial activity of 1018-K6 is connectable to its affinity for bacterial membranes. The study highlights differences in peptide-lipid interaction and partitioning between model membranes of bacteria, suggesting that peptides have to establish more deeper interactions for binding to Gram-positive membrane that could be favored with Arg residues. Moreover, the peptide ability to interact with bacterial model liposomes was confirmed by the formation of vesicular aggregates at ratios of lipid to peptide lower than 80. In conclusion, it is worth pointing out the higher affinity of 1018-K6 for bacterial membranes than those of the respective multilamellar vesicles (MLVs). The knowledge gathered on the mechanism of action of this peptide shows a high specificity against anionic liposomes and provides valuable information on the time of action. By calculating that the peptide needs only about 30 minutes or 2 hours to enter in the membranes of Gram-negative and Gram-positive, respectively, the prospect of using 1018-K6 in the formulation of active packaging becomes more concrete.

Keywords: antimicrobial peptide, partition constant, quencher.

7.1 Introduction

The growing emergence of antimicrobial resistance is mainly due to the used of the same classes of antimicrobial compounds in food-producing animals and in human medicine. Moreover, resistant bacteria could spread to humans via ingestion of contaminated food, as has been reported for the zoonic bacteria *Salmonella* ed *E. coli* (EFSA, 2021), and cause severe foodborne disease and treatment failures. It is important to underline the role of commensal bacteria in food, which could be reservoir of resistance genes (EFSA, 2008). In this contest, the search for alternative antimicrobial agents is gaining the attention of the whole scientific world.
In recent decades, antimicrobial peptides (AMPs) have conquered the dominance among the many natural preservatives proposed (Muzzarelli, 2011; Giacometti *et al*., 2018; Ambrosio *et al*., 2020). Due to their naturalness, and broad spectrum of action, AMPs are considered a promising alternative capable of killing pathogenic bacteria.
Many mechanistic models were proposed to explain their mechanism of action, contemplating membrane and intracellular targets (Guilhelmelli *et al.,* 2013). Commonly, cationic peptides interact with anionic lipids of membrane. Electrostatic forces place peptides and lipids near, making the binding and the subsequent partitioning possible. At this point, peptides could kill the bacterial cell by damaging the lipidic membrane or by modifying the cellular metabolism (Lee *et al*., 2016). The AMPs are also immunomodulators (Hancock *et al*., 2016) and promote bacterial aggregation

(Petrlova *et al*., 2017) through nonspecific H-bonding, and electrostatic cross-linking between them and specific components of lipopolysaccharide and bacterial peptidoglycan (Robert *et al*., 2015; Torrent *et al*., 2012).
Although cases of resistance to AMPs have been described, it is worth pointing out that peptide have been associated with less common resistance than conventional drugs. Obviously, a better comprehension of their mechanisms of action is essential for promoting their use in the food industry.
In previously studies, a cationic peptide composed of 12 residues and called 1018-K6 was described and characterized (Palmieri *et al*., 2018; Colagiorgi *et al*., 2020; Festa *et al*., 2021). It was engineered from the sequence of the innate defense regulator peptide-1018 (IDR-1018) (Mansour *et al*., 2015), which derived from the bovine host-defense peptide (HDP) bactenecin. The results reveled that 1018-K6 is a peptide with high stability and capable of adopting mixed α-helical/β-sheet conformations when in contact with bacterial membranes (Palmieri *et al*., 2018). Moreover, it has shown a great antimicrobial and antibiofilm activity against various microorganisms responsible for food spoilage and foodborne diseases. At micromolar concentrations, it is capable of killing dangerous and resistant food bacteria, such as *Salmonella enterica*, *Listeria monocytogenes,* and *Staphylococcus aureus*, including MRSA (Palmieri *et al*., 2018; Colagiorgi *et al*., 2020). Although a lot is known about its antibacterial properties, the information on its mechanism of action is fragmented. Some hypotheses have been formulated in previous studies (Palmieri *et al*., 2018; Colagiorgi *et al*., 2020), when an effect on the membrane cell was observed using scanning electron microscopy (SEM). The authors reported the presence of small cellular aggregates characterized by blebbing on the membrane surface, that could be associated with phenomena of destabilization. However, the mechanism of action of 1018-K6 at the molecular level is still unclear and a better comprehension of their mechanisms of action is essential for promoting the use in the food industry.
This study aimed to provide more information on the specific mechanism of action of 1018-K6 using bacterial model membranes that resemble different pathogenic bacteria and lipid membranes extracted from wild strains of bacteria. For this purpose, strains of *Salmonella* spp., *Escherichia coli* and *Staphylococcus aureus* isolated from food matrices. Furthermore, to gather a more detailed understanding of the potential toxicity, the interaction between AMPs and model membrane with composition similar to eukaryotic cells were investigated.

7.2 Materials and Methods

7.2.1 Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (**POPE**), L-α-lysophosphatidylcholine (Egg Lecithin) (**PC**), L- α-phosphatidylethanolamine (Liber Bovine) (**PE**), 1′,3′-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-glycerol (Cardiolipin, 18:1) (**CL**), phosphatidyl serine (**PS**), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (**DOPE**) were purchased from Avanti Polar Lipids (Alabaster, AL); while 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (**POPG**), and N-Acyl-D-sphingosine-1-phosphocholine (chicken egg yolk) (**SM**) were provided by from Larodan AB (Sweden) and Sigma-Aldrich (St. Louis, MO), respectively. Tris-HCl, Tris base, and acrylamide were purchased from and Sigma-Aldrich (St. Louis, MO). Sodium Dodecyl Sulfate (SDS) 20% Solution and Luria (LB) Broth were bought from Thermo Fischer (Germany). Ammonium heptamolybdate tetrahydrate ($(NH_4)Mo_7O_{24}\cdot 4H_2O$), di-sodium hydrogen phosphate anhydrous (Na_2HPO_4), L(+)-Ascorbic acid, and the all other reagents were obtained from Scharlau (Spain).
The derivative 12-mer peptide 1018-K6 was purchased from SynPeptide Co., LTD (Shanghai, China).

7.2.2 Preparation of Multilamellar vesicles (MLVs)

Model membranes were prepared from lipid stock solution of 10 mM (POPE, PE, DOPE, PC, SM, PS, CL and POPG) dissolved in chloroform:methanol (2:1, *v/v*). Lipid films were prepared in glass tubes by mixing an appropriate volume of phospholipids to obtain the desired final concentrations (Table 7.1). The solutions thus prepared were dried under Argon (Ar) flow and then subjected to vacuum (at least for 3 hours) to remove traces of solvent. A sufficient volume of binding buffer (Tris HCl 50 mM/ NaCl 100 mM; pH=7.4) was used to resuspend the lipidic film, yielding 2 mM working solutions. New lipid suspensions were prepared before each experimental trial.

Table 7.1 *Membrane lipid composition in eukaryotic and prokaryotic organisms (% mol).*

MEMBRANE	PC	SM	PS	PE	DOPE	POPE	POPG	CL
Eukaryotic membrane[1]	40	15	5			40		
Zwitterionic membrane	100							
Salmonella Typhimurium[2GN]					78		18	4
Escherichia coli[3GN]				80			15	5
Staphylococcus aureus[3GP]							58	42

[1]Casares *et al*., 2019; [2]Barbosa *et al*., 2019; [3]Epand and Epand, 2011.
PC: phosphatidylcholine; SM: sphingomyelin; PS: phosphatidylserine; PE: phosphatidylethanolamine; DOPE: dioleoyl-phosphatidylethanolamine; POPE: Palmitoyl-oleoyl-phosphatidylethanolamine; POPG: Palmitoyl-oleoyl-phosphatidylglycerol; CL: cardiolipin.
GN: Gram-negative bacteria; GP: Gram-positive bacteria

7.2.3 Lipid Binding Assay—MLVs

To assess the grade of peptide-MLVs interaction, reaction mixtures were prepared in Eppendorf tubes by combining each lipid solution (at fixed molar concentration of 1800 μM) with different amounts of peptide ranged from 15.63 to 78.13 μM. When saturation did not occur, higher peptide concentrations were tested. Solutions were vigorously vortexed and incubated at room temperature for 30 minutes to allow binding. To produce negative controls, analogous samples were made using buffer solution instead of peptide stock solution.
The solutions were carefully transferred in the corresponding polycarbonate centrifuge tubes (1 mL, 8x51 mm; Beckman Coulter, U.S.A.) and centrifuged at 59181 xg (20000) rpm at 20°C for 1 hour (Beckman LE-80 Ultracentrifuge; rotor type 25) (Alvarez *et al*., 2017). Upon completion, the resulting supernatant was isolated, collected in an Eppendorf tube and mixed with the above buffer in the presence of SDS to a final concentration of 1-2%. The pellet, after being washed once with binding buffer, was resuspended using the same SDS containing buffer. To calculate the amount of peptide detected in the supernatant and pellet solutions, a calibration curve was generated for each experimental run by adding different known concentrations of peptides to control samples prepared with supernatants o pellets of vesicles prepared without peptides.
The peptide binding affinity for MLVs was measured by exploiting the fluorescence and absorbance properties of the tryptophan residue of the peptide 1018-K6.
-*Steady-state tryptophan fluorescence.* Tryptophan (Trp) fluorescence spectra were recorded for each sample of supernatant and pellet after 30 min of stirring at 930 rpm in Thermo-Shaker, TS-100 (Biosan) at room temperature. The variation in Trp emission was recorded between 300 and 450 nm, exciting at λ_{ex} = 280 nm, using a Cary Eclipse Fluorescence spectrophotometer (Varian, Australia). Slit widths were 5 nm for both excitation and emission. Each spectrum was corrected by subtracting liposome background.
-*UV-Vis absorption spectrophotometry.* Peptide quantification was also performed by UV absorbance of Trp residues at 280 nm using UV-Vis Cary 300 Bio Spectrophotometer (Varian, Australia). To this purpose, the samples were treated as described above for fluorescence measurements.
-*Data analysis.* Saturation binding analyses was performed with GraphPad Prism 8.0.1 using the Scatchard analysis method and estimating the K_d (equilibrium dissociation constant) and B_{max}

(maximum specific binding) values (Eq.1). B_{max} is the total number of binding sites and is expressed in the same units as the *y* values (nmol/mg), while K_d represents the concentration of ligand which at equilibrium occupies 50% of the receptors present in the biological preparation and is expressed in the same units as ligand (peptide, P), $[P]_{total}$ (µM).

(1) $$[P]_{bound} = \frac{B_{max} \times [P]_{total}}{K_d + [P]_{total}}$$

Plotting the ratio between the quantity of bound ligand and free ligand ($[P]_{bound}/[P]_{free}$) with respect to the quantity of bound ligand ($[P]_{bound}$), a straight line with slope of - $1/K_d$ is obtained. The intercept on the abscissa axis (($[P]_{bound}/[P]_{free} = 0$) allows to calculate the receptor density, B_{max}. In other words, this analysis allows the determination of the peptide binding affinity and density of binding sites in lipid bilayers for these peptides.
Furthermore, the partition coefficient (the equilibrium distribution of proteins between the membrane and the solution), K_p, was calculated by fitting the experimental data with Eq. (2) as described by White *et al*., 1998. This equation takes into account the centrifuge methods principle which consist in the sedimentation of vesicles with bound peptides and the persistence of free molecules in the supernatant (Roussel *et al*., 2020). For this reason, considering that the amount of peptide bound is the result of the subtraction of free peptide from the total amount, the partition coefficient can be calculated as follow:

(2) $$K_p = \frac{\frac{[P]_{total} - [P]_{free}}{[L]}}{\frac{[P]_{free}}{[W]}}$$

where $[P]_{total}$ and $[P]_{free}$ are the aqueous peptide concentrations measured before the addition of vesicles and after the centrifugation, respectively; and [L] and [W] are the molar lipid concentration of lipids and water (55.3 M).

7.2.4 Lipid sedimentation assay

To gather more information on the mechanism of action of 1018-K6, the ability to cause membrane rupture was evaluated by detecting the presence of a fragment of membranes in the supernatant samples. The method consisted in measuring the lipid phosphorus concentration in the supernatant after sedimentation of 1800 µM of MLVs by centrifugation, considering the assumption that in presence of intact vesicles no lipids were detected in the supernatant. In this regard, the same samples used for the lipid-binding assay were included in the studies, using both supernatant and pellet solutions. The lipid quantification was performed by evaluating the lipid phosphorus concentration via the Fiske assay (Fiske *et al*., 1925; Bartlett, 1959; Böttcher *et al*., 1961) wit same modifications. Briefly, a calibration curve with different volumes of 1 mM Na_2HPO_4 was generated for each experiment. 30 µL per sample were placed in a glass tube, and then 500 µL of 70% perchloric acid were added to each tube. After being vortexed, tubes were incubated at 180°C for 45 minutes in thermoblock (Multiplaces, Selecta). At the end of the incubation period, the tubes were allowed to cool and 4 mL of molibdate ($(NH_4)Mo_7O_{24}\cdot 4H_2O$ 1.89 mM and 14.3 mL of H_2SO_4 at 95-97%, diluted to 1 L with purified water) and 500 µL of 10% ascorbic acid (*w/v*) were added to each tube. Vortexed samples were incubated in boiling water for 5 minutes. When the tubes cooled down, 250 µL of each sample and standard were transferred to 96-well plates and the absorbance was read using a UV Spectrophotometer (FLUOstar Omega, BMG LABTECH, Germania). The optical density was determinated at 800 nm, and the standards ODs were plotted versus the known lipid concentration and used as a calibration curve to determine the lipid concentrations in samples.

7.2.5 Bacterial strains

Wild strains of *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* spp., isolated from foods, were used to evaluate the binding affinity of 1018-K6 towards bacterial membrane lipids. These bacteria belong to the Type Culture Collection of the Laboratory of Food Microbiology of the Department of Veterinary Medicine and Animal Production, Federico II, University of Naples. In particular, *S. aureus* has been isolated from buffalo cheese, while *E. coli* and *Salmonella* spp. from wild boar carcass and meat, respectively. The bacterial identification was performed by the "direct colony identification method" (Alatoom *et al*., 2011) and processed by MALDI Biotyper® sirius System (Peruzy *et al*., 2020). The identification was obtained by comparing each bacterial mass spectra to the Bruker MSP database using the Bruker Compass software (Bruker Daltonics). Then, bacterial cultures were freeze-dried in 20% (*v/v*) glycerol and stored at -80 °C.

7.2.6 Whole lipid extract of bacterial membranes

Lipid extracts of bacterial membranes were obtained following the experimental protocol of Dennison *et al*., 2006, with some modifications. Briefly, cultures of *S. aureus*, *S. enterica*, and *E. coli* were grown overnight in Luria Broth (formulation for 1 liter: 10 g SELECT Peptone 140, 5 g SELECT Yeast Extract, and 5 g NaCl), incubated at 37° C. Reached the desired cellular concentrations (exponential phase), the total amount of bacterial culture was separated in 50 mL falcon tubes and centrifuged (Sigma, 2-16 K, rotor 231/F) at 8603 xg (9000 rpm) for 10 minutes at 4° C. Then, the supernatant was removed, and each resulting pellet resuspended in 1 mL of Tris Buffer (25 mM, pH 7.5) and the final solution transferred into a 10 mL glass tube. 3.57 mL of a 1:2 (*v/v*) chloroform:methanol mixture was added to cell suspension and the cell/solvent sample was vigorously vortexed for 5 min. Then, 1.25 mL of chloroform and 1.25 mL of distillated water were added, vortexing between each addition step. After the water addition, the solution was again vortexed for 5 minutes and then centrifuged (Thermo Scientific ST 8R, rotor 75005701) at low speed (1000 xg) for 10 minutes at 4°C, in order to obtain three separated phases: organic, aqueous and protein phases (bottom, upper and central phases, respectively). The organic phase was transferred into a clean 10 mL centrifuge tube and 4.75 mL of a 1:1 (*v/v*) methanol-buffer mixture were added, while 500 µl of chilled chloroform were added to the remaining aqueous phase. The two solutions were vigorously vortexed for 5 minutes, centrifuged as described above and the respective organic phases collected. The organic phase was dried under Argon (Ar) flow and then submitted to high vacuum for at least for 3 hours. The lipid films were resuspended in binding buffer (Tris HCl 50 mM/ NaCl 100 mM; pH=7.4) and Fiske assay was performed to determinate the lipid phosphorus concentration.

7.2.7 Lipid Binding Assay and Lipid sedimentation assay - Bacterial membranes

Bacterial lipid extracts were used to perform a peptide binding assay to lipid bilayers. The experimental design was built in order to test the binding capacity of 1018-K6 when incubated with a bacterial lipid solution. To this purpose, the reaction mixtures were prepared as described above for MLVs. Lipid solutions at fixed concentrations (µg/µL) were incubated with different amounts of peptide. Due to the low lipid extraction yield from bacterial cultures, the peptide binding analysis were performed calculating and maintaining the same ratio lipid concentration (C_L, phosphorus concentration (µg/µL)) and peptide concentration (C_P (µg/µL)) tested for model membranes of *S. aureus*, *S. enterica*, and *E. coli*. This approach aimed to compare binding results on bacterial membranes with those of respective MLVs.
Tryptophan (Trp) fluorescence spectra and measurements of UV absorbance of Trp residues were recorded for each sample of supernatant and pellet as described above. The B_{max} and the K_d values were calculated by Scatchard analysis of the data using GraphPad Prism 8.0.1. Furthermore, the

presence of lipid phosphorous was also investigated in bacterial supernatant samples using the same protocol described above for MVLs.

7.2.8 Fluorescence properties of 1018-K6 in aqueous and lipid solutions

The peptide affinities and interactions with MLV model bacterial membrane were also investigated by analyzing the degree of blue shifts in emission spectra (nm) of the Trp fluorescence. In particular, the peptide partition in the lipid bilayer is reflected on the emission wavelength at the maximum intensity, allowing to discriminate the extent of lipid-peptide interaction. The experiment was performed by titrating the peptide 1018-K6 (fixed concentration of 20 μM) with vesicle suspensions (final lipid concentration ranged from 0-2 mM). Fluorescence measurements were measured in quartz cuvettes with 1 cm path length on a Cary Eclipse Fluorescence spectrophotometer (Varian, Australia). Trp emission intensity and wavelength were recorder between 305 and 490 nm, and the integrated area of each fluorescence spectrum were corrected by subtracting liposome background and artifacts. All the samples were incubated 30 minutes before the spectra scanning.
To evaluate the exposure of Trp residue of 1018-K6 to the aqueous environment, fluorescence emission quenching upon titration with acrylamide was performed and Stern–Volmer constants (K_{sv}) were calculated (Lakowicz, 1999). 5 μL aliquots of a 4.0 M stock acrylamide solution were added to the cuvette containing samples of 30 μM of MLVs and 1 μM of peptide (total volume of 1 mL).
The K_{sv} was calculated from the equation (Eq.3):

$$F_0/F=1+K_{SV}(Q) \quad (3)$$

where F_0 is the initial fluorescence of the peptide and F is the fluorescence intensity following the addition of different concentration of soluble quencher, Q.

7.2.9 Vesicle aggregation

The potential ability of the antimicrobial peptide to induce vesicle aggregation was followed by optical density measurements at 436 nm (OD_{436}) (Henriques and Castanho, 2004). Different concentrations of the peptide (6.6, 12.5, 20, 25 and 50 μM) were incubated in the presence of MLVs at 500 μM. This assay was performed in flat-bottom 96-well plate and the turbidity variations were followed during the first 35 minutes of peptide-lipid incubation using UV Spectrophotometer (FLUOstar Omega, BMG LABTECH, Germania).

7.2.10 Statistical analyses

Statistical analyses were performed using SPSS version 26 (IBM Analytics, Armonk, NY, USA) and GraphPad Prism®, version 8.0.1 (GraphPad, San Diego, California, USA). All experiments were performed at least three times and data were presented as the mean (M) ± standard error (sem).
Scatchard analyses, student's t-test ($p < 0.05$) and Anova two ways analysis were performed using GraphPad Prism in order to analyze data from peptide binding analyses, peptide fluorescence properties (blue shift and quenching assay), and vesicle aggregation, respectively.
Partition constant values were analyzed using SPSS with generalized linear mixed models (GLMMs) and the means were compared using the Tukey test ($p < 0.05$).

7.3 Results and Discussion

7.3.1 Peptide binding to MLVs and lipid sedimentation assay

In the studies on the mechanism of action of any membrane-binding compound, its interaction with a model or biological membrane system can be calculated by determining the amount or proportion of ligand bound to the lipid bilayer and free in the aqueous medium. The experimental determination of the binding parameters of molecules into membranes can be performed through different methods, such as the physical separation of bound and free molecules and the titration methods (White *et al*., 1998). In the present study, the binding affinity of the peptide, 1018-K6, was evaluated by separating the bound and free peptides through centrifugation (Alvarez *et al*., 2017). This separation technique allows to immediately calculate the amount and fraction of peptide partitioned, ranging from 0 to 100%. However, the density of lipids very close to that of the water could interfere with the vesicle sedimentation. This problem was avoided by centrifuging the samples at high speed and using MLVs instead of small unilamellar vesicles (SUVs), commonly used in partitioning studies (Fox *et al*., 1970).

The ways useful to describe the interactions between peptides and lipids are different. Albeit determination of the mole-fraction partitioning coefficient is commonly used in binding studies, a more accurate means to define the binding mechanism is the Scatchard analysis (Scatchard, 1949). This analysis allows determination of dissociation constant K_d and the number of lipid site of binging, B_{max}, when the bound and the free ligand concentrations have been determined experimentally (Jain *et al*., 1985; Ito *et al*., 1993). However, several authors believe that the latter method alone cannot describe the phenomenon, due to the absence of specific receptors on the membranes for peptides. Lipid membranes are complex systems, and the peptide-lipid interaction is controlled by the lipidic ensemble (Nuno *et al*., 2003). Furthermore, it is important to take into account that no direct comparisons are possible between the partition constant (K_p) and dissociation constant (K_d) and because of this it is necessary to calculate both. However, the Scatchard method can be considered to be appropriate to analyze this type of interactions if the lipid bilayer is considered itself as a receptor and the peptide as a ligand (Escribá *et al*., 1990).

For all these reasons, I exanimated the ability of 1018-K6 to bind to phospholipid bilayers by carrying out saturation binding experiments and fitting the experimental data with Eq. 1 and Eq. 2 (Materials and Methods section). Moreover, the presence and the concentration of peptides in the two phases, aqueous (supernatant-free ligand) and lipidic (pellet-bound ligand), were measured using spectroscopic techniques such as UV-Vis absorption and fluorescence based on the analysis of the Tryptophan signals originated in both phases. Indeed, 1018-K6 has one Trp residue which enable its characterization using spectroscopc techniques (Palmieri at *al*., 2018).

Saturation binding assay allows to calculate the receptor number and the affinity by determining the binding at various concentration of peptides. This method considers the achievement of an equilibrium among aqueous and lipidic phases during the period of incubation, which has been fixed at 30 minutes for these experiments.

Data from binding saturation experiments were analyzed using Graph Pad Prism 8.0.1 software to prepare saturation binding and Scatchard plots. Specifically, in the Figures 7.1 are reported the results of the data elaboration starting from the values obtained with the fluorescence measurements, while the in the Figure 7.2 are shown the experimental binding data from absorption spectroscopy results.

As it can be seen in both figures, the behavior of the cation peptide clearly differs from one type of model membrane to another. Its affinity for the lipid mixtures seems to be influenced by their difference in lipid composition, mainly related to the membrane surface charge. It is known that the first step of any AMP mechanism of action is the interaction, and the cationic residues of CAMPs are attracted via electrostatic forces from negatively charged lipids of the bacterial target membrane, such as phosphatidylserine, phosphatidyl glycerol, or cardiolipin (Bessalle *et al*., 1992; Matsuzaki *et al*., 1997; Dathe *et al*., 2001; Melo *et al*., 2009). Knowing that the 12-mer peptide, 1018-K6, has net. positive charge (Palmieri *et al*., 2018), the potential interaction of this molecule with lipids containing net negative charges had already been hypothesized. This hypothesis had been confirmed by the results shown in the present study where membranes composed of zwitterionic PC (Matsuno *et al*.,

2011), which simulates the bulk fluid phases in eukaryotic cell, were compared with those that mimic the anionic bacterial cells.

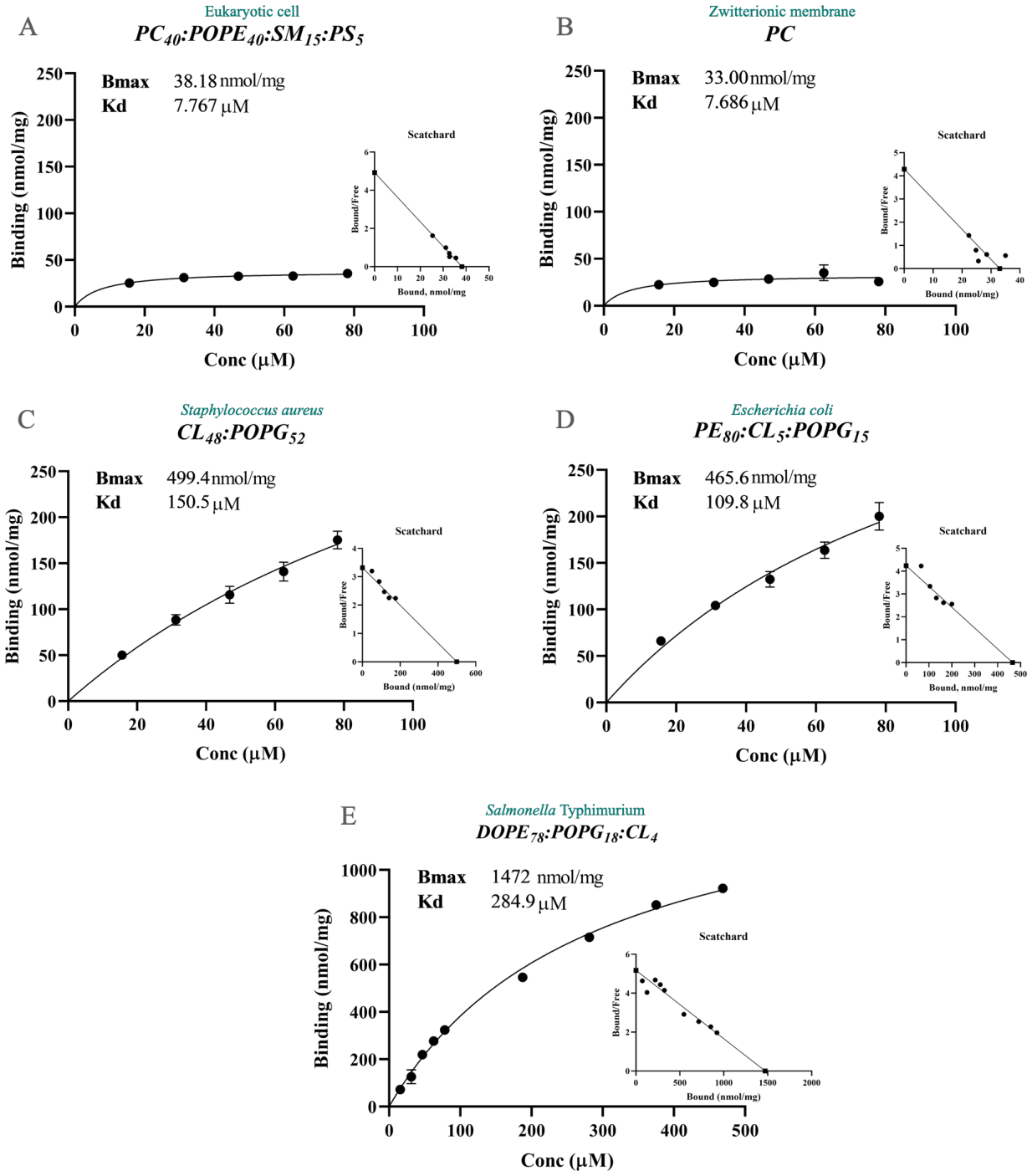


Figure 7.1 *Saturation binding assay curves and Scatchard plots transformation for florescence spectroscopy data of 1018-K6 in model membranes of* ***(A)*** *eukaryotic cell,* ***(B)*** *zwitterionic lipids,* ***(C)*** *S. aureus,* ***(D)*** *E. coli, and* ***(E)*** *S. Typhimurium. Results are means of three independent experiments.*

The receptor density assessment affirms the very low affinity of 1018-K6 for the membranes with net neutral charge or low negative charge (PC and PC_{40}:$POPE_{40}$:SM_{15}:PS_5, respectively). Furthermore, the peptide seems to prefer the model membranes that resemble that of *Salmonella* Typhimurium, reaching the highest value of B_{max} after binding determination using both spectroscopy methods (Figures 7.1 and 7.2). Moreover, in order to carry out saturation binding experiments, it was necessary to increase the maximum peptide dose more than 5 times for this type of membrane (graphs 7.1E and

7.2E). This finding could suggest the propensity of the peptide to bind to Gram-negative bacterial cells much more easily than to Gram-positive ones. From the interactional point of view, it can be argued that the *Salmonella*-like membrane contains more binding sites and/or that its affinity is higher than that of the eukaryotic-like membrane. In this context, the results obtained using the model membranes of *E. coli* could refute the hypothesis (graphs 7.1D and 7.2D), being characterized by B_{max} values more similar to *S. aureus* model membranes than those of the other Gram-negative bacterium. However, it is necessary to highlight the difficulties encountered in the spectroscopic measurements of the Trp residues of peptides in this type of model membrane, slightly contained increasing the SDS final concentration from 1 to 2 %.

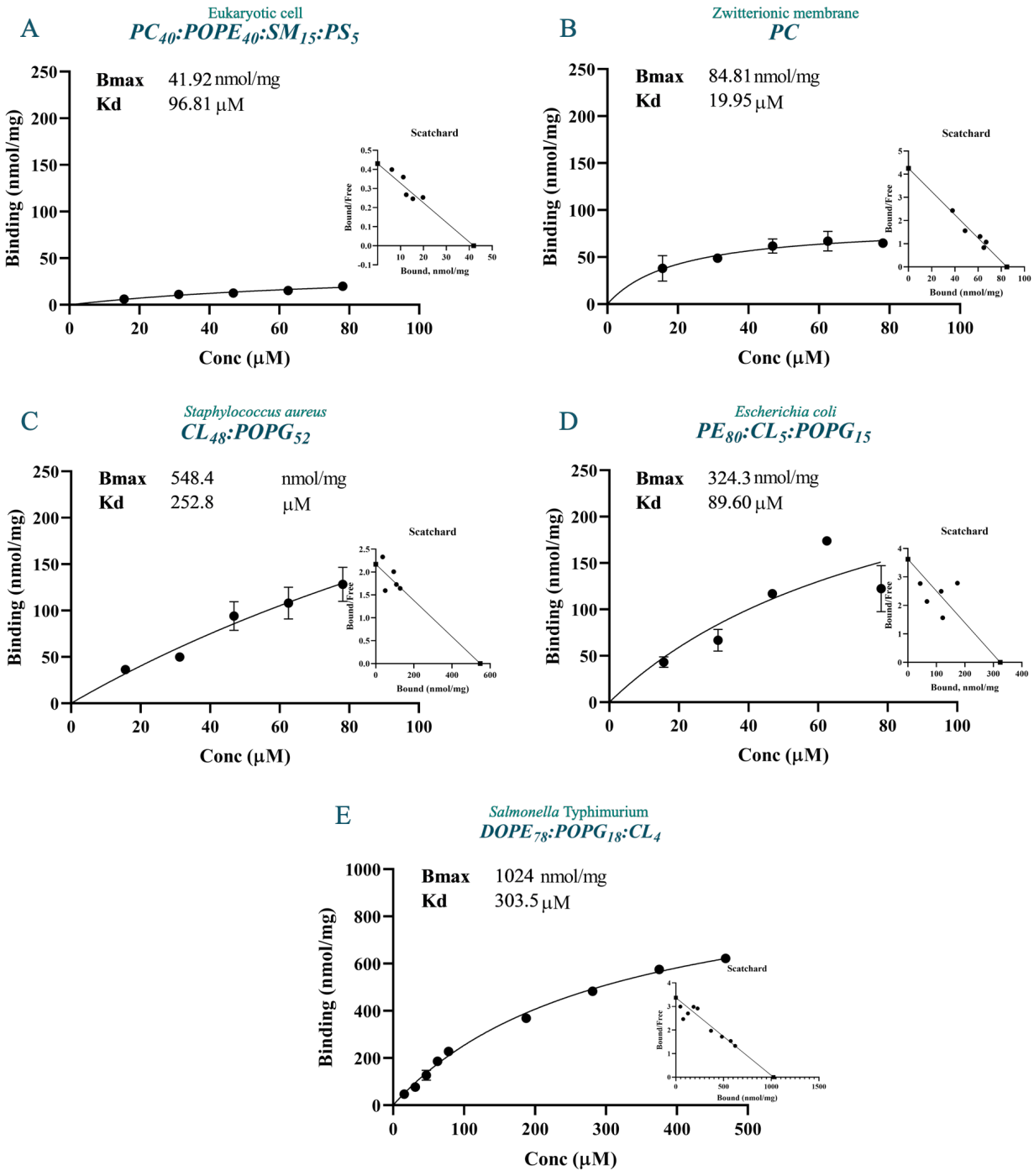


Figure 7.2 *Saturation binding assay curves and Scatchard plots transformation for UV-Vis absorption spectroscopy data of 1018-K6 in model membranes of* ***(A)*** *eukaryotic cell,* ***(B)*** *zwitterionic lipids,* ***(C)*** *S. aureus,* ***(D)*** *E. coli, and* ***(E)*** *S. Typhimurium. Results are the mean of three independent experiments.*

Despite this correction in the protocol, in fact, on the basis of the different functioning of the two spectroscopic methods, the measurements with the UV-Vis absorption spectrophotometry of the *E. coli* membrane model are significantly different from the fluorescence spectra ($p<0.01$). However, it is not the first time that the scientific community collides with phenomena similar to those just described. The answer could be found in the natural type of lipid structure that forms the main constitutive lipid of the model membrane, PE. It is known that phosphatidylethanolamine isolated from several sources tends to form hexagonal phases (H_{II}) *in vitro* below physiological temperature, and that Boggs *et al*. (1981) determined that the temperature of the lamellar to hexagonal (L-H) phase transition of the bovine white matter PE is 18 °C. Moreover, it has been demonstrated that short peptides are capable to induce a reversed hexagonal (H_{II}) phase and, to this purpose, Morein *et al*. (2000) have studied the effect of hydrophobic peptides at several length on the lipid phase behavior of a model membrane of *E. coli* (dioleoylphos-phatidylethanolamine (DOPE)/ dioleoylphosphatidylglycerol (DOPG) (7:3 molar ratio)). Therefore, since many of the conditions described above occur in the experimental tests of this study (the incubation temperature close to 18°C and use of a short peptide) it can be concretely hypothesized that the recorded B_{max} and K_d values are influenced by the PE lipid transition to the hexagonal phase. The *in vivo* behavior of PE differs from that observed *in vitro* in the present study, as it forms lipid bilayers instead of H_{II} phases, and the methodological changes introduced in this study aim to maintain PE in its lipid bilayer organization.

The partitioning of the peptide into lipid vesicles is obviously dependent on the MLVs composition since the presence of negatively charged lipids (Table 7.2) such as CL, POPG and DOPE/PE is associated with high values of K_p. Results again showed a significant difference ($p<0.01$) between the coefficient values of the model membrane of *E. coli* when measured with different spectroscopic methods. Interestingly, unlike the previously described datasets focused on B_{max} and K_d values, the affinity of the 12-mer peptide appears to be stronger for *E. coli* MLVs than the other model bacterial membranes. This observation could be explained by taking into account the difference among the two equations, Eq. 1 and Eq. 2. The first method calculates B_{max} and K_d values by fitting the data regarding the concentration of peptides detected in the lipidic environments (pellets), while the second evaluates the K_p *values* by fitting the amount of 1018-K6 presents in the aqueous phases (supernatants). Therefore, the absence or the very low concentration of lipid mixture in the supernatants may have allowed the measurement of the Trp residues without interference even in *E. coli* MLVs samples.

Table 7.2 *Affinity of 1018-K6 for model membranes of (**A**) eukaryotic cell, (**B**) zwitterionic lipids, (**C**) S. aureus, (**D**) E. coli, and (**E**) S. Typhimurium, measured by florescence and UV-Vis absorption spectroscopies.*

Cellular type	Lipid mixture	K_p (x 10^4)		
		Fluorescence	UV-Vis absorption	
Eukaryotic membrane	PC_{40} : $POPE_{40}$: SM_{15} : PS_5	0.37 ± 0.27^{A}	0.10 ± 0.10^{A}	
Zwitterionic membrane	PC	0.76 ± 0.21^{A}	0.83 ± 0.22^{B}	
Staphylococcus aureus	CL_{48} : $POPG_{52}$	19.69 ± 2.90^{B}	16.11 ± 2.88^{C}	
Escherichia coli	PE_{80} : CL_5 : $POPG_{15}$	36.50 ± 4.67^{C}	14.65 ± 1.68^{C}	**
Salmonella Typhimurium	$DOPE_{78}$: $POPG_{18}$: CL_4	30.07 ± 4.53^{C}	26.23 ± 4.87^{C}	

Data analyses were performed using SPSS version 26 (IBM Analytics, Armonk, NY, USA). The results were analyzed with generalized linear mixed models (GLMMs) and the means were compared using the Tukey test. All data were presented as the mean (M) ± standard error (sem). $^{A, B}$ values within column differ $p<0.01$; $^{a, b}$ values within row differ $p<0.05$. *****, ****** values within column differ $p < 0.05$, $p < 0.01$ respectively.

Because of many of the mechanistic models of action referred to AMPs result in the membrane rupture (Lee *et al*., 2016), the possible fragmentation of the membrane into peptide-lipid micelles was evaluated. The same samples used to calculate all parameters referring to the affinity of the peptide for the model membrane were subjected to the modified Fiske protocol to measure the lipid

phosphorus concentration. As shown in the Figure 7.3, only a very small percentage of the total lipid concentration could be detected in the supernatant of all control samples (0 µM 1018-K6), demonstrating the effectiveness of the separation technique. Moreover, it is worth noting that no significant differences were found among MLVs samples incubated with increasing peptide doses for 30 minutes and controls. These findings could suggest two different hypotheses: 1) the mechanism of action of 1018-K6 foresees the formation of pore according to the "toroidal wormhole" model (Lee *et al*., 2016), which does not cause the membrane disruption; 2) the incubation period necessary to detect the final effect on the membranes is more than 30 minutes. For these reasons, further analyses are necessary to better clarify these aspects.

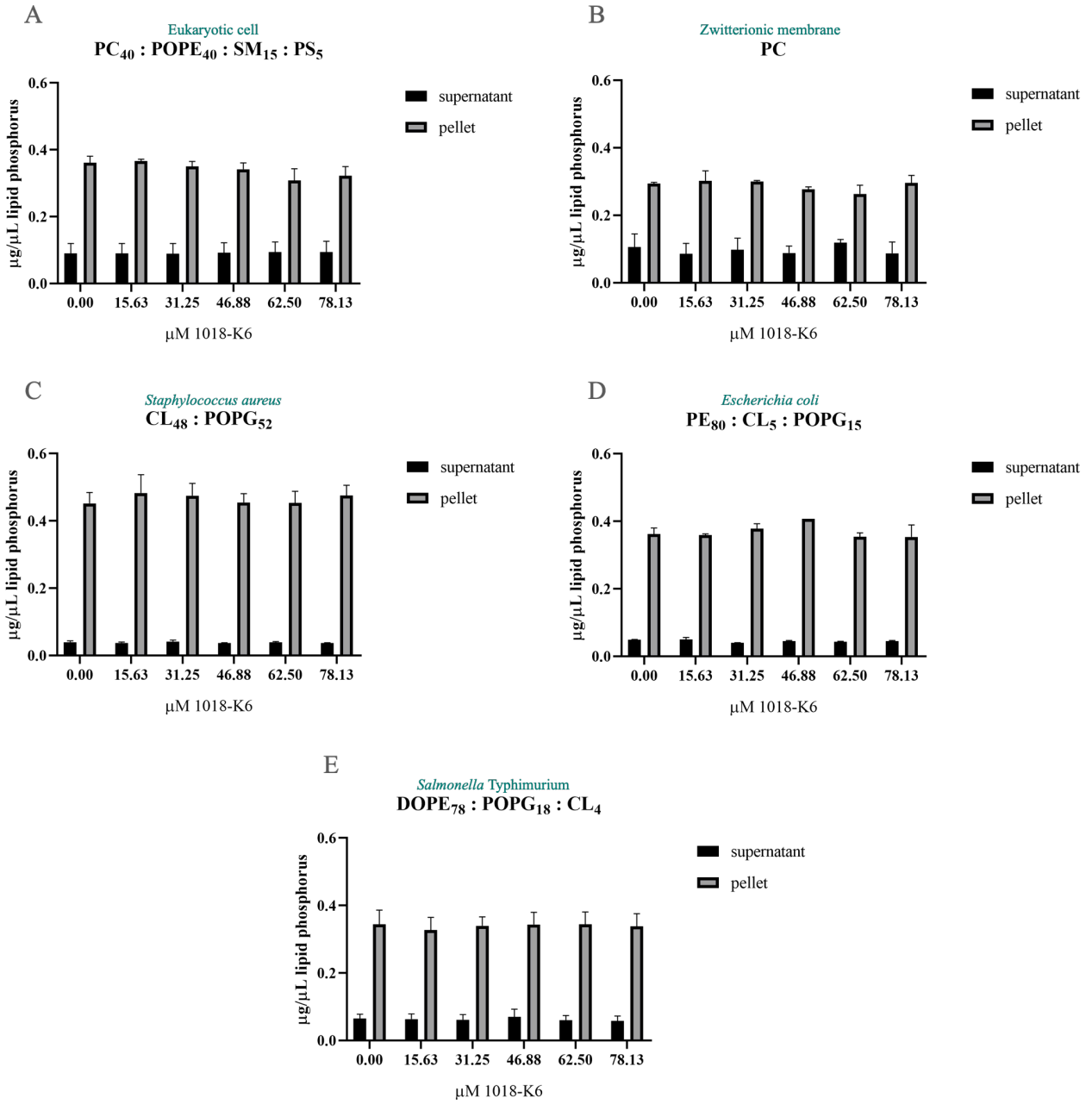


Figure 7.3 *Lipid phosphorus concentration in the supernatant and pellet after centrifugation of each lipidic sample subsequently incubation with several concentrations of peptide 1018-K6. Results are means of three independent experiments per model membranes ((**A**) eukaryotic cell, (**B**) zwitterionic lipids, (**C**) S. aureus, (**D**) E. coli, and (**E**) S. Typhimurium), and error bars represent the standard errors (sem).*

7.3.2 Peptide binding to Bacterial Membranes

The interaction of the peptide with membrane surfaces involves several events. First of all, the attraction of cationic residues of CAMPs by electrostatic forces from negatively charged lipids of the bacterial target membrane (Melo *et al*., 2009), and, once nearby to them, peptides change their conformations, commonly thought a coil-helix transition (Bello *et al*., 1982; Dathe *et al*., 1999). However, this is not true for β -sheet AMPs which have a more rigid structure already in solution. Hence, following this electrostatic interaction, a critical concentration of peptides is necessary to induce the molecule self-association and fully or partially lipid bilayer penetration. Once located in the membrane core, AMPs can exert their action via a variety of mechanisms (Huang *et al*., 2006; Hall *et al*., 2012).

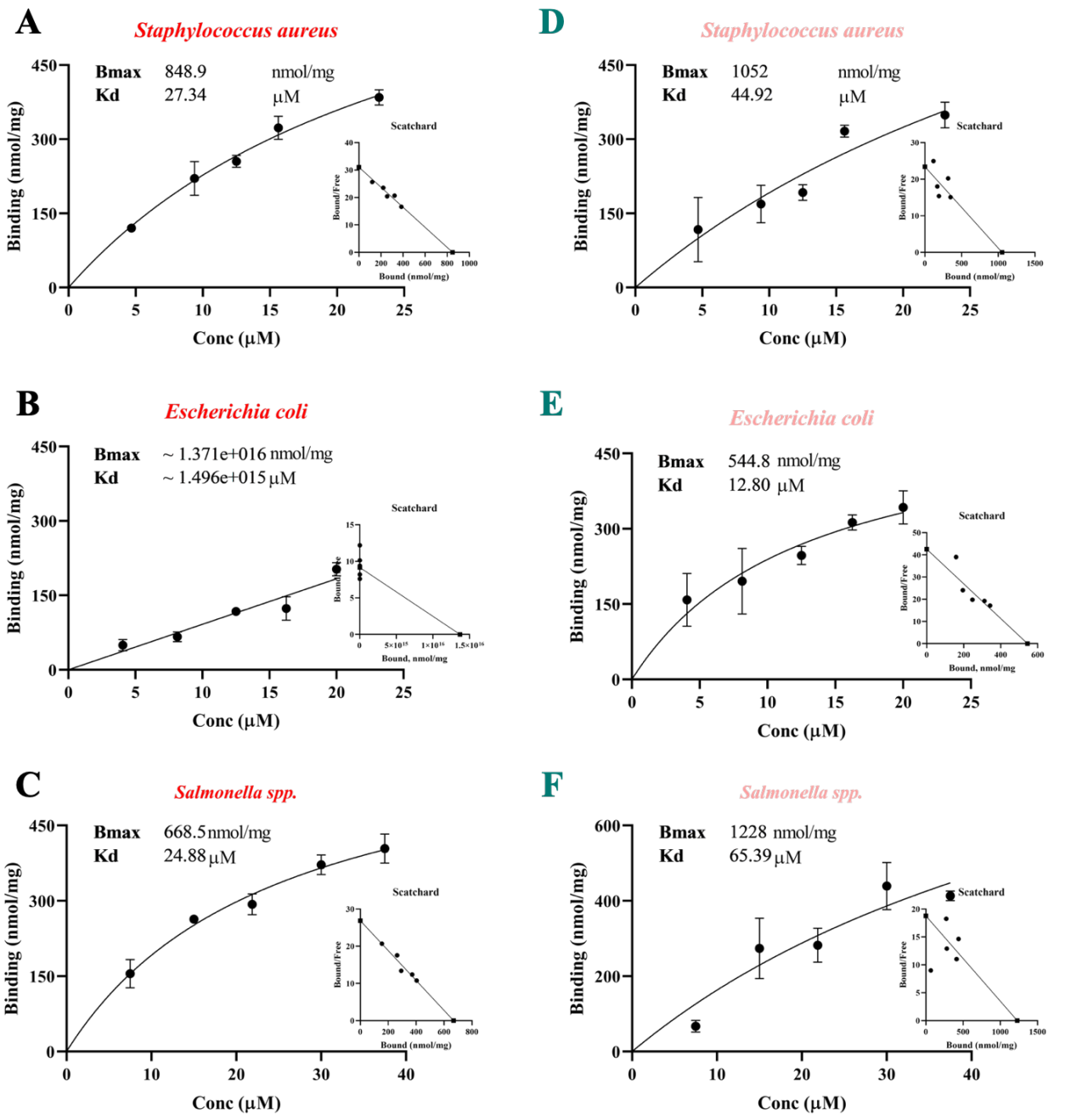


Figure 7.4 *Saturation binding assay curves and Scatchard plots transformation for florescence (**A-B-C**) and UV-Vis absorption (**D-E-F**) spectroscopy data of 1018-K6 in bacterial membranes of S. aureus, E. coli, and Salmonella spp., isolated from foods. Results are means of three independent experiments.*

Figure 7.4 shows the binding of 1018-K6 to the membranes of Gram-positive and Gram-negative bacteria as a function of peptide concentrations. In order to recreate the same experimental condition of the MLVs studies, the doses of added peptide were calculated respect to the ratio between lipid and peptide concentrations.
Membrane saturation was achieved for *Salmonella* spp. and *S. aureus*, for which it was possible to calculate the maximum receptor density and the dissociation constant. As displayed in the graph 7.4B, the peptide doses used were not sufficient to reach the saturation of the binding sites of *E. coli* membranes and, for this reason, further studies are needed to correctly calculate the values of B_{max} and K_d against this species. Specifically, the results of the fluorescence measurements underlined such a high affinity of the antimicrobial peptide toward these Gram-negative bacteria, even if this observation is not confirmed by the results of the UV-Vis absorption. However, if these data are analyzed taking into consideration what has already been discussed for the behavior of the model membrane of *E. coli* in the binding studies, it is possible to note how the results overlap, reinforcing the hypotheses formulated to explain the phenomenon. The presence of an interference in the composition of *E. coli* membranes that behaves like a quencher for Trp is evident. Furthermore, the lipid compositions of the two membranes of Gram-negative bacteria appear to be more similar than those of model membranes, so much so as that a difference between the two spectroscopic methods was also noted for *Salmonella* spp. (graphs 7.4C-F).
Overall, the comparison of the results of bacterial membranes with those of the respective MLVs reveals a strong difference among the dissociation constant values, which are lower in the interaction peptide-bacterial lipids. It is known that when the K_d is low and the peptides has a high affinity for the receptor (high B_{max}), fewer molecules are required to bind 50% of the receptors.
This important result supports the studies on the antimicrobial ability of the 1018-K6. In this regard, the authors have already proved the bactericidal and antibiofilm efficacy of the peptide toward strains of *Salmonella* spp. (Festa *et al*., 2021) and *Staphylococcus aureus* (Colagiorgi *et al*., 2020). In particular, several serotyped wild strains were selected on the base of their antibiotic resistance to common antibiotics and used to evaluate the 1018-K6 antimicrobial efficacy. The results have demonstrated that the AMP is capable to kill resistant Gram-positive and Gram-negative microorganisms ($5x10^5$ cfu/mL) at concentration ≤ 20 μM.
Regarding the potential effect of 1018-K6 to cause the fragmentation of the bacterial membranes, some hypotheses have been formulated by Palmieri *et al*. (2018) and Colagiorgi *et al*. (2020). Authors demonstrated the antibiofilm activity of the peptide by using scanning electron microscopy (SEM), which provided more information also on the possible mechanism of action. Indeed, the treatment of preformed biofilm of *S. aureus* and *Listeria monocytogenes* with 1018-K6 at the concentration of 80 and 50 μM, respectively, results in a significant biofilm eradication within 16 hours. Moreover, at magnification of 5000x it was possible to see coating matrix material and signs of damage to the cell membranes. The authors reported the presence of small cellular aggregates characterized by blebbing on the membrane surface, that could be associated with phenomena of destabilization.
The experimental protocol chosen in the present study could not be used to describe any possible effect of the peptide on the membrane of bacterial cells; however, the difference in time of the incubation periods (only 30 minutes in the binding studies) could explain the discordance in the results.

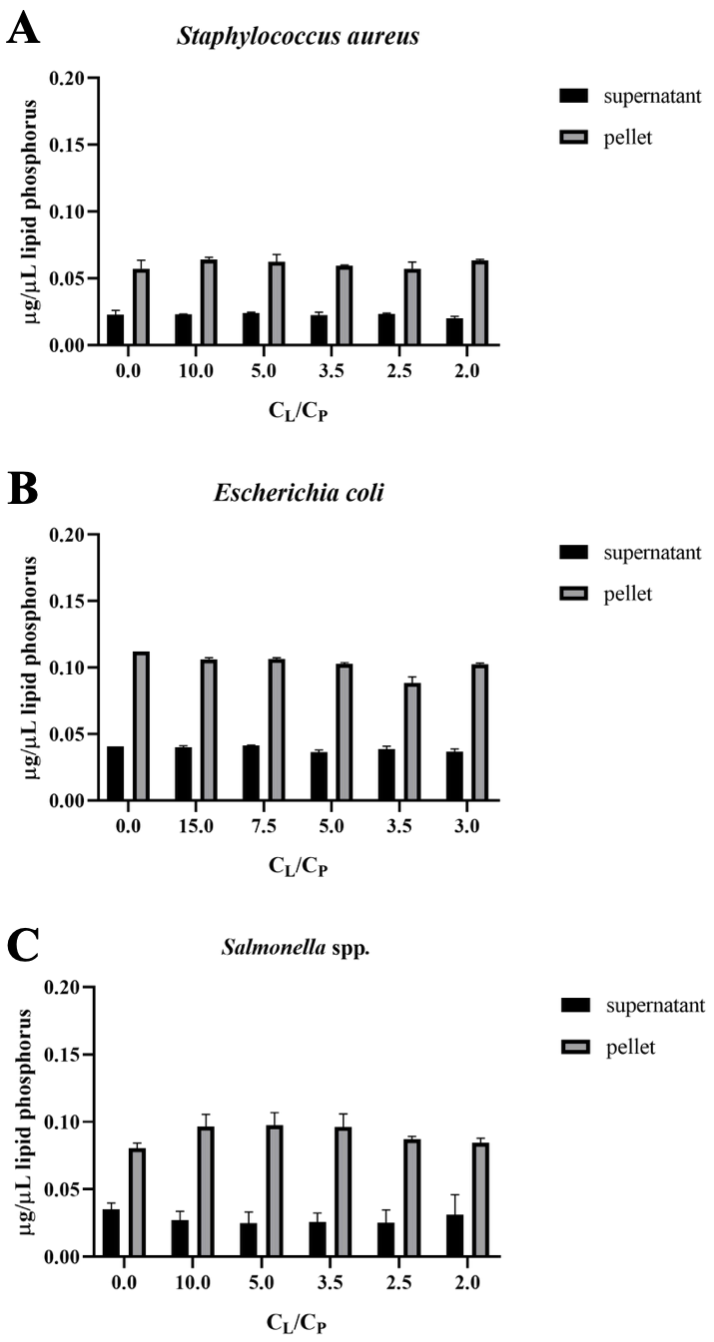


Figure 7.5 *Lipid phosphorus concentration in the supernatant and pellet after centrifugation of each lipidic sample subsequently incubation with several concentrations of peptide 1018-K6. Results are means of three independent experiments per bacterial membrane of S. aureus, E. coli, and Salmonella spp. (isolated from foods), and error bars represent the standard errors (sem).*

7.3.3 Fluorescence properties of 1018-K6 in aqueous and lipid solutions

The peptide 1018-K6 owes its fluorescence to the presence in its sequence of the Trp residue, which aromatic ring is sensitive to the environment. Hence, the intrinsic properties of steady-state fluorescence can provide information on the organization and position of peptides in the aqueous environment. For this reason, the titration of aqueous suspension of the 1018-K6 at 20 µM with increasing concentration of lipidic vesicles (ranged from 0 to 2mM) of MLVs allows recording information on the peptide partition into the membrane.

The 12-mer peptide emission spectra have a characteristic blue shift when titrated with bacterial membrane models (Figure 7.6). This typical shift is associated with the transition of Trp residue from

aqueous to more hydrophobic environment (Santos *et al.*, 2002). The $\Delta\lambda_{max}$ shift was greatest for the interaction of the peptide with the model membrane of *S. aureus* at a lipid phosphorus concentration of 1.8 mM with the specific maximum shift value of ~16.55 (Figure 7.7).

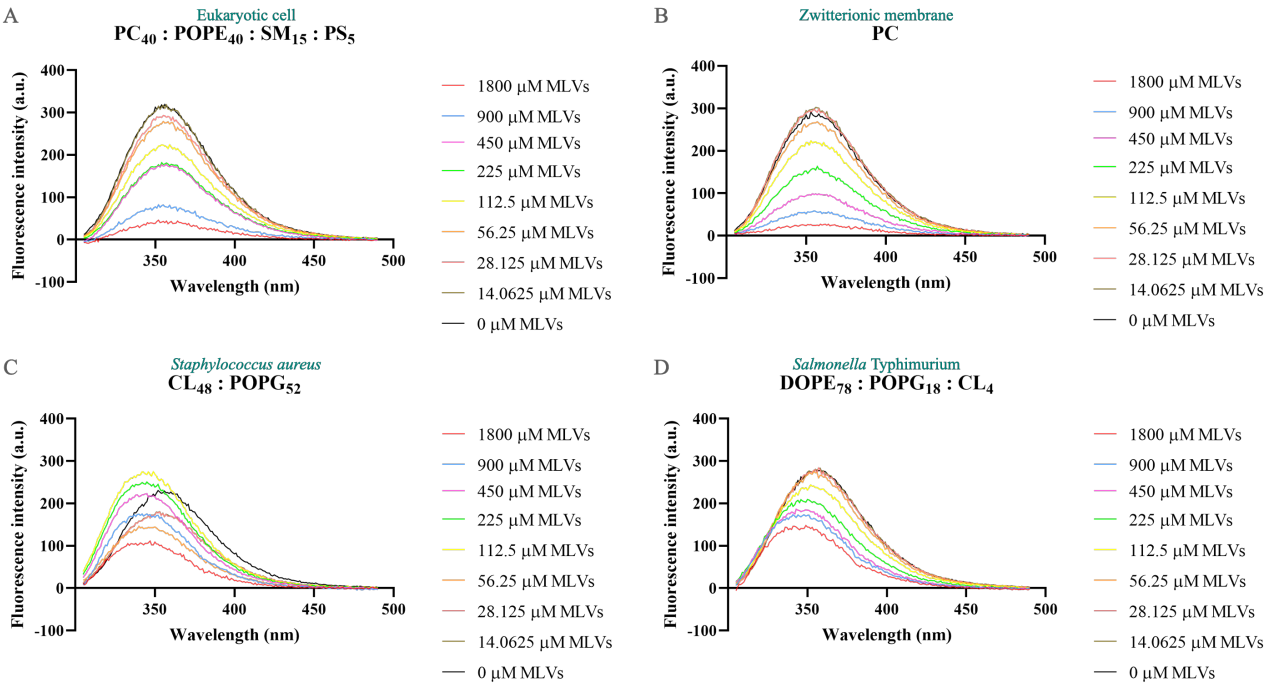


Figure 7.6 *Fluorescence emission spectra of 20 µM 1018-K6 in buffer before and after the addition of MLVs (up to 2 mM) of (**A**) eukaryotic cell, (**B**) zwitterionic lipids, (**C**) S. aureus, and (**D**) S. Typhimurium. Results are means of three independent experiments.*

The Figure 7.7 shows that no significant differences are found between the model membranes from Gram-positive and Gram-negative bacteria. However, significant differences among zwitterionic membranes and anionic ones were detected starting at a concentration of 0.225 mM of MLVs.

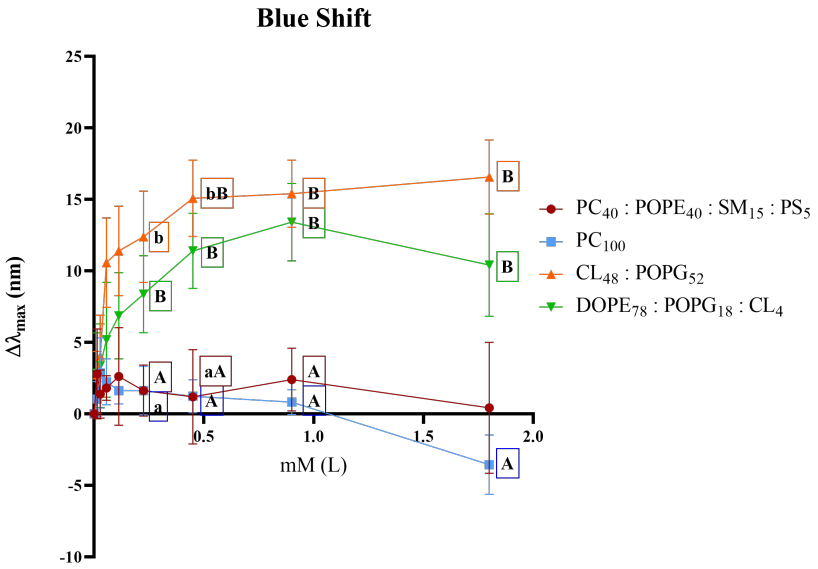


Figure 7.7 *$\Delta\lambda_{max}$ shift of 20 µM 1018-K6 in buffer before and after the addition of MLVs (up to 2 mM) of (**A**) eukaryotic cell, (**B**) zwitterionic lipids, (**C**) S. aureus, and (**D**) S. Typhimurium. Results are means of three independent experiments and error bars represent the standard errors (sem).*

Fluorescence-quenching experiments were performed using acrylamide quencher to detect the relative position of the 1018-K6 peptide in the lipid bilayer. Indeed, due to the incapability of the soluble quencher to enter into the lipidic bilayer, when the peptide is located in the membrane its Trp residue cannot be quenched. The quenching of the fluorophore is directly connected to the concentration of the added quencher and the exposure of Trp residue to the aqueous environment. Thus, only the 1018-K6 molecules not inserted into the membrane will be quenched by acrylamide, which includes free peptide molecules and peptide molecules adsorbed on the surface of the model membrane. In contrast, Trp residues from those 1018-K6 molecules inserted into the lipid bilayer will not be accessible to acrylamide and will still maintain fluorescence emission. To evaluate the relative position of the peptide in its interaction with membranes, the quenching of the fluorescence emission after titration with acrylamide was performed and *Stern-Volmer* constants (K_{sv}) were calculated (Lakowicz, 1999).

As shown in the Figure 7.8, changes in the fluorescence intensity of the solution peptide-MLVs spectra were recorded by increasing the concentration of the acrylamide. However, the fluorescence of the Trp rapidly decreased when the peptide has been surrounded by the Buffer or incubated in zwitterionic model membranes. Specifically, the fluorescence of free peptides in the buffer was quenched almost completely by adding only 0.02 mM of acrylamide, while twice the dose was needed to quench Trp emission of peptides incubated with eukaryotic cells membrane models. This finding suggests a location of the peptide on the lipid vesicle-water interface or the absence of AMP molecules-lipids interaction, conditions that allow the exposure of all Trp residues to the quencher.

In the same way, in PC membranes all peptides have Trp residues more accessible to the quencher than the other model membranes. In $DOPE_{78}$:$POPG_{18}$:CL_4 membranes, the quenching efficacy was very low, probably due to the deeper insertion of the peptides in the hydrophobic region of the phospholipid bilayer in only 30 minutes. A different condition has been described for the model membranes of *S. aureus* in which the peptide insertion appears to be slower than in those of Gram-negative bacteria. Tang *et al.* (2008) have already described the phenomenon, reporting that more than 20 minutes are needed to obtain the binding between a peptide and model membranes of *S. aureus* respect to *E. coli* model membranes. They underlined the importance of the membrane surface potential that could interfere with the permeation of the peptide into membrane of different lipid compositions.

The Stern–Volmer constants (K_{sv}) in the Tables 7.3 and 7.4 confirm the higher membrane-binding affinity of 1018-K6 with anionic membrane and better describe the case of *S. aureus* MLVs. Indeed, at 120 min the constant was reduced by more than 100 times compared to the first interval, reaching values similar to *S*. Typhimurium.

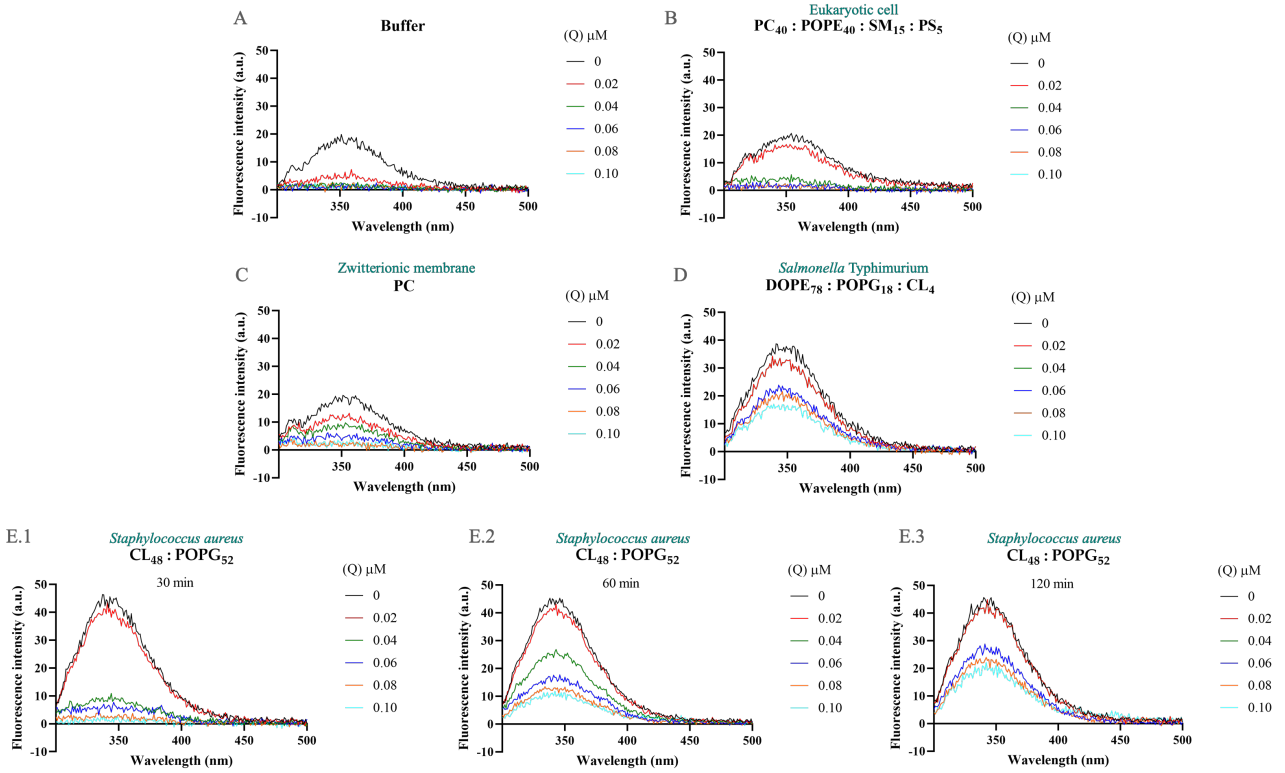


Figure 7.8 *Fluorescence spectra of 1 µM 1018-K6 before and after the addition of the acrylamide (up to 0.1 mM) in (**A**) buffer, (**B**) eukaryotic cell, (**C**) zwitterionic lipids, (**D**) S. Typhimurium and (**E**) S. aureus. Results are means of three independent experiments per each time of incubation peptide-lipidic solution (30-60-120 minutes).*

For many years the scientific community has been aware of the role of numerous physicochemical features of AMPs in determining their general activities. Molecule charge, length and amino acid composition of the sequence, hydrophobicity and amphipathicity are some of the main influencing specifications (Guilhelmelli *et al*., 2013; Auvynet and Rosenstein. 2009) of antimicrobial peptides. Torcato *et al*. (2013) attributed the binding affinity of their peptide for Gram-positive membranes to the presence of the arginine (Arg-R) in its amino acid sequence, pointing out the important role of the guanidinium side chain in the binding process. Authors suggested that this may be due to the strong ability of guanidinium group to create solid bidentate H-bond with phosphate moieties and cation–π interactions with aromatic residues (Gallivan and Dougherty, 1999). This hypothesis was based on the differences described among the model membranes of Gram-positive and Gram-negative bacteria. In particular, they compared the binding properties of three peptides that defer in their amino acid sequence. On one hand, the changes in sequence residues did not affect the affinity of peptides for Gram-negative membrane; on the other hand, the replacement of lysine (Lys-K) with Arg intensely improved the affinity for Gram-positive bacteria, marking the difference in action among peptides. Moreover, White and Wimley (1998) demonstrated that the addition of Trp in the peptide sequence confers greater affinity for lipid membrane, improving its insertion in the lipid core by inducing the penetration of Arg through cation–π interactions (Chan *et al*., 2006).
The peptide 1018-K6 has 4 Arg residues and 1 of Trp, positive charge and α-helix conformation, all positive features that could explain its high affinity for anionic membranes.

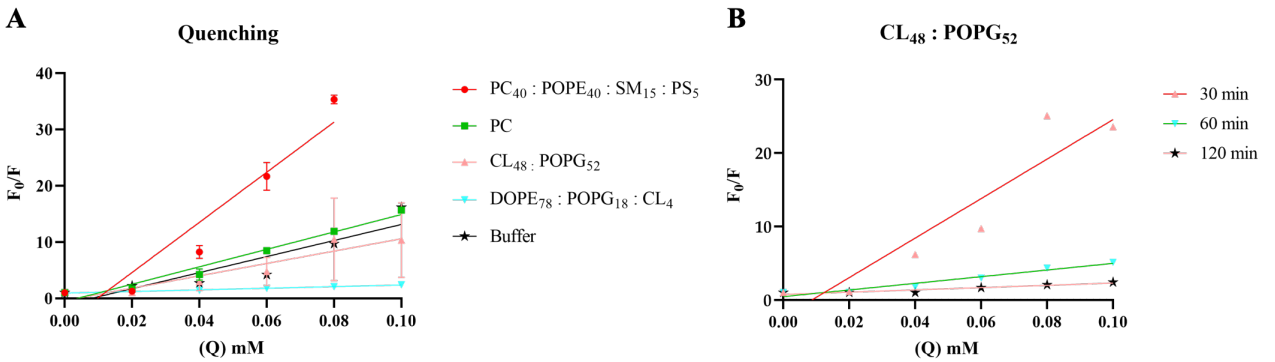


Figure 7.9 *The Stern-Volmer plots for the quenching of 1018-K6 with acrylamide at 280 nm, in (**A**) lipid solutions of all model membranes (eukaryotic cell (red), zwitterionic lipids (green), S. Typhimurium (light blue) and S. aureus (pink)) and buffer (black), and in (**B**) lipid solutions of membrane model of S. aureus. Results are means of three independent experiments per each time of incubation peptide-lipidic solution (30-60-120 minutes).*

Table 7.3 *Emission fluorescence quenching of 1018-K6 induced by acrylamide in presence of absence of MLVs. K_{SV} were calculated by fitting the Stern-Volmer equation.*

Cellular type	Lipid mixture	$K_{SV,\ acrylamide}$ M^{-1}	Equation	*R square*
Eukaryotic membrane	PC_{40}:$POPE_{40}$:SM_{15}:PS_5	445.4 ± 43.7^{aA}	Y = 445.4*X - 4.302	0.8887
Zwitterionic membrane	PC	154.8 ± 8.8^{bA}	Y = 154.8*X - 0.5730	0.9504
Staphylococcus aureus	CL_{48}:$POPG_{52}$	109.8 ± 44.6^{B}	Y = 109.8*X - 0.3776	0.2747
Salmonella Typhimurium	$DOPE_{78}$:$POPG_{18}$:CL_4	14.4 ± 0.9^{aB}	Y = 14.4*X + 0.9384	0.9333
	Buffer	142.7 ± 16.3^{b}	Y = 142.7*X - 1.124	0.8279

Data analyses were performed using GraphPad Prism®, version 8.0.1 (GraphPad, San Diego, California, USA). The results were analyzed with *t test*. All data were presented as the mean (M) ± standard error (sem). $^{A, B}$ values within column differ $p<0.01$; $^{a, b}$ values within row differ $p<0.05$.

Table 7.4 *Emission fluorescence quenching of 1018-K6 induced by acrylamide in presence of S. aureus model membranes at 30-60-120 minutes of peptides-lipids incubation. K_{SV} were calculated by fitting the Stern-Volmer equation.*

CL_{48}:$POPG_{52}$	$K_{SV,\ acrylamide}$ M^{-1}	Equation	*R square*
30 min	268.7 ± 50.8^{a}	Y = 268.7*X - 2.3330	0.8748
60 min	45.2 ± 5.1^{bA}	Y = 45.2*X + 0.4529	0.9509
120 min	15.5 ± 2.8^{bB}	Y = 15.5*X + 0.7478	0.8870

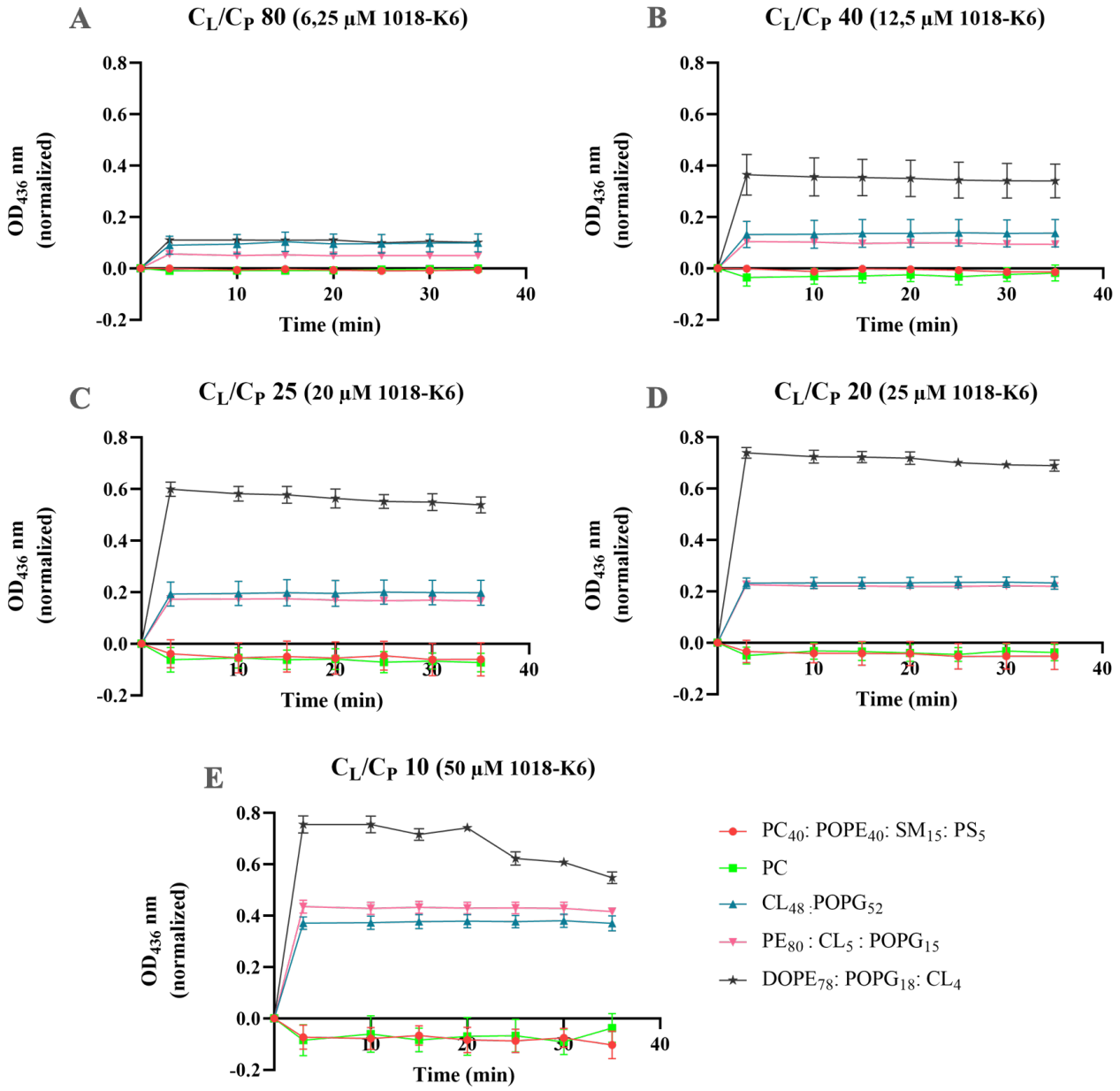
Data analyses were performed using GraphPad Prism®, version 8.0.1 (GraphPad, San Diego, California, USA). The results were analyzed with *t test*. All data were presented as the mean (M) ± standard error (sem). $^{A, B}$ values within column differ $p<0.01$; $^{a, b}$ values within row differ $p<0.05$.

7.3.4 Vesicle aggregation

The antimicrobial peptides could interact simultaneously with one or more vesicles, resulting in the formation of lipid vesicle aggregates. Normally, the lipid vesicles are dispersed in the solution due to the repulsive electrostatic forces. Their binding to cationic peptides could break this equilibrium, affecting the surface stability of membrane (Mulgrew-Nesbitt *et al.*, 2006) and reducing the repulsions among MLVs.

The peptide 1018-K6 has been shown to be capable to induce vesicle aggregation in all the model bacterial membranes, both Gram-negative and Gram-positive. Already at very low concentration (Figure 7.10A) the difference between zwitterionic and anionic vesicles is noticeable. Clearly this significant difference (Figure 7.10E) was higher with increasing peptide doses and decreasing the ratio C_L/C_P. Furthermore, in support of the findings discussed above, the peptide appears to prefer

more negatively charged membranes than the neutral ones and it shows a very high affinity for *S.* Typhimurium MLVs.



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		Fixed effect		
		Time (min)	Lipid Mixtures	Time x Lipid Mixtures
C_L/C_P 80	P value	0.0352	0.0878	0.0978
	p	*	ns	ns
C_L/C_P 40	P value	0.0364	0.0153	0.0192
	p	*	*	*
C_L/C_P 25	P value	0.0127	0.0268	0.0365
	p	*	*	*
C_L/C_P 20	P value	<0.0001	<0.0001	<0.0001
	p	****	****	****
C_L/C_P 15	P value	0.0049	0.0106	0.0082
	p	**	*	**

Figure 7.10 *Vesicle aggregation studies. OD_{346nm} was monitored for 35 minutes after addition of 6.25 (**A**), 12.5 (**B**), 20 (**C**), 25 (**D**) and 50 (**E**) μM of 1018-K6 to 500μM of MLVs of eukaryotic cell (red), zwitterionic membrane (green), S. aureus (blue), E. coli (pink) and S. Typhimurium (black). Anova two-way analysis was performed using GraphPad Prism 8.0.1 (**F**). Results are the mean of three independent experiments and error bars represent the standard errors (sem).*

7.4 References

Alatoom AA, Cunningham SA, Ihde SM, Mandrekar J, Patel R, 2011. Comparison of direct colony method versus extraction method for identification of gram-positive cocci by use of Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry. J cli microbiol 49(8), 2868–2873.

Alvarez R, Casas J, López DJ, Ibarguren M, Suari-Rivera A, Terés S, Guardiola-Serrano F, Lossos A, Busquets X, Kakhlon O, Escribá PV, 2017. Triacylglycerol mimetics regulate membrane interactions of glycogen branching enzyme: implications for therapy. J lipid res 58(8), 1598-1612.

Ambrosio RL, Gratino L, Mirino S, Cocca E, Pollio A, Anastasio A, Palmieri G, Balestrieri M, Genovese A, Gogliettino M, 2020. The bactericidal activity of protein extracts from Loranthus europaeus berries: a natural resource of bioactive compounds. Antibiotics 9(2), 47.

Auvynet C, Rosenstein Y, 2009. Multifunctional host defense peptides: antimicrobial peptides, the small yet big players in innate and adaptive immunity. FEBS J. 276:6497–6508.

Barbosa SC, Nobre TM, Volpati D, Cilli EM, Correa DS, Oliveira ON, 2019. The cyclic peptide labaditin does not alter the outer membrane integrity of Salmonella enterica serovar Typhimurium. Sci rep 9(1), 1-10.

Bartlett GR, 1959. Phosphorus assay in column chromatography. J Biol Chem 234: 466-468

Bello J, Bello HR, Granados E, 1982. Conformation and aggregation of melittin: dependence on pH and concentration. Biochemistry 21, 461-465.

Bessalle R, Haas H, Goria A, Shalit I, Fridkin M, 1992. Augmentation of the antibacterial activity of magainin by positive-charge chain extension. Antimicrob Agents Chemother 36, 313- 317.

Boggs JM, Stamp D, Hughes DW, Deber CM, 1981. Influence of ether linkage on the lamellar to hexagonal phase transition of ethanolamine phospholipids. Biochemistry, 20(20), 5728-5735.

Böttcher CJF, van Gent CM, Pries C, 1961. A rapid and sensitive sub-micro phosphorus determination. Analytica Chimica Acta 24: 203-204.

Casares D, Escribá PV, Rosselló CA, 2019. Membrane lipid composition: Effect on membrane and organelle structure, function and compartmentalization and therapeutic avenues. Int J Mol Sci 20(9), 2167.

Chan DI, Prenner EJ, Vogel HJ, Tryptophan- and arginine-rich antimicrobial pep- tides: structures and mechanisms of action, Biochim. Biophys. Acta 1758 (2006) 1184–1202.

Colagiorgi A, Festa R, Di Ciccio PA, Gogliettino M, Balestrieri M, Palmieri G, Anastasio A, Ianieri A, 2020. Rapid biofilm eradication of the antimicrobial peptide 1018-K6 against Staphylococcus aureus: A new potential tool to fight bacterial biofilms. Food Control 107, 106815.

Dathe M, Nikolenko H, Meyer J, Beyermann M, Bienert M, 2001. Optimization of the antimicrobial activity of magainin peptides by modification of charge. FEBS Lett 501, 146-150.

Dathe M, Wieprecht T, 1999. Structural features of helical antimicrobial peptides: their potential to modulate activity on model mem- branes and biological cells. Biochim Biophys Acta 1462, 71-87.

Dennison SR, Howe J, Morton LH, Brandenburg K, Harris F, Phoenix DA, 2006. Interactions of an anionic antimicrobial peptide with Staphylococcus aureus membranes. Biochem Biophys Res Commun 347(4), 1006-1010.

EFSA (European Food Safety Authority), 2008. Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal Escherichia coli and Enterococcus spp. from food animals. EFSA Journal 2008;6(4):141, 44 pp. https://doi.org/10.2903/j.efsa. 2008.141r

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2021. The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. *EFSA Journal*2021;19(4):6490, 179 pp.

Epand RM, Epand RF, 2011. Bacterial membrane lipids in the action of antimicrobial agents. J Pept 17(5):298-305.

Escriba PV, Ferrer-Montiel AV, Ferragut JA, Gonzalez-Ros JM, 1990. Role of membrane lipids in the interaction of daunomycin with plasma membranes from tumor cells: implications in drug-resistance phenomena. Biochemistry 29(31), 7275-7282.

Fiske CH, Subbarow I, 1925. Determination of Phosphorus. J Biol Chem 66: 375-400

Fox CF, Law JH, Tsukagoshi N, Wilson G, 1970. A density label for membranes. P Natl A Sci, 67(2), 598-605.

Gallivan JP, Dougherty DA, 1999. Cation-pi interactions in structural biology. Proc Natl Acad Sci U. S. A. 96 (1999) 9459–9464.

Giacometti J, Bursać Kovačević D, Putnik P, Gabrić D, Bilušić T, Krešić G, Stulić V, Barba FJ, Chemat F, Barbosa-Cánovas G, Režek Jambrak A, 2018. Extraction of bioactive compounds and essential oils from mediterranean herbs by conventional and green innovative techniques: A review. Food Res Int 113:245-262.

Guilhelmelli F, Vilela N, Albuquerque P, Derengowski Lda S, Silva-Pereira I, Kyaw CM, 2013. Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. Front Microbiol 4:353.

Hall K, Lee TH, Daly NL, Craik DJ, Aguilar MI, 2012. Gly6 of kalata B1 is critical for the selective binding to phosphatidylethanolamine membranes. Biochim Biophys Acta 1818, 2354-2361.

Hancock REW, Haney EF, Gill EE, 2016. The Immunology of Host Defence Peptides: Beyond Antimicrobial Activity Nat Rev Immunol 16, 321−334.

Henriques ST, Castanho MA, 2004. Consequences of nonlytic membrane perturbation to the translocation of the cell penetrating peptide pep-1 in lipidic vesicles. Biochemistry 43,9716–9724.

Huang HW, 2006. Molecular mechanism of antimicrobial peptides: the origin of cooperativity. Biochim Biophys Acta 1758, 1292- 1302.

Ito AS, Castrucci AML, Hruby VJ, Hadley ME, Krajcarski DT, Szabo AG, 1993. Structure – activity correlations of melanotropin peptides in model lipids by tryptophan fluorescence studies. Biochemistry 32, 12264 – 12272.

Jain MK, Rogers J, Simpson L, Gierasch LM, 1985. Effect of tryptophan derivatives on the phase properties of bilayers. Biochim Biophys Acta 816, 153 – 162.

Lakowicz JR, 1999. Principles of Fluorescence Spectroscopy, Kluwer Academic, New York.

Lee TH, Hall KN, Aguilar MI, 2016. Antimicrobial Peptide Structure and Mechanism of Action: A Focus on the Role of Membrane Structure. Curr Top Med Chem 16(1):25-39.

Mansour SC, de la Fuente-Núñez C, Hancock RE, 2015. Peptide IDR-1018: modulating the immune system and targeting bacterial biofilms to treat antibiotic-resistant bacterial infections. J Pept Sci 21(5):323-9.

Matsuno R, Ishihara K, 2011. Integrated Functional Nanocolloids Covered with Artificial Cell Membranes for Biomedical Applications. Nano Today 6, 61−74.

Matsuzaki K, Nakamura A, Murase O, Sugishita K, Fujii N, Miyajima K, 1997. Modulation of magainin 2-lipid bilayer interactions by peptide charge. Biochemistry 36, 2104-2111.

Melo MN, Ferre R, Castanho MA, 2009. Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations. Nat Rev Microbiol 7, 245-250.

Mulgrew-Nesbitt A, Diraviyam K, Wang J, Singh S, Murray P, Li Z, Rogers L, Mirkovic N, Murray D, 2006. The role of electrostatics in protein-membrane interactions. Biochim Biophys Acta 1761, 812–826.

Muzzarelli RAA, 2011. Formation and diagenesis. In Chitin nanostructures in living organisms (1st ed., pp. 1–34). Dordrecht, The Netherlands: Gupta, N., Ed.; Springe.

Palmieri G, Balestrieri M, Capuano F, Proroga YT, Pomilio F, Centorame P, Riccio A, Marrone R, Anastasio A, 2018a. Bactericidal and antibiofilm activity of bactenecin-derivative peptides against the food-pathogen Listeria monocytogenes: New perspectives for food processing industry. Int J Food Microbiol 279, 33–42.

Peruzy MF, Houf K, Joossens M, Yu Z, Proroga YTR, Murru N. Evaluation of microbial contamination of different pork carcass areas through culture-dependent and independent methods in small-scale slaughterhouses. Int J Food Microbiol 336:108902.

Petrlova J, Hansen FC, van der Plas MJA, Huber RG, Mörgelin M, Malmsten M, Bond PJ, Schmidtchen, A, 2017. Aggregation of Thrombin-Derived C-Terminal Fragments as a Previously Undisclosed Host Defense Mechanism. Proc. Natl. Acad. Sci. U. S. A. 114, E4213−E4222.

Robert E, Lefevre T, Fillion M, Martial B, Dionne J, Auger M, 2015. Mimicking and Understanding the Agglutination Effect of the Antimicrobial Peptide Thanatin Using Model Phospholipid Vesicles. Biochemistry 54, 3932−3941.

Roussel G, White SH, 2020. Binding of SecA ATPase monomers and dimers to lipid vesicles. Biochim Biophys Acta Biomembr. 1862(2):183112.

Santos NC, Castanho MARB, 2002. Fluorescence spectroscopy methodologies on the study of proteins and peptides. On the 150th anniversary of protein fluorescence. Trends Appl Spectrosc 4,113–125.

Santos NC, Prieto M, Castanho MA, 2003. Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1612(2), 123-135.

Scatchard G, 1949. The attractions of proteins for small molecules and ions. Ann N Y Acad Sci 51(4), 660-672.

Tang YL, Shi YH, Zhao W, Hao G, Le GW, 2008. Insertion mode of a novel anionic antimicrobial peptide MDpep5 (Val-Glu-Ser-Trp-Val) from Chinese traditional edible larvae of housefly and its effect on surface potential of bacterial membrane. Journal of pharmaceutical and biomedical analysis, 48(4), 1187-1194.

Torrent M, Pulido D, Nogueś MV, Boix E, 2012.ExploringNew Biological Functions of Amyloids: Bacteria Cell Agglutination Mediated by Host Protein Aggregation. PLoS Pathog 8, e1003005.

White SH, Wimley WC, 1998. Hydrophobic interactions of peptides with membrane interfaces, Biochim Biophys Acta 1376, 339–352.

White SH, Wimley WC, Ladokhin AS, 1998. Energetics of Peptide–Bilayer Interactions. Methods in enzymology 295, 62-87.

8. Conclusion

8.1 General conclusion

The food sector is facing new important challenges. Food loss and food waste as well as antimicrobial resistance (AMR) are recognized as the main issues that have affected the sector in the last decades. Although the connection between the food industry and the food losses is more intuitive, the spread in foods of pathogenic bacteria which are resistant to common antimicrobial compounds worries Food Business Operators. In fact, these pathogenic bacteria could be responsible for food spoilage as much as foodborne diseases. The absence of compounds capable of controlling and limiting the growth of resistant bacteria could affect the durability, in terms of shelf-life, and the food safety. The food industry needs to be prepared for these events, which inevitably impact on the food lost and affect the public health.

It is worth pointing out that the market is able to meet the exigences of the food industry by offering a selection of chemical compounds capable to control the common spoilage bacteria; however, FBOs have to deal with the rejection of chemical additives by the consumers. Still now, the prevention of foodstuff was mainly based on the application of chemical preservatives, whose adverse effects on human health have increased the demand for finding effective, healthy, safer and natural compounds (Bondi *et al*., 2017).

In this context, the plants and their products and the antimicrobial peptides (AMPs) are gaining a wide interest in the food industry. The antimicrobial compounds derived from plant organisms are sought-after for their potential as decontaminating agents and due to their classification as Generally Recognized as Safe (GRAS) (Delesa, 2018). AMPs have been demonstrated to be valid natural alternatives to conventional antibiotics to control and combat bacterial infections (Festa *et al*., 2021). To this purpose, the potential antimicrobial activities of yellow berries extracts, and small peptide have been investigated in Chapter 1 and in Chapter 2, respectively.

In Chapter 1, the antimicrobial activity of different protein extracts from *Loranthus europeaus* yellow berries was examined against fungal phytopathogens, Gram-positive and Gram-negative bacteria isolated from foods. The findings clearly revealed that the protein sample containing bioactive constituents exhibited a remarkable inhibitory activity against two Gram-positive bacteria, *L. monocytogenes* and the methicillin-resistant *S. aureus* M7 strain. In addition, a partially purification of this plant fruit extract allowed to identify at least two protein compounds responsible for the efficient bactericidal activity against *S. aureus* M7, which has been classified as the most sensitive bacterium.

To the best of our knowledge, this is the first study aimed at the identification of proteins with bactericidal activity in the fruits of *L. europeaus*. This work represents a pilot study and confirms that the traditional medicinal plants can be considered an important source of economic and safe alternative to treat human diseases.

In Chapter 2, we showed that the peptide 1018-K6 displayed a potent efficacy against wild and reference strains belonging to *Salmonella enterica* subsp. *enterica*, including pathogens found in fish and poultry processing plants. Specifically, results revealed that 1018-K6 was effective at low concentrations, both in planktonic cells and biofilms of all strains investigated, including the multi-drug resistance bacteria. Therefore, due to the wide susceptibility profile, 1018-K6 could be proposed as a promising candidate for developing bio-sanitizing formulations or active packaging, making it applicable at several points in the food chain. Indeed, the remarkable stability and the broad-spectrum activity of this peptide was previously demonstrated, even in strong alkaline and acid environments (Palmieri *et al*., 2018), thus suggesting its use in sanitizer formulations equipped with acid or alkaline chemical ingredients to improve food sanitation and safety. Hence, the peptide could help in preventing the bacterial contamination during the transformation and production phases, being able to act downstream and upstream of the food chain.

A good milestone has been reached by the scientific community with the creation of antimicrobial-active packaging by using natural antimicrobial compounds. The innovative packaging represents an emerging solution that can confer many preservation profits on many food products. Several authors (Agrillo *et al*., 2020; Gogliettino *et al*., 2020) have been proved the AMPs ability to bind to PET polymer materials. We had to prove their antimicrobial activity once bond to polymeric surfaces.
In Chapters 3 and 4, the antimicrobial activities of two antimicrobial peptides bond to PET polymer materials were evaluated. The devices were tested on three different food matrices and microbiological, chemical and organoleptic analyses were performed.
The Chapter 3 revealed that a 15-mer AMP could be covalently bound to the surface of PET materials, to produce highly stable antimicrobial packaging, which could be successfully used to improve the quality and safety of fresh products. The results obtained demonstrated the potential applicability of the MTP1-PET materials as not cytotoxic and safe active antimicrobial packaging through inhibition of the growth of spoilage microorganisms in relevant food model systems, such as the easily perishable ricotta cheese and fresh meat. The significant suggest a possible increase compared to the samples in contact with the control PETs. Although no significant variation was observed against yeast in meat samples and bacteria in ricotta cheese, we considered notable to underline the constant bacteria/yeast decrease in all samples stored on the PETs activated.
In the Chapter 4 was evaluated the antimicrobial activity of 1018-K6 bond to the surface of PET materials used to package fish burgers. Specific Spoilage Organisms (SSOs) were the bacterial targets of the innovative active packaging. The results showed an important antimicrobial activity of the 1018-K6 – PET, slowing down the growth of many bacteria responsible for deteriorative processes. Specifically, the finding is in agreement with *in vitro* studies which have demonstrated the efficacy of the free 1018-K6 of killing bacteria belonging to the Enterobacteriaceae family (Festa *et al*., 2021) and *Staphylococcus* genus (Colagiorgi *et a*l., 2020). Moreover, similarly to MTP1-PET (Gogliettino *et al*., 2020), the results pointed out the significant effect against the total bacteria counts, considered as indicators of the progress status of alteration. 1018-K6 – PET showed potent antibacterial and anti-adhesion properties, and ability to control the alteration processes in food matrices, probably by reducing the formations of microbiological metabolites that affect the odor and the general appearance of fish burgers.
The presented technology holds a great prospective for the development of highly effective bio-active and appealing packaging for food, preserving the microbiology quality and improving the safety of fresh products, with a concomitant extension of their shelf-life without adversely affect the organoleptic properties. However, long-term stability studies will be necessary in order to assure that the developed AMP-PETs maintain stable performances over longer storage periods, which constitute an essential requirement for an innovative food packaging.
Both these works represent a pilot study, which provides a starting point to evaluate further the real potential application of AMP- PETs as antimicrobial packaging in the food market. FBOs could meet the consumer's requests by offering natural antimicrobial compounds and, at same way, take part in the research of antibacterial molecules with low capacity to induce antimicrobial resistance. To this purpose, it is important to investigate the mechanism of action of these natural compounds.
The membrane permeability and destabilization have been previously published as the mechanism to describe the activity of the cationic AMPs, such as the peptide 1018-K6 investigated in Chapter 2, Chapter 4 and Chapter 5. Specifically, the extracellular membrane of Gram- negative bacteria comprises negatively charged LPS. The cationic AMPs can replace the divalent cations linked to this unique lipopolysaccharide, cause a breakage or a pore on the cell outer membranes and finally go through extracellular membranes, inducing their disintegration and cell death (Lei *et al*., 2019; Guo *et al*., 2018). It was previously demonstrated that the peptide 1018-K6 could possess a membrane destabilization and pore-forming activity against two different Gram-positive microorganisms (Palmieri *et al*., 2018; Colagiorgi *et al*., 2020). These hypotheses had to be confirmed by carrying out a specific study.

The Chapter 5 revealed that the antimicrobial activity of 1018-K6 towards both Gram-positive and Gram-negative bacteria appears to depend on the peptide affinity to bind the bacterial membranes. The results suggest a strong attraction between the cationic peptide and the anionic lipidic vesicles, typical of bacterial cells. It is worth underlining that whatever was the method of measuring the binding capacity, the 12-mer peptide appeared to prefer the mimetic membrane of *S*. Typhimurium, in which penetrate until 30 minutes of contact. Moreover, the binding experiment with bacterial membranes of *E. coli*, *S. aureus* and *Salmonella* spp. pointed out the higher affinity of peptide to bind to them than the mimetic ones. This finding provides important information of the peptide properties useful to design a novel peptide effective and capable of not activating bacterial defense mechanisms.

8.2 References

Agrillo B, Balestrieri M, Gogliettino M, Palmieri, G, Moretta R, Proroga YT, Rea I, Cornacchia A, Capuano F, Smaldone G, De Stefano L, 2019. Functionalized Polymeric Materials with Bio-Derived Antimicrobial Peptides for "Active" Packaging. Int J Mol Sci 20, 601.

Bondi M, Lauková A, de Niederhausern S, Messi P, Papadopoulou C, 2017. Natural Preservatives to Improve Food Quality and Safety. J Food Qual 2017, 3.

Colagiorgi A, Festa R, Di Ciccio PA, Gogliettino M, Balestrieri M, Palmieri G, Anastasio A, Ianieri A, 2020. Rapid biofilm eradication of the antimicrobial peptide 1018-K6 against Staphylococcus aureus: A new potential tool to fight bacterial biofilms. Food Control 107, 106815.

Delesa DA, 2018. Traditional Medicinal Plants for Industrial Application as Natural Food Preservatives. Int J Adv Res Biol Sci 5, 85–94.

Festa R, Ambrosio RL, Lamas A, Gratino L, Palmieri G, Franco CM, Cepeda A, Anastasio A, 2021. A Study on the Antimicrobial and Antibiofilm Peptide 1018-K6 as Potential Alternative to Antibiotics against Food-Pathogen *Salmonella enterica*. Foods 10(6):1372.

Gogliettino M, Balestrieri M, Ambrosio RL, Anastasio A, Smaldone G, Proroga YTR, Moretta R, Rea I, De Stefano L, Agrillo B, Palmieri G, 2020. Extending the Shelf-Life of Meat and Dairy Products via PET-Modified Packaging Activated With the Antimicrobial Peptide MTP1. Front Microbiol 10:2963.

Guo Y, Xun M, Han J, 2018. A bovine myeloid antimicrobial peptide (BMAP-28) and its analogs kill pan-drug-resistant acinetobacter baumannii by interacting with outer membrane protein A (OmpA). Medicine 97, e12832.

Lei J, Sun L, Huang S, Zhu C, Li P, He J, Mackey V, Coy DH, He Q, 2019. The antimicrobial peptides and their potential clinical applications. Am J Transl Res 11, 3919–3931.

Palmieri G, Balestrieri M, Capuano F, Proroga YT, Pomilio F, Centorame P, Riccio A, Marrone R, Anastasio A, 2018. Bactericidal and antibiofilm activity of bactenecin-derivative peptides against the food-pathogen Listeria monocytogenes: New perspectives for food processing industry. Int J Food Microbiol 279, 33–42.