
**UNIVERSITÀ DEGLI STUDI DI CAMERINO**

**School of Advanced Studies**

**DOCTORAL COURSE IN**

***Pharmaceutical Sciences***

**XXXIV cycle**

# STUDY OF NEW ANTIBACTERIAL PACKAGING SYSTEMS THROUGH THE MONITORING OF SHELF LIFE MARKERS

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# CHAPTER I

# GENERAL INTRODUCTION

## 1.1. Antibacterial packaging

### 1.1.1. Fundamental conception

With the improvement of living standards, the consumers are paying more and more attention to the food safety and corruption prevention functions of food packaging. **Food packaging** generally refers to a technology that protects food from harmful environmental disturbances, such as moisture, light, oxygen, dust, and chemical and microbial contamination (Vilela, et al., 2018). Food safety is the basic guarantee of people's health and has become a global issue in modern society. Microbial contamination may occur immediately after harvesting, and it is one of the biggest causes of food spoilage and food poisoning. As a result, there is an increasing demand for developing antimicrobial food packaging. **Antibacterial packaging** can inhibit the growth and reproduction of food microorganisms, and even kill them, thereby reducing food spoilage and waste, and prolonging the shelf life of food (Zhao, et al., 2022). **Food shelf life** refers to the period during which the product maintains its microbiological safety and sensory qualities at a specific storage temperature (Corradini, 2018). In addition, packaging also helps to ensure the acceptability of food by consumers before and after consumption among consumers (Piergiovanni & Limbo, 2019). During the shelf life period, a set of chemical, physical, and biological reactions occur in food matrix, leading to decreased safety and quality until the

product is considered unacceptable (Soro, et al., 2021). Contamination introduced during processing increases the microbial burden in food that favors the growth of many different microorganisms. As a consequence, high microorganisms load in meat and fish can manifest as mucus, as well as unwelcome colors, flavors, and odors (Soro, et al., 2021).

The reproduction of microorganisms is the most important factor in the deterioration of perishable food (COMA, 2008). Therefore, package with antimicrobial ability seems to be a strategy for controlling and preventing microbial contamination and proliferation, thereby maintaining the safety and quality of food during transport and storage. Antimicrobial materials are mainly bases of antibacterial packaging technology, which are primarily composed on substrate/matrix materials, supplemented by the addition of the antibacterial agent as the functional core (Liu, Jin, Coffin, & Hicks, 2009).

### 1.1.2. Construction of Antibacterial packaging systems

The antibacterial packaging system is generally composed by two parts: substrate/matrix material and antibacterial agent. Generally, antimicrobials are incorporated into packaging material through molding and fixing.

Substrate/matrix materials for developing packaging are synthetic petroleum-based polymers or biodegradable polymers (Pandey, Sharma, & Gundabala, 2022). The former is a classic packaging material with superior mechanical properties and barrier properties, but is not biodegradable. Petroleum-based packaging systems included polyethylene (PE), polyesters, conventional polyolefins, metals and paraffins. Biodegradable polymers compose natural and renewable polymers extracted from

plants, microorganisms or animals. The most extensively used biopolymers in food packaging industry are cellulose, chitosan, starch, gelatin, pectin, alginate, zein and soy protein, polyhydroxyalkanoates (PHA), and polylactic acid (PLA). Although environmentally friendly but exhibiting poor water vapor permeability and mechanical properties, plasticizers and/or micro or nano-fillers may be required (Pandey, Sharma, & Gundabala, 2022).

Antibacterial packaging technology is made by adsorbing antibacterial agents in the film by technologies such as surface modification, coating and direct incorporation (Mukurumbira, Shellie, Keast, Palombo, & Jadhav, 2022). It should be noted that the selection of the appropriate processing technology and active compound are essential to the final efficiency of the active packaging. Because of the high temperature encountered during thermal processing, temperature may negatively affect the packaging efficiency by inactivating heat sensitive agent such as bacteriocins, plant extracts and enzymes. To overcome this limit, methods, including microencapsulation technology (protection of active compounds) (Martins, Rodrigues, Barreiro, & Rodrigues, 2009), the addition of plasticizers (lower processing temperatures) (Liu, Jin, Coffin, & Hicks, 2009) and the use of suitable compounding methods, have been investigated. Studies have shown that the extrusion method is suitable for the production of PLA films containing heat-resistant substances such as metals and nanoparticles (Kuorwel, Cran, Sonneveld, Miltz, & Bigger, 2011); the solvent casting method is another appropriate method because it only requires a suitable solvent to dissolve the antibacterial agent; novel technology nanomaterials could not only improve the intrinsic properties of packaging, but also control the release of active agents, thus achieving extended food protection (Hardiansyah, Tanadi, Yang, & Liu, 2015).

In order to ensure the activity of antibacterial packaging during storage, attention should be paid to various factors that could potentially affect its efficiency. There are mainly four factors, including the properties of the active compound (thermal sensitivity, size, high volatility and compatibility with packaging materials), the incorporation method coating method showing limited interactions and potential effects, then the processing technique (shear force, acidity, high temperature), finally the food characteristics and storage environment (light, temperature fluctuations) that may affect the efficiency of antibacterial packaging (Sadaka, et al., 2014).

### 1.1.3. Antibacterial agents

The function of antibacterial packaging comes from the addition of inhibitors to the matrix include chemical sources, microbial, plants (Figure 1-1) (Liu, Jin, Coffin, & Hicks, 2009).

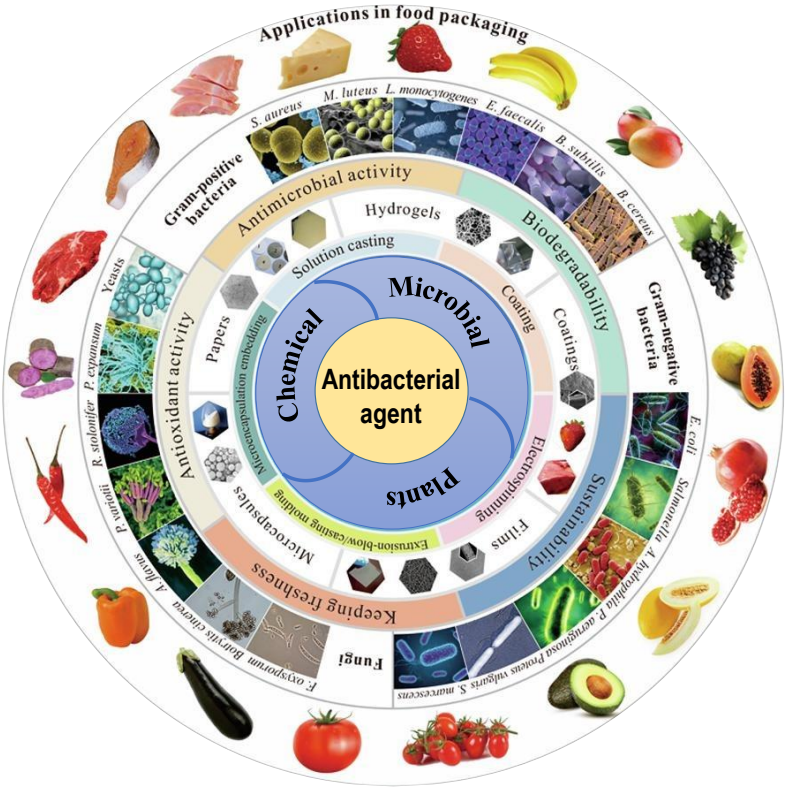



**Figure 1-1.** Schematic of antimicrobial packaging.

**(1) Chemical antibacterial agents**

At present, the most important commercial antibacterial material is the addition of chemical components as antibacterial agents. There are a variety of chemical antimicrobial agents, most of which are petroleum- or fossil-based organic and inorganic antimicrobials, such as traditional commercial halides, EDTA, nanocomposites, metal ions, metal oxides, graphene oxide, ethanol and quaternary ammonium salts (Zhao, et al., 2022). Early Antibacterial packaging also mostly fell into this category. For example, the antimicrobial wax coating on fruits and vegetables were composed by mixing wax with antibacterial agent or mixing the cellulose with sorbic acid and wax paper wrap sausage, etc., the antibacterial film with carboxymethyl cellulose as the carrier can reduce the moisture loss of fresh cut fruits, inhibit oxidation, delay maturity and inhibit the growth of microorganisms, and package fresh strawberries with potassium sorbate starch film to reduce the number of harmful microorganisms, will prolong the storage cycle, etc.

Nanoparticles (mainly silver, gold or metal oxide nanoparticles) are another popular chemical antibacterial agents due to their stability and antimicrobial capacity (Youssef, EL-Sayed, EL-Sayed, Salama, & Dufresne, 2016). The antimicrobial mechanism of silver nanoparticles is through damaging and penetrating bacterial cells, leading to cell leakage and DNA damage (Honarvar, Hadian, & Mashayekh, 2016).

Other chemical antibacterial agents include combination of ferrous carbonate or a mixture of ascorbic acid and citric acid act as carbon-dioxide emitters that suppress microbial growth leading to a longer shelf life of packaged food. Calcium alginate inhibits the growth of pathogenic bacteria in steak due to the presence of $CaCl_2$. Antimicrobial polymers copolymerized from acrylamide monomers can be used as

packaging materials for the preservation of fruits and vegetables. In addition, polymers containing guanidine groups are also antibacterial (Suppakul, Miltz, Sonneveld, & Bigger, 2003).

**(2) Microbial antibacterial agents**

Commonly used Microbial antibacterial agents are peptides, bacteriocins, enzymes, organic acids, polyamines and polysaccharide.

Antimicrobial peptides (AMPs) are the most commonly used inhibitory substances in antibacterial packaging, encapsulating them in various nanocarriers to ensure their controlled release for food preservation during storage. In recent years, AMPs have received increasing attention as potential ingredients in packaging as they exhibit a wide range of antibacterial and immunomodulatory activities against foodborne pathogens and infectious bacteria. Since antimicrobial agents are generally heat-sensitive, they can be added only after the packaging material is formed. For example, Nisin-methylcellulose coating film on a polypropylene film material, Nisin adsorbed on the functionalized silicon surface and polyethylene (PE), ethylene-vinyl acetate copolymer (EVA), Polypropylene (PP), Poly (Ethylene Terephthalare) (PET), Polyvinylchlorid (PVC) (Liu, Sameen, Ahmed, Dai, & Qin, 2021).

Polysaccharides are the most popular basic packaging material, due to their richness and useful physico-chemical properties. Polysaccharides are carbohydrates in which carrageenan, starch, cellulose, hemicellulose, alginate, chitosan are widely used for packaging purposes. Chitosan is widely used as an edible coating material due to its non-toxic and biodegradable properties. Chitosan fights a variety of Gram-positive and Gram-negative bacteria (Pandey, Sharma, & Gundabala, 2022).

**(3) Plants antibacterial agents**

Commonly used plant antibacterial agents are essential oils and extracts. Studies

have shown that essential oils demonstrate a broad inhibitory spectrum (bacteria, fungi and viruses). Gram-positive bacteria are more susceptible to the effects of essential oils than Gram-negative bacteria, and rod-shaped bacteria are more susceptible than coccidioides. The antibacterial properties of some essential oils have replaced traditional antimicrobial agents. The antibacterial mechanism of essential oils is mainly attributed to its lipophilicity, and bacterial cell membranes are the main target site, which can also cause degradation of bacterial cell walls, reduced levels of cellular energy synthesis, and decreased proton motility (Mukurumbira, Shellie, Keast, Palombo, & Jadhav, 2022).

## 1.2. Lactic acid bacteria and bacterial metabolites as bacteriostasis

### 1.2.1. Lactic acid bacteria as bacteriostasis

Lactic acid bacteria (LAB) are spore free and Gram-positive bacteria whose main product of fermentation sugars is lactic acid (Paul, 2017). LAB are a heterogeneous group of microorganisms commonly found in various foods and human bodily sites. LAB are widely used in food, mainly involving *Lactobacillus, Streptococcus, Aerococcus*, *Leuconostoc, Pediococcus* (Gopal, 2022), usually used as a starter for fermented foods, as an auxiliary additive for the production of additional flavor or to improve taste, as a bacteriostatic agent for maintaining food safety (Bintsis, 2018).

LAB is widely used for the production of fermented foods and is also part of intestinal microflora. Recently, the bioconcretion of LAB and their metabolites have become a focus of attention because they can improve the safety of various foods and

extend their shelf life (Kuley, et al., 2021). These bacteria have a long history of food application. They produce some antagonistic compounds that are able to control pathogenic bacteria and undesirable spoilage microflora (Dalie, Deschamps, & Richard-Forget, 2010). Antagonistic compounds have both a positive effect on host metabolism and an inhibitory effect on pathogens. These bacteriostatic substances can inhibit the growth and reproduction of spoilage bacteria and foodborne pathogenic bacteria in food, but they themselves will not cause damage to the human body.

**(1) Mechanisms of LAB action**

Bacterial antagonism or antimicrobial activity mechanisms of probiotics play a major role in the equilibrium between competing beneficial and potentially pathogenic microorganisms (Balcazar, Vendrell, Blas, Ruiz-Zarzuela, & Muzquiz, 2004). Many LAB strains have probiotic properties with well-characterized genomes and secretory systems (Huang, Nzekoue, Silvi, Sagratini, & Verdenelli, 2020). Probiotic microorganisms apply this mechanism by secreting a variety of antimicrobial compounds, including organic acids, hydrogen peroxide, bacteriocins, and biosurfactants, which can inhibit the growth of pathogenic bacteria. However, bacteriocins have stronger antimicrobial activity against pathogens under acidic conditions (Kanmani, et al., 2013). For example, the probiotic bacterium *Lactobacillus reuteri* releases an antimicrobial agent, reuterin, that has broad-spectrum activity against a variety of pathogens, including bacteria, fungi, protozoa, and viruses (Spinler, et al., 2008). By releasing antimicrobial compounds, probiotics can suppress the growth of foodborne pathogens, and may also reduce the biofilm development by pathogens and defend against infection.

The production of organic acids lowered the surrounding pH value, thus creating unfavourable growth environment for spoilage bacteria (Paul, 2017). LAB are tolerant

to low temperatures and oxygen levels under storage conditions, which is another technical advantage as a food bacteriostatic agent. In addition, certain LAB strains can secrete active antimicrobial compounds which showed high efficacy and broad-spectrum activity (Arrioja-Breton, Mani-Lopez, Palou, & Lopez-Malo, 2020), considering as ideal antibacterial agents to be applied in food packaging materials (Huang, et al., 2020).

**(2) Health benefits of LAB**

The healthy benefits of LAB are mainly reflected in their antibacterial, antioxidant and immunoprotective abilities. The prebiotic properties of LAB are that organic acids, special enzyme lines, acid bacteria and other substances produced through fermentation have special physiological functions. The immunomodulatory effects driven by LAB regulate not only intestinal responses, but also systemic responses in the body, which explains their role in influencing the prognosis of non-congenital diseases (Zhao, Liu, Kwok, Cai, & Zhang, 2020). A good probiotic candidate must fulfill the following criteria: it must be an organism that is capable of exerting a beneficial effect on the hosts and resistance to disease; it must be nonpathogenic and non-toxic; it must be capable of surviving and metabolizing in the gut environment by resisting the low pH of the stomach, organic acids, bile acid and enzymes present in the intestines; and it should be stable under storage and field conditions. At present, the mechanism of antibacterial properties of LAB is gradually clear, and its health-related benefits and the potential for food preservation have promoted the application of its live bacteria or its metabolites as bacteriostatic agents to prolong the shelf life of food (Hossain, Sadekuzzaman, & Ha, 2017).

### 1.2.2. LAB metabolites as bacteriostasis

LAB exert antagonism mainly through cell-to-cell competition and the production of active metabolites. Depending on the bacteria species and available substrates, LAB fermentation produces various valuable metabolites. As of now, most of the identified antibacterial substances are low molecular weight compounds consisting of hydrogen peroxide, exopolysaccharides (EPSs), organic acids, short-chain fatty acids, biosurfactants, ethanol, bacteriocins and bacteriocin-like substances (Barzegari, Kheyrolahzadeh, Khatibi, Sharifi, & Vahed, 2020). Cellular metabolites of LAB are often used as bacteriostatic agents in food, including heterogeneous mixtures of multiple products-cell-free supernatants, or purified forms of antioxidant enzymes, bacteriocins, extracellular polysaccharides, etc (Mora-Villalobos, Montero-Zamora, Barboza, Rojas-Garbanzo, & JP, 2020).

#### 1.4.2.1. Cell-free supernatant of LAB

Cell-free supernatants (CFS) are liquid broth with exometabolites produced by bacteria after culture. The major bioactive exometabolites secreted by Lactobacillus spp. are hexadecanoic acid methyl ester, phenol, 6-octadecenoic acid methyl ester, hydrocarbons, and 2,4-bis(1,1-dimethylethyl). Direct application of LAB CFS was effective in controlling the growth of undesirable bacteria in various foods, including chicken breast, beef, bread, and cheese (Arrioja-Breton, Mani-Lopez, Palou, & Lopez-Malo, 2020). The CFS of *Lactiplantibacillus plantarum* is characterized by valeric acid, acetic acid, alcohol, furanone and maltol derivatives with antimicrobial properties and has been found to be effective against the fish spoilage bacteria tested (Kuley, et al., 2021). Although *Lactobacillus* CFS has antibacterial effect on common food pathogens and spoilage species on the agar surface (George-Okafor, Ozoani,

Tasie, & Mba-Omeje, 2020), the complexity of the food matrix hampers the efficiency of CFS, and thus higher amount of CFS is required to achieve the same efficacy on food models (Moradi, Kousheh, Almasi, Alizadeh, & Lotfi, 2020). In addition, CFS also possesses properties - antioxidant, biofilm scavenging, and immunomodulatory - that contribute beneficial roles in food preservation and functions (Barzegari, Kheyrolahzadeh, Khatibi, Sharifi, & Vahed, 2020). Although the majority of studies have demonstrated the preservation ability of probiotic strains and CFS in the form of food additives (Yong, Hamidon, Rajangan, Soh, & Min, 2016), the application of CFS as part of packaging system remain scarce. Reasons at the top of the list include its sensitivity to alkaline conditions (the production of organic acids) and strong color (normally prepared in broth medium). Another limitation is CFS generally contains multiple metabolites that need to be further identified and characterized in order to better understand its mechanisms and efficacy.

#### 1.4.2.2. Exopolysaccharides of LAB

The Lactic acid bacterial exopolysaccharides (LAB EPS) is a class of carbohydrate secreted by LAB in the cell wall during growth and metabolism, either loosely bound to the cell surface or released into the surrounding environment during growth (Tao, et al., 2022). EPS is the product of bacterial adaptation to the environment, with the aim of forming biofilm structures that facilitate population sensing between species and gene exchange, protecting the bacterial community from adverse conditions and antibiotics (Zannini, Waters, Coffey, & Arendt, 2016). EPS is commonly used as an additive by the food industry due to its abilities to retain moisture and improve the rheological properties of food and beverages.

EPS produced by LAB have recently received increasing attention because of their health benefits to the consumers (Mejia-Gomez & Balczar, 2020). LAB are

generally recognized as safe microorganisms and produce EPS with a wide diversity of structures with no health risk (Thummar & Ramani, 2016). EPS produced by LAB possess various potential health benefits and have important functional roles in human or animal health including immunomodulatory properties, anti-cancer, antioxidant activity, anti-ulcer, anti-biofilm agents to prevent adhesion of pathogenic bacteria, blood glucose and cholesterol lowering properties, and antihypertensive activity (Tao, et al., 2022). Although some EPS form biofilms that cause hygiene problems, other EPS derived from LAB play a crucial role in improving the rheology, texture, and mouthfeel of fermented food formulations in the food industry (Lee, Kim, Jung, Park, & Kim, 2011). However, the extraction and purification process of EPS is complex, the yield is low, and the instability under storage conditions, sensitivity to different food compounds, inhibition spectra, etc. may weaken the preservation effect. Therefore, food safety control in combination with other hurdle techniques, such as antibacterial packaging, is considered a good strategy to improve its effectiveness and preservation effectiveness (Silva, Silva, & Ribeiro, 2018).

#### 1.4.2.3. Bacteriocins of LAB

Bacteriocins are small antimicrobial peptides synthesized by ribosomes that are naturally produced by a variety of bacteria capable of inhibiting growth and/or directly killing the target organism in a specific way (Goldbeck, et al., 2021). Bacteriocins can help the producer organism to outcompete other bacterial species. Although bacteriocins are produced by many microbial species, the bacteriocins produced by lactic acid bacteria (LAB) are the most studied and well-known group (Punia Bangar, Chaudhary, Singh, & Ozogul, 2022). Bacteriocin obtained from LAB has sparked a lot of interest in research fraternity owing to its capability of acting as

natural antimicrobial preservative in food (van Heel, Montalban-Lopez, & Kuipers, 2011). Although bacteriocins have been widely tested in foods such as seafood, meat, beverages, and dairy, their protein properties make their activity susceptible to proteolytic enzymes in foods, which may limit the efficiency of bacteriocin preservation. To date, the most studied and widely used bacteriocins of LAB are nisin, and pediocin.

**(1) Nisin**

Nisin is a member of class I bacteriocins produced by *Lactococcus lactis*, which is characterized by extensive antibacterial activity against Gram-positive bacteria, namely *Staphylococcus aureus* and *Listeria monocytogenes*. This characteristic, together with its low toxicity and safety in food, have contributed to the success of nisin as a natural food preservative in the world (Malaczewska & Kaczorek-Lukowska, 2021). The main antimicrobial mechanism of nisin is the pore-formation on cell membranes and the destruction of cell wall synthesis (Cavera, Arthur, Kashtanov, & Chikindas, 2015). The World Health Organization, United States Food and Drug Administration and the European Union have approved nisin as a food preservative (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016).

**(2) Pediocin**

Pediocins belong to the pediocin family of class IIa bacteriocins, is thermostable and pH-resistant bacteriocin, produced by LAB of *Pediococcus* sp. and *Lactobacillus* sp. species and genes involved in production and immunity of naturally occurring bacteria have been identified (Goldbeck, et al., 2021). Pediocin is effective against most studied spoilage species and food pathogens, especially *Listeria monocytogenes*, such as PA-1 also called Listeria-active bacteriocins (Pisoschi, et al., 2018). The main inhibition mechanisms of pediocin are through binding to cell

membrane, forming pores and leading to cell leakage and death (Villalobos-Delgado, Nevarez-Moorillon, Caro, Quinto, & Mateo, 2019). The main bottleneck preventing new pediocins from entering the market and preventing their clinical applications is the simple and cost-effective production and purification of industrially relevant titers (Goldbeck, et al., 2021).

However, compared to antibiotics, there are still fewer bacteriocins to be marketed and approved for use in food preservation and treatment for infectious diseases by the FDA and WHO (Punia Bangar, Chaudhary, Singh, & Ozogul, 2022). This may be due to the lack of clinical trials of bacteriocins in humans or animals. In order to validate bacteriocins inviolability in vitro and in vivo toxicity and the immunogenicity must be investigated. Therefore, before using bacteriocins in the food, pharmaceutical or veterinary industries, diverse tests and their interaction with other food constituents must be carried out to ensure their safety. Furthermore, the development of bacterial resistance to bacteriocin after repeated administration must be assessed. Further studies are necessary to characterize potent bacteriocins thoroughly and clinically test them.

In summary, LAB cells and their metabolites have great potential as food bacteriostatic agents to maintain food quality and prevent food spoilage. However, there is less research on the direct use of cell and their metabolites of LAB in food packaging, requiring a careful assessment of the effect of food matrix and storage conditions on the active compounds, as well as a thorough understanding of issues related to added amount, stability and safety of additions.

## 1.3. GRAPHENE AND FOOD APPLICATIONS

### 1.3.1. Graphene antibacterial properties

Since 2004, graphene has held a special position in nanotechnology, which has increased its functionality in various fields. Many efforts have been made to fabricate graphene on a large scale for use in several industries, especially the modern composite industry (Azizi-Lalabadi & Jafari, 2021). Graphene is fabricated from a single layer of graphite in hexagonal rings with $sp^2$-hybridized carbons and sigma connections. Moreover, the remaining p-orbitals form delocalized π-type bonds (Alam & Moussa, 2020). Over the past few years, various methods for synthesizing graphene have been developed, including top-down and bottom-up methods. Top-down methods can be synthesized from graphitic sources via chemical exfoliation, micromechanical cleavage, reduction of graphite oxide. Bottom-up methods can be obtained from chemical vapor deposition and non-graphite sources by epitaxial growth (Gurunlu, Tasdelen Yucedag, Bayramoglu, & Munroe, 2020). Graphene particles can form graphene derivatives by chemical modification, such as graphene chemical oxidation and micro-mechanical stripping (Dreyer, Park, Bielawski, & Ruoff, 2010) or reduction of graphene oxides to form graphene oxides. Graphene has a unique high surface area, excellent electron mobility, thermal conductivity, flexibility and optical properties. All of these properties make graphene an ideal material for manufacturing and designing a variety of devices, such as biosensors, electronic parts, biomedical instruments, transistors (Bhuyan, Uddin, Islam, Bipasha, & Hossain, 2016).

One of the most attractive features of graphene composites is their antimicrobial

properties, as the main inhibition mechanism is through physical interactions that are less likely to cause resistance. With the growing interest in the antimicrobial mechanisms of graphene-based antimicrobials and publications, researchers use a variety of instruments to visualize graphene's interactions with microorganisms, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to visualize morphological changes after interactions (Dreyer, Park, Bielawski, & Ruoff, 2010). Graphene composites showed a relatively wide antimicrobial profile, which has inhibitory effects on *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Streptococcus mutans, Candida albicans, Klebsiella pneumoniae* and *Porphyromonas gingivalis* (Xia, et al., 2019) (Figure 1-2). Their results showed that graphene oxide had good antibacterial activity (time dependence) against *Staphylococcus aureus*, while antibacterial activity against *Pseudomonas aeruginosa* was weak and transient, and effectiveness against *Candida albicans* was observed late in growth (24 h). In most studies, the bactericidal activity of graphene polymer membranes against *Staphylococcus aureus* was higher than that of *Escherichia coli* because of the attachment and accessibility of complex cell structure inhibiting complexes of Gram-negative bacteria (Al-Jumaili, Alancherry, Bazaka, & Jacob, 2017). In addition, graphene composites can also active against mycelium synthesis and protozoans by oxidative stress, inhibition of fungi by ultrastructural changes (Xie, et al., 2016).

Recently, with the harm caused by the coronavirus worldwide, the antiviral properties of graphene-based materials against this epidemic have been widely studied and concerned. It was found that graphene composites can interact with negative groups on the surface of the virus, resulting in physical damage and local rupture of the virus. Therefore, the number of negatively charged groups on the virus and the

sharpness of the graphene composite are decisive factors in its final efficiency (Srivastava, et al., 2020).

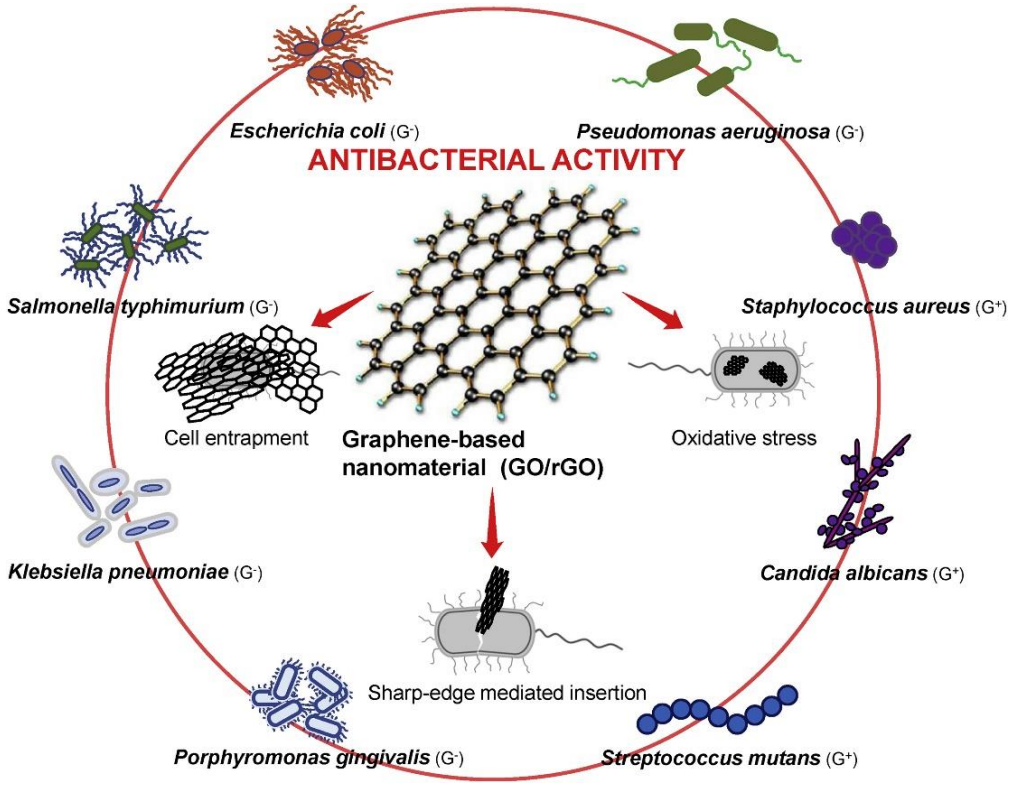



**Figure 1-2.** Multiple antibacterial mechanisms of graphene-based nanomaterials against various pathogens (Xia, et al., 2019).

In short, the main bactericidal effects of graphene can be summarized as follows: ① the sharp edges of graphene composites destroy cell membranes; ② induce reactive oxygen species production and oxidative stress; ③ deprive bacterial membranes of electrons and cause loss of integrity; ④ encapsulate cells, cells are separated from air and nutrients; ⑤ interferes with bacterial DNA replication (Salomoni, Leo, Montemor, Rinaldi, & Rodrigues, 2017; Webster, 2010).

### 1.3.2. Discussion on bacteriostatic properties of blended graphene-polymers

Graphene composites have excellent mechanical, optical, thermal and antimicrobial properties and are ideal fillers for the preparation of antibacterial food packaging films. Currently, common polymers used to blend with graphene composites with strong antibacterial properties include polylactic acid (PLA), polyvinyl alcohol (PVA), polyethylene carbazole (PVK), polyamide (PA), polyalrylamine hydrochloride (PAH), polyurethane (PU), polysulfone (PSU), polyvinylidene fluoride (PVDF) and polydopamin (PDA) (Khandelwal, Kumar, & Kumar, 2019). The functional groups present on polymer and graphene composites can interact, affecting the overall properties of the graphene-polymer mixture. Polymers prevent agglomeration/self-assembly of graphene composites, while graphene composites can enhance the physicochemical and functional properties of polymer-based materials. In addition, blended graphene-polymers showed better antibacterial ability and less cytotoxicity compared to using graphene composites alone (Arriagada, Palza, Palma, Flores, & Caviedes, 2018).

The addition of graphene composites improved mechanical properties, gas impermeability, stiffness and strength of graphene PLA membranes compared to pure PLA membranes (Pinto, Carotenuto, & Meo, 2015). Some authors believe that the improvement in performance was attributed to the covalent and nonvalent interactions between the functional groups of graphene nanocomposites and the polymer matrix (Allahbakhsh, 2017). The addition of graphene nanosheets improves the thermal, toughness and strength properties of plasticized PLA films. Active graphene- PLA film also successfully inhibited the proliferation of *Staphylococcus aureus*, *E. coli,*

*Listeria monocytogenes* and *Salmonella typhimurium* as the high aspect ratio of nanocomposites inhibits inactive microorganisms (Chieng, et al., 2015). In another study, although the graphene nanoplatelet-modified PLA-based film was inactive to the growth of gram-positive bacteria (*Micrococcus luteus*) by agar diffusion test, the graphene nanoplatelets achieved sustained release of antibiotics and improved the stiffness and elastic modulus of the PLA film (Scaffaro, Botta, Maio, Mistretta, & La Mantia, 2016).

In summary, the factors that affect the final antimicrobial efficiency of graphene matrix composites include: ① the inherent properties of the composite material - particle size, carbon radical density, layer, arrangement, functional group (oxygenated) intermolecular interactions, concentration, compatibility, dispersibility, processing methods and distribution in different substrates; ② target microbial species, membrane characteristics and growth stage (Wang, Hu, & Shao, 2017).

In order to improve the performance of graphene composites and enhance their antibacterial efficiency, titanium dioxide, metal nanoparticles-silver, zinc oxide, gold, copper are used to form graphene metal composites or graphene metal oxide nanoparticles (Mohammed, et al., 2020). $TiO_2$ is a suitable candidate for graphene functionalization because its high photocatalytic activity can the ROS generation performance of graphene composites, thereby improving antibacterial properties. In addition, $TiO_2$ is an economical material with low toxicity and high stability, but it is less reactive under visible light, and the modification of graphene composites improves the photocatalytic efficiency in visible light, thereby improving antibacterial activity (Kandasamy, Selvaraj, Kumarappan, & Murugesan, 2022). However, the ratio of graphene to metal in composites is decisive for final efficiency, as composites have

less functional groups and unbalanced proportions can lead to agglomeration or weakening of functionality. Moreover, to enhance antibacterial ability, organic molecules can also be combined with graphene composites. Bacteriocin (nisin), protein-lactoferrin, chitosan and Enzyme (lysozymes) (Yuan, Zhu, Zhang, Jiang, & Ma, 2011), have been used in functional graphene-based materials that have improved bacteriostatic or bactericidal effects on *Staphylococcus aureus, E. coli* and *Pseudomonas species* (Duan, Wang, Zhang, & Liu, 2015).

## 1.4. FRESHNESS MARKERS

### 1.4.1. General remarks

Microorganisms proliferate, consume nutrients, and produce many undesired metabolites, which are the main reason for the spoilage of food. Detecting microbial activity during food safety monitoring is critical. Because of the complexity of the food matrix, special chemical and biological markers can be screened for analysis to determine the activity of microorganisms in the food matrices (Jayan, Pu, & Sun, 2020). Over the past few decades, a variety of recognition elements and detection technologies have been developed for detecting microbial activity in food systems. While many methods for microbial activity detection have been developed, the complexity of various food systems leads to great differences in the detection environment, which hinders great sensitivity and specificity of the detection (Pinto, et al., 2021).

Nowadays, the increasing attention to foodborne epidemics requires on-site, sensitive and rapid methods to detect microorganisms in food matrix (Jayan, Pu, & Sun, 2020). The most commonly used methods for microbial identification and

quantification are microscopy, plate culture colony counting, polymerase chain reaction (PCR), and immunological analysis.

Plate culture colony counting is classic but time-consuming and labor-intensive. Microscopic analysis has great limitations to only observing larger cells, is more demanding on the sample, and is not accurate in species identification. The PCR method is specific and sensitive as a molecular method, but there are false positive problems and quantitative detection difficulties. Improved real-time PCR methods may be more specific, but the processing steps are more complex and require prior sequence data for specific target genes (Smith & Osborn, 2009). Immunological analysis is rapidly evolving due to their speed, precise quantitative target organisms, and high throughput capabilities, including enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), enzyme-linked fluorescence assays (ELFA), flow-based immunoassays, and a few other serological assays (Alahi & Mukhopadhyay, 2017). However, these methods lack selectivity and sensitivity for samples, and the high price of consumables and the need for skilled personnel are not widely used in all fields (Wang, Sun, Pu, & Wei, 2019).

As an alternative, biometric technology with multiple biometric elements has been shown to be applied to food matrices to meet the requirements of rapid monitoring in the field and ensure food safety (Umesha & Manukumar, 2018). The most commonly used is to analyze microbial activity in food by detecting specific biomarkers contained by microorganisms such as proteins, metabolites, antigens, and nucleic acids.

**(1) Proteins**

Several proteins expressed on the surface of microorganisms and cells, are commonly used in medical diagnosis as biomarkers to detect microorganisms.

Proteins often exhibit strong interactions with the cell matrices, and bind to lipid bilayers making them difficult to extract. To overcome this limitation, specific ligands like antibodies and nucleic acids are often selected for the selection of target proteins (Takahashi, 2018).

**(2) Metabolic products**

All microorganisms exhibit metabolic activity during life activities, and living cells absorb oxygen and sugar through catabolism and release by-products such as organic acids and carbon dioxide to obtain the energy needed for survival and reproduction. The metabolites of microorganisms vary greatly from strain to strain, mostly metabolites such as nucleotides, amino acids and organic acids, so they can be detected in food according to the metabolic state of microorganisms (Liu, et al., 2017).

**(3) Adenosine triphosphate**

Adenosine triphosphate (ATP) is a coenzyme that is the primary source of energy in living cells. A larger number of living cells results in higher ATP levels, especially in bacteria and yeasts where there is a linear relationship between the total number of colony-forming units and the ATP level, so ATP is often used to detect the number of viable cells in a sample (Hyun, et al., 2018).

**(4) Nucleic acids**

Nucleic acid is a general term for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), and is a biological macromolecule that stores cytogenetic material. The continuous development and improvement of the genetic detection technology platform at the molecular level has greatly promoted the rapid development of DNA detection technology. DNA testing relies on target amplification, which can greatly improve the efficiency of food microbiological detection, but requires complex

equipment and reagents (Palecek, et al., 2002).

**(5) Antigens**

Antigens are usually present on the surface of pathogens and sometimes in proteins, lipids, and polysaccharides. Each type of pathogen has one or more unique antigens on its surface and even inside the strain that induce an immune response in the host body, so that the pathogen can be identified with the help of specific antibodies (Slavov, Alusta, Buzatu, & Wilkes, 2017).

Therefore, while developing antibacterial packaging materials to inhibit the growth of foodborne pathogens in food, this project is also committed to finding evaluation markers for different foods that meet the food packaging industry standards. In particular, the composition of meat and meat products is complex and rich in nutrients, resulting in problems such as the current unknown harmful characteristics of microorganisms in meat, the lack of hazard identification ability, and insufficient active prevention and control safeguard measures. To evaluate the shelf life of meat and meat products, levels of biogenic amine (BA), adenosine triphosphate (ATP) and microorganisms can be analyzed as important information for food freshness during storage.

## 1.4.2. Biogenic Amines

### 1.4.2.1. Biogenic Amines as Markers of Freshness and Safety

Spoilage is a complex process characterized by food becoming inedible. Food spoilage is essentially a process of certain biochemical changes in food in which proteins, carbohydrates, fats, etc. are metabolized and decomposed by microorganisms or by their own tissue enzymes, leading to physical and chemical

changes such as color, odor and oxidation (Mariutti & Bragagnolo, 2017). Throughout the metamorphism, several compounds are produced, especially BA. BA is nitrogen compounds with low molecular weight generated mainly by microbial decarboxylation of amino acids. This enzymatic reaction can be transamination, reductive amination, degradation and decarboxylation of certain precursor amino compounds. BAs are found in a variety of foods, especially fermented foods (such as cheese, wine, beer, rice wine, fermented sausages, condiments), aquatic products and meat products (Guo, Yang, Peng, & Han, 2015).

BAs can be considered polar or semi-polar, or they can be classified as "natural polyamines" according to biosynthetic pathways (Erdag, Merhan, & Yildiz, 2018). BAs have aliphatic, aromatic, or heterocyclic structures (Liu, Xu, Ma, & Guo, 2018). Aliphatic BAs include cadaver amine (CAD), putrescine (PUT), spermine (SPM) and spermidine (SPD); aromatic BAs include 2-phenylethylamine (PHE), tyramine (TYR), guanidine (AGM), and dopamine (DOP); heterocycle BAs include tryptamine (TRY), histamine (HIS) and serotonin (5-HT) (Jaguey-Hernandez, et al., 2021; Melnikov, Rogovskii, Boyko, & Pashenkov, 2018).

BAs are used as shelf life marker of food spoilage, as high concentrations of BAs are found when the hygienic quality of a product decreases (Hazards, 2011). The most relevant BAs that arise due to food spoilage are TRY, PHE, PUT, CAD, HIS, TYR, SPD, and SPM (Figure 1-3).

BAs are synthetized by enzymatic decarboxylation of free amino acids (Yoon, Park, Choi, Hwang, & Mah, 2015), or by amination and transamination of aldehydes and ketones (Doeun, Davaatseren, & Chung, 2017). BAs present in plant, microbial, and animal cells, and can be detected in both raw and fermented foods (Tittarelli, Perpetuini, Di Gianvito, & Tofalo, 2019). BAs are formed by the decarboxylation of

amino acids by living organisms (Ozogul & Ozogul, 2019). Factors that form BAs in foods include the presence of free amino acids in foods, and decarboxylase positive in microorganisms. Biosynthesis of BA and amino acid precursors are represented in Figure 1-4.

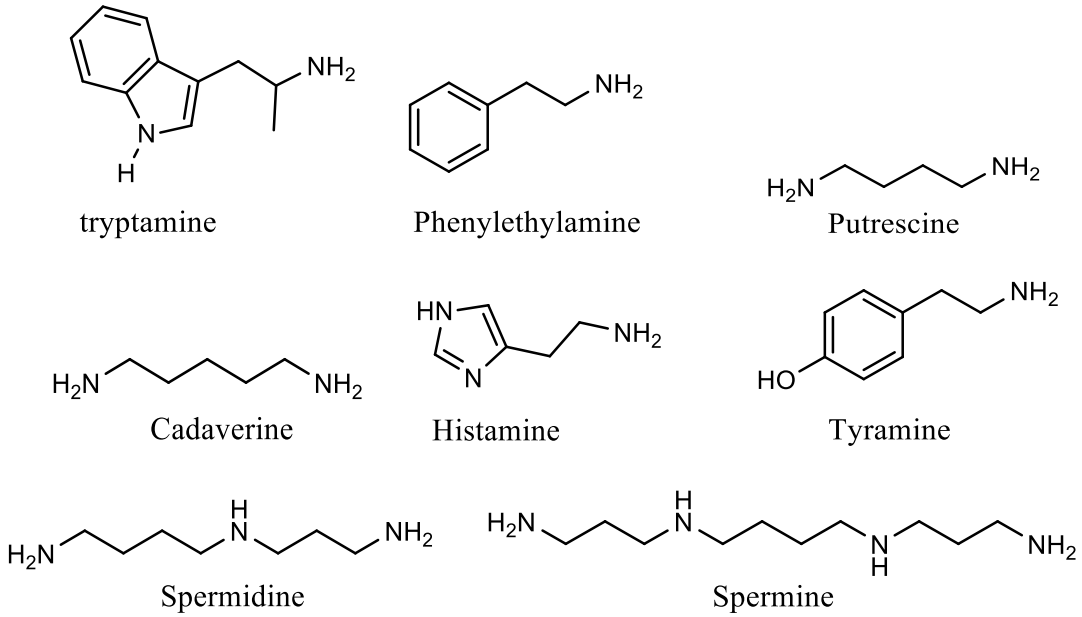



**Figure 1-3.** Main biogenic amines in food spoilage.

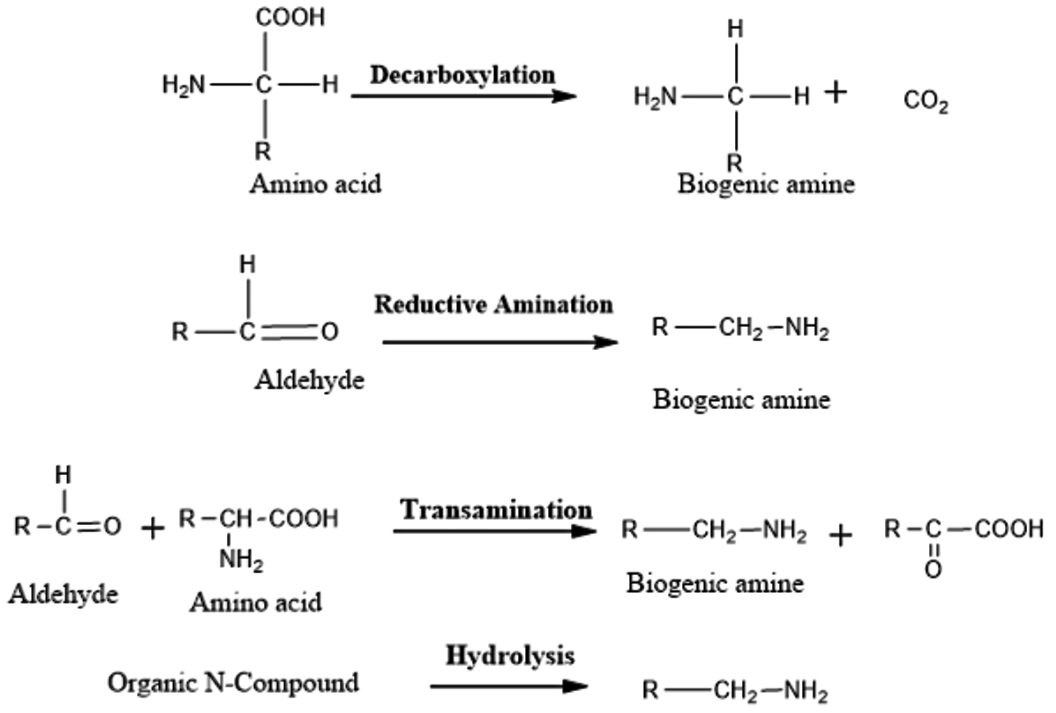



**Figure 1-4.** BAs formation.

Since the accumulation of BA indicates that food has deteriorated, finding a way to determine its concentration in food will be an important issue (Ruiz-Capillas & Herrero, 2019). The first naturally occurring BAs observed when storing fresh meat are SPM and SPD, which range from 20-60 mg/kg and about 10 mg/kg, respectively (Triki, Herrero, Jimenez-Colmenero, & Ruiz-Capillas, 2018). The only amines present at significant levels in fresh meat are spermidine and spermine. The significant increase in PUT and CAD (>15 mg/kg) observed during storage of raw pork has been considered a corruption index (Siripongpreda, Siralertmukul, & Rodthongkum, 2020). Due to the importance of harmful effects of BAs on food hygiene and health, it was concluded that BAs alone or in combination can be used as an important indicator of freshness, quality and spoilage of food (Triki, et al., 2018).

Biogenic Amine Index (BAI) is calculated according to the following formula:

$$\text{BAI} = \text{PUT} + \text{CAD} + \text{HIS} + \text{TYR}$$

Therefore, it is suggested that cooked meat products should be classified into four levels. If the BAI <5 mg/kg, the meat is fresh and of good quality; BAI between 5-20 mg/kg, it is still acceptable, but there are some signs of spoilage; between 20-50 mg/kg and >50 mg/kg, the quality of the meat is lower, the quality of hygiene is poor (spoiled).

The Chemical Quality Index (CQI) is calculated according to the following formula:

$$CQI = \frac{\text{HIS} + \text{PUT} + \text{CAD}}{1 + \text{SPD} + \text{SPM}}$$

where HIS is histamine, PUT is putrescine, CAD is cadaverine, SPD is spermidine, SPM is spermine.

The spermidine spermine ratio SPD/SPM can be considered as one of the most

important indices for evaluating the quality of meat, and the levels of TYR and CAD were proposed to control poultry spoilage and beef during storage (Schirone, et al., 2022). However, fermented or heat-treated meat products are too complex in their matrix, and the effectiveness of BA indicators may vary due to many factors, so they may be more suitable for fresh meat freshness assessment (Ruiz-Capillas & Herrero, 2019).

The number and variety of BAs formed in food is strongly influenced by external parameters such as microbiota growth, storage time and temperature during food storage and processing, as well as the inherent characteristics of food, including moisture activity and pH (Ruiz-Capillas & Herrero, 2019). Table 1-1 shows data on BAs in different raw meats and meat products.

The main BAs found in red meat and white meat are TYR and CAD, but the concentration of TYR in white meat is much lower (Feddern, Mazzuco, Fonseca, & De Lima, 2019). The concentrations of PUT, CAD, HIS, SPM and SPD in raw pork were 7.8, 13.3, 4.7, 67.1 and 7.0, respectively (Jairath, et al., 2015). The edible muscle tissue of animals is sterile. However, during processing, the surface of this tissue can be contaminated with microorganisms, which is why it is common to detect BAs on the surface of such products (Erkmen & Bozoglu, 2016).

**Table 1-1.** Biogenic amines levels in the different meat and meat products reported by the literatures.

| Category | Biogenic Amines (mg/kg) | PUT | CAD | HIS | SPD | SPM | TYR | PHE | TRY | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| **Raw meat** | Beef | 6.6–90.9 | <0.5–295.6 | <0.5 | | | 11.1–65.5 | | | (Rosinska & Lehotay, 2014) |
| | Pork | 12.7–131.5 | 13.6–440.2 | <0.5 | | | 34.4–55.2 | | | |
| | Poultry | <0.5–382.7 | <0.5–764.2 | <0.5–180.5 | | | <0.5–171.2 | | | |
| | Sardine (µg/mL) | nd | 1.67-2.21 | | | 27.9-105.9 | 1.12-1.99 | nd | 1.35 | (Munir, Inayatullah, & Badrul, 2021) |
| | Pork leg | 0.6–14.6 | nd-16.2 | nd | 2.6–3.9 | 25.2–27.6 | 0.7–16.6 | nd-1.7 | nd-6.6 | (Triki, et al., 2018) |
| | Lamb leg | 1.2–10.1 | nd-5.1 | nd | 8.1–12.0 | 31.4–40.9 | 0.1–10.7 | 0.8–9.1 | nd | |
| | Turkey leg | 1.2–68.7 | nd-13.3 | nd | 7.3–18.3 | 32.6–49.2 | nd-6.9 | 0.2–15.1 | nd | |
| | Chicken breast | 1.2–52.0 | nd-14.3 | 0.5–2.1 | 6.2–9.8 | 41.9–53.6 | nd-35.2 | nd-16.9 | 0.4–15.8 | |
| | Beef leg | 1.3–7.4 | nd | nd-0.5 | 2.3–5.4 | 25.1–33.0 | 0.3–1.6 | 0.5–2.6 | nd | |
| | Chicken breast muscle | 1.0–1.8 | <LOQ-10.5 | 1.4–4.3 | | | <LOQ-4.2 | | | (Wojnowski, Kalinowska, Majchrzak, Plotka-Wasylka, & Namieśnik, 2019) |
| | Red meat | 1.9-1.9 | 0.9-2.3 | | | 0.2-33.8 | 1.2-20.0 | 1.1-48.3 | 3.7-7.1 | (Motaghifar, Akbari-Adergani, Rokney, & Mottalebi, 2020) |
| **Processed meat products** | Dry fermented meat | nd-225.1 | nd-16.8 | nd-151.8 | | | nd-228.1 | nd-42.7 | | (Franti, Ludmila, R, & V, 2012) |
| | Sausages | 1.0-24.6 | 23.8-681.2 | | | 53.5-273.9 | 0.87-343.9 | 2.74-38.28 | 13.2-68.7 | (Ekici & Omer, 2018) |
| | Brazilian commercial salamis | 91.5–818.5 | 37.9–166.4 | nd-500.2 | 51.2–55.8 | 96.7–151.9 | 91.3–346.9 | nd-375.9 | nd-123.9 | (Roselino, et al., 2020) |
| | Italian commercial salamis | nd-381.2 | nd-215.9 | nd-240.9 | nd-99.7 | 102.8–141.2 | nd-270.0 | nd-316.4 | nd-297.1 | |

Legend: PUT = Putrescine; CAD = Cadaverine; HIS = Histamine; SPD = Spermidine; SPM = Spermine; TYR = Tyramine; PHE = Phenylethylamine; TRY = Tryptamine; nd = not detected.

The accumulation of BAs in fermented meat products is more complex than in fresh meat, and protein hydrolysis can be affected by intrinsic factors in meat products, such as dehydration, the role of sodium chloride and increased acidity, as well as by microbial groups in meat production (Ashaolu, Khalifa, Mesak, Lorenzo, & Farag, 2021; De Mey, et al., 2014). Fermented products usually contain higher concentrations of BAs because the fermentation process leads to increased free amino acid precursors of BAs (Jairath, Singh, Dabur, Rani, & Chaudhari, 2015). TYR is the most representative amine in cured meat products. However, in accordance with European legislation (Regulation EC No 2073/2005 of the European Commission and further amendments), maximum limits only the for HIS in fish and fish products, especially fish with high free HIS content in muscle tissue, while no standards or guidelines for reporting meat products.

BAs were also detected in salami, adult bovine meat samples, dry sausages, onion sausage, smoked turkey fillets and pepperoni sausage, with the highest values of CAD, TYR and HIS for dry sausages being 790, 320 and 200 mg/kg, respectively. BAs were also detected in European dry ripened sausages made with horse, beef or turkey meat, where the average total BAs content of Turkish sausages was highest at 730 mg/kg, while the average content of beef sausages and horse sausages was 500 mg/kg and 130 mg/kg, respectively (Lorenzo, Munekata, & Dominguez, 2017).

BAs have been extensively studied, and their respective concentrations in food are an important area of research. According to statistics, between 2018-2020, there were about 430 articles on BAs, of which 38% studied the quantification and identification, 23% of control and eduction and degradation, 15% of bioactivity, quick detection 9%, BA formation or BA inhibition of microbial culture 5%, 1% of other applications such as design of delivery materials, synthetic precursors or thermodynamic research, and

the remaining 9% were review papers (Jaguey-Hernandez, et al., 2021). All of these studies prove that BAs formation in meat and meat products is a suitable and useful marker for monitoring meat safety and predicting meat quality.

#### 1.4.1.2. Effects of BA on health

BAs are commonly found in plants, animals, and humans, where they exhibit basic physiological functions such as neurotransmission, regulation of growth and blood pressure, and other important roles in the intestinal immune system (Erdag, Merhan, & Yldz, 2019). However, when introduced in large quantities by eating contaminated foods and/or beverages, they may cause adverse effects and/or allergic reactions to the nervous, respiratory, and cardiovascular systems, especially in individuals deficient in monoamine oxidase and diamine oxidase, or assuming that drugs inhibit such enzymes (Visciano, Schirone, & Paparella, 2020).

BAs are precursors of nitrosamines and have been linked to carcinogenic and mutagenic activity (Papageorgiou, Lambropoulou, Morrison, Namieśnik, & Plotka-Wasylka, 2018). BAs can lead to an increased risk of a variety of diseases, from food poisoning to other diseases such as cancer (Latorre-Moratalla, Comas-Baste, Bover-Cid, & Vidal-Carou, 2017). Figure 1-5 shows the main harmful effects of BA, such as scombroid poisoning, carcinogenesis and cheese reaction.

Microbial activity in food can form N-nitrosamines, which are formed by a reaction between secondary amines and nitrosizing agents, in most cases has a strong carcinogenic effect and can be found in the environment and various foods (Park, Seo, Lee, & Kwon, 2015). Scombroid poisoning events are formed due to the high concentration of free histidine in muscle tissue, which after decarboxylation leads to the formation of HIS by the action of endogenous or bacterial histidine decarboxylase, and occurs mostly in fish such as tuna, saury and mackerel, and related products (Lee,

et al., 2016). Another important dietary BA is TYR, which is relatively abundant in aged cheeses and often causes a reaction called the “cheese reaction”. This reaction is associated with high levels of tyrosine causing poisoning (Jaguey-Hernandez, et al., 2021). The main BAs in fermented meat products are TYR, HIS, PUT and CAD, which are usually detoxified by amine oxidase produced by the intestinal mucosa (Lorenzo, et al., 2017).

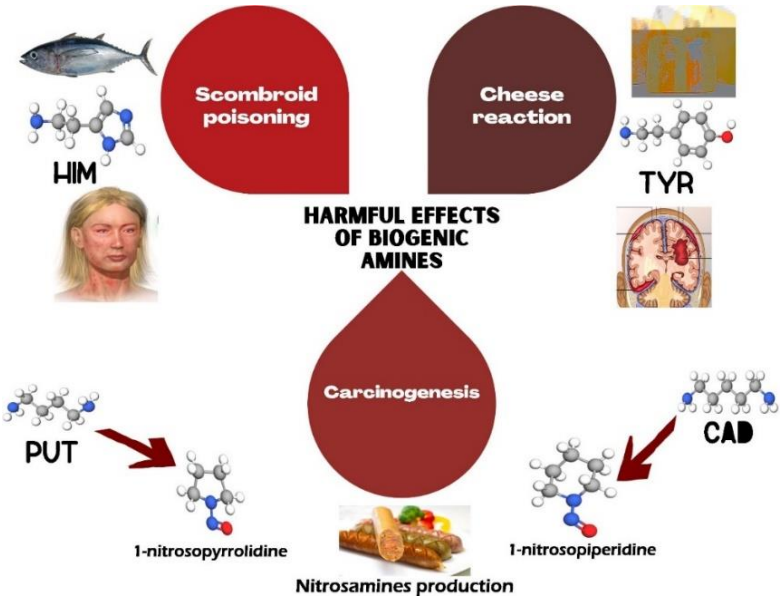



**Figure 1-5.** The main harmful effects of BA. HIM: histamine, PUT: putrescine, TYR: tyramine and CAD: cadaverine (Jaguey-Hernandez, et al., 2021).

Recently, the correlation of these values was assessed in a study evaluating the cytotoxicity of TYR and HIS in human gut cells. The results of this study show that TYR (301.8 mg/kg) exhibits a higher ability to induce toxic effects in intestinal cells compared to HIS (440.6 mg/kg). Interestingly, the mechanisms involved are specific to each compound: TYR-induced necrosis (accidental, disordered, and accidental forms of cell death), while HIS-induced apoptosis (programmed and organized sequences of key events leading to cell death) (Linares, et al., 2016). At concentrations of up to 219.49 mg/kg of TYR and HIS of 1445 mg/kg, these two BAs

can also have a synergistic toxic effect on human intestinal cells in vitro (Del Rio, et al., 2017). Currently, information related to BAs with different physiological functions indicates how their toxic effects (dependent on concentration and synergy) affect human health. The European Food Safety Authority assessed the risks associated with the intake of BAs out of public health concerns, but limits for PUT and CAD have not been determined due to limited scientific information. It is important to note that fish with HIS levels in the range of 200-1000 mg/kg are considered improperly handled and may be toxic(Alvarez & Moreno-Arribas, 2014).

#### 1.4.1.3. Main microbiota associated with BAs in meat

The meat products has a short shelf life around 3-5 days at 4 °C (Mehdizadeh, Tajik, Langroodi, Molaei, & Mahmoudian, 2020). High levels of moisture, oxygen and enzymes facilitate the lipid oxidation, as well as high protein content promotes microbial growth during the storage time. The major spoilage bacteria of meat are psychrotropic strains of *Pseudomonas*, Enterobacteriaceae and Gram-positive Lactobacillus and *Brochothrix thermosphacta*. Furthermore many pathogens with public health significance such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enteritidis, Salmonella typhimurium*, and *Yersinia enterolitica*, also isolated from meat and meat products (COMA, 2008).

The formation of BAs in meat depends on different factors, such as the presence of bacteria containing genes encoding decarboxylase, the availability of specific free amino acids, and favorable conditions for enzyme activity and bacterial growth. Decarboxylase is produced by certain groups of bacteria, such as *Streptococcus,* Enterobacteriaceae, *Clostridium, Pseudomonas* and *Lactobacillus* (Ozogul, Kacar, & Hamed, 2015), leading to the formation of BAs in a range of foods. The strain-specific BA-producing genes can spread among bacterial species through

horizontal transfer, increasing the risk of poisoning. For fresh and fermented products as well as animal products, the types of BAs found and their concentrations are variable. These changes are mainly due to differences in positive decarboxylase microorganisms, processing type, conditions, storage time and temperature, availability and type of amino acids in food, and packaging type. Meat environments are typically characterized by Lactic Acid Bacteria (LAB), Enterobacteriaceae, *Pseudomonads*, *Brochothrix thermosphacta*, and some clostridia. Obviously, the gaseous composition of the environment and storage conditions have a direct impact on the final ecology of microorganisms, enhancing specific groups (Chmiel, et al., 2022).

In fermented meat products, different microflora intentionally added (cultured in starter cultures) or parts of the natural microbiota can produce BAs (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015). The conversion of meat and fats into fermented meat products involves the activity of microorganisms in complex ecosystems (Cocolin, Dolci, & Rantsiou, 2011). The main bacterial groups that form BAs in meat fermented products are Enterobacteriaceae (i.e., *Escherichia, Citrobacter, Klebsiella, Proteus, Salmonella, and Shigella*), *Micrococcaceae* (*Staphylococcus* and *Micrococcus* genera), *Pseudomonas*, some strains belonging to *Bacillus* spp., as well as LAB (Li, Yu, Zhu, & Cao, 2020).

#### 1.4.1.4. Methods for detection of BA

The determination of BAs is both challenging and laborious, in part because of the physico-chemical properties of these compounds. Numerous analytical methods have been developed to the detection of BAs, such as High-performance liquid chromatography (HPLC), capillary electrophoresis (CE), thin layer chromatography (TLC) and gas chromatography (GC) (Fu, et al., 2016). Chromatography is the most

common method for determining BAs in food samples.

**(1) Capillary electrophoresis**

Capillary electrophoresis (CE) is considered a reliable method that can analyze a large number of samples with fewer reagents (He, Ren, Shi, & Xu, 2016). This technique can use a selected buffer system without the need for derivatization or use of specific kits, and the method used will depend on the expected concentration of BAs in the food sample being analyzed. However, it is not one of the most commonly used techniques for detecting BAs, as its sensitivity is generally lower (Papageorgiou, et al., 2018).

**(2) Thin layer chromatography**

Thin layer chromatography (TLC) is a fast and easy-to-implement method that does not require heavy equipment and expensive while allowing multiple samples to be analyzed simultaneously (Sherma & Rabel, 2018). This appears to be a general-purpose technique that can be used to determine BAs in different food samples with μg/mL LOD. Moreover, the detection limits for using this method in different procedures and different foods are very close. This technique has been used to detect different BAs in beef stored aerobically at 4 °C, among which the detection limits of PHE, PUT, TRY, CAD, SPD, SPM, HIS, and TYR are 0.30, 0.2, 0.1, 0.03, 0.03, 0.15, 0.14 and 0.06 μg/mL, respectively (Li, Johansson, Vidanarachchi, Pickova, & Zamaratskaia, 2016). Recently, rapid self-visualization nanomaterials based on highly efficient thin layer chromatography strips have been developed for the detection of HIS in fish samples with an LOD of 5.0 mg/kg (Zhang, et al., 2021).

**(3) Liquid chromatography-tandem mass spectrometry**

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) showed good improvements for the determination of BAs in biological and food samples because

no derivatization process was required (Manetta, et al., 2016). Information on the molecular weight, structure, and concentration of organic compounds can usually be obtained in conjunction with MS. It is more suitable for detecting low-volatile, highly polar mixed organic compounds such as BAs, especially for isolating strongly retained amines such as SPM and SPD (Zhang, et al., 2019). This analytical method has the characteristics of high sensitivity, low background interference, relatively low standard sample requirements, and is more accurate for the analysis of compounds of unknown structure, which is an ideal method for analyzing complex matrix and trace samples. For mass analysis techniques, detectors such as ion traps, triple quadrupoles (QQQ), time-of-flight, etc. can provide a view of the analyte structure with significant resolution. Among them, QQQ is the most quantitatively reproducible and sensitive (Zhang, et al., 2019). Comparing the determination of dansulfonyl derivatives by MS/MS method and fluorescence detection (FD) method, the results show that there is a significant difference in sensitivity between the two methods, and the MS/MS method has higher precision and sensitivity (Sagratini, et al., 2012).

**(4) High-performance liquid chromatography**

High-performance liquid chromatography (HPLC), an analytical technique for identifying, isolating, and quantifying each ingredient in a mixture, is the most commonly used technique for determining BAs in food (Papageorgiou, et al., 2018). HPLC facilitates the highly sensitive and selective determination of BAs in food. Before the final separation, a solvent extraction step and a chemical derivatization step are required, followed by fluorescence or UV detection (Ishimaru, Muto, Nakayama, Hatate, & Tanaka, 2019). The polarity of BAs needs to be reduced in order to be more soluble in the organic solvents often used in most technologies. The first phase involves removing potentially interfering compounds and concentrating the

associated analytes, while the second phase reduces the polarity of BAs and improves the resolution of the reversed-phase column, making it more sensitive to detection (Ishimaru, et al., 2019).

In order to detect BAs in wine, derived and improved LLE with HPLC-FLD and the LOD obtained obtained ranged from 0.001-0.050 mg/L (Liu, Han, Liu, & Wang, 2020). Ishimaru used the same technique and used a column switching system with a fluorescence detector to detect BAs content in fish and fermented foods (cheese and soy products). In cheese samples, 15 BAs were successfully determined simultaneously by in-line solid phase extraction capillary high performance liquid chromatography, and the LOD obtained was in the range of 0.05-0.25 mg/L (Ishimaru, et al., 2019).

Currently, UHPLC is common method as the BAs detection in the most published studies. This technique has higher sensitivity and resolution than HPLC with less solvent consumption. Overall, the LODs values for BAs analysis methods are typically mg/L levels (Neofotistos, Tsagkaris, Danezis, & Proestos, 2019).

**(5) Enzyme-based biosensors for the detection of BA**

New research have focused on biosensors as modern, robust, sensitive and computerized devices to provide an instant, economical and straightforward solution for the detection of BAs (Verma, Hooda, Gahlaut, Gothwal, & Hooda, 2020). Catalytic biosensors can use microorganisms, tissues, enzymes, cell receptors, and antibodies as biological recognition elements. Among them, enzymes are the most common biometric recognition elements in biosensor fabrication, with market low cost, simple operation, and accessibility. Enzyme-based biosensors are used to detect BAs, and the interaction of biological recognition element with BAs produces a determinable response, such as depleting a substance or creation of a product without

consuming the catalyst (Ahangari, Kurbanoglu, Ehsani, & Uslu, 2021). To date, many nanozyme-based biosensors have been successfully expanded according to modes of nanoenzyme signal amplification patterns such as fluorescence, chemiluminescence, electrochemistry, and colorimetry.

In the future, research into various aspects of biosensors, such as intelligent nanofilm biosensors, multifunctional nanocomposite biosensors and nanoelectrode development, will meet customer's needs for lower-cost, mobile and ultrasensitive nanozymes-based methods for BAs detection and enhancement of food safety.

## 1.4.3. ATP as shelf life markers

### 1.4.3.1. ATP in living cells

ATP is an organic molecule that is the primary energy source in living cells for the transport of molecules and enzymatic activity (Sang, et al., 2020). It consists of three main components, a triphosphate, an adenine base and a 5-carbon ribose sugar. The triphosphate moiety is the reactive site of ATP and directly participates in different cellular processes and combination of two organic molecules of ribose sugar and adenine constructs the adenosine (AD) moiety of ATP (Agteresch, Dagnelie, van den Berg, & Wilson, 1999). Typically, ATP is produced by living organisms such as animal and plant cells. In plant cells, it is formed by the use of light energy in photosynthetic processes and photosynthetic phosphorylation reactions, while in animal cells it is typically formed in the chloroplasts and mitochondria of living cells through the participation of many different cellular aerobic respirations and enzymes (Agteresch, Dagnelie, van den Berg, & Wilson, 1999). In prokaryotes, ATP is synthesized on the inner membrane of the cell membrane and is synthesized mainly in

mitochondria in eukaryotes. The special properties of ATP are attributed to its active triphosphate site, where two phosphoanhydride bonds connect three phosphate groups. The breakdown of phosphoanhydride bonds of ATP requires lower activation energy, therefore, ATP is easily hydrolyzed and converted to ADP by simultaneously releasing energy. The resulting ADP can also be transferred back to ATP through cellular aerobic respiration, and the ATP-ADP cycle is constantly regenerated as energy is released (Ng, Lim, Ma, & Gao, 2016). Under standard conditions, removing a phosphate group produces 7.3 kilocalories per mole energy (Figure 1-6).

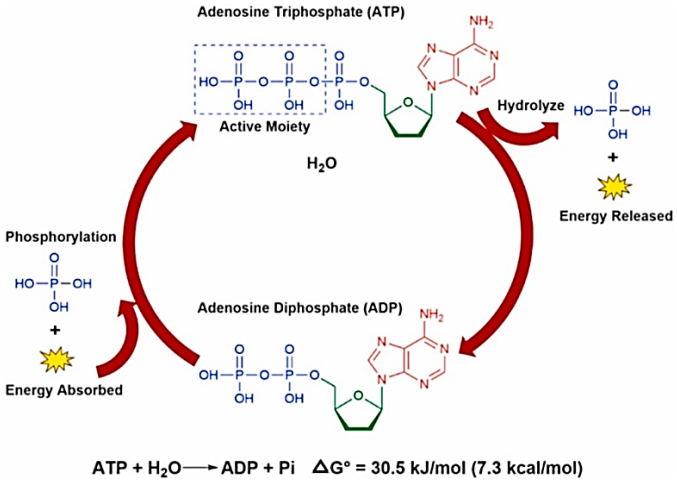



**Figure 1-6.** ATP-ADP cyclie and energy release mechanisms in the cell.

ATP levels in living cells are constant (Sottofattori, Anzaldi, & Ottonello, 2001), but after cell death, ATP is rapidly degraded into adenosine diphosphate (ADP), and subsequently into adenosine monophosphate (AMP) and inosine monophosphate (IMP). IMP is further degraded into inosine (Ino) and hypoxanthine (Hx) (Mora, Hernandez-Cazares, Aristoy, & Toldra, 2010). The ATP degradation process is shown in Figure 1-7.

ATP is the source of energy for living organisms, which is essential not only for microbial growth and viability, but also for other cellular activities. This energy performs most of the processes that occur in living organisms, and this phenomenon

plays a fundamental role in the arrangement of cell physiology and cell metabolism (Cook, Vulchanova, Hargreaves, Elde, & McCleskey, 1997).

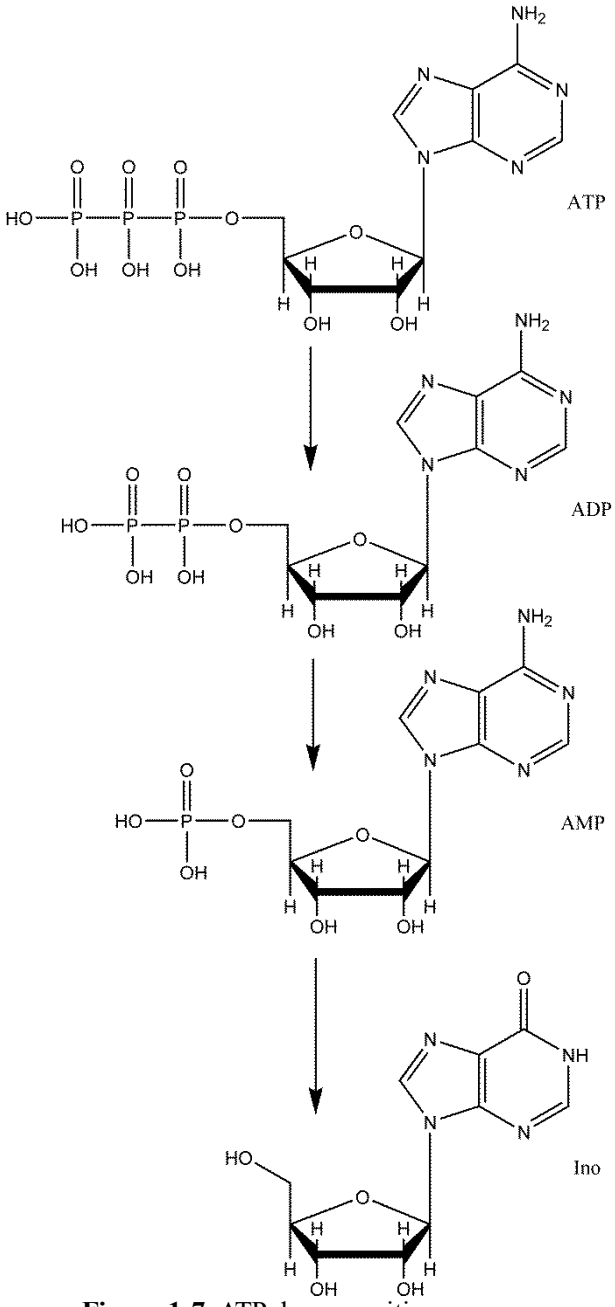



**Figure 1-7.** ATP decomposition process.

### 1.4.3.2. Application of ATP detection in food safety

Providing consumers with safe food is becoming increasingly challenging for the food industry. In addition to nutrition and attractiveness, food must first and foremost be safe (Vasconcelos, et al., 2021). In addition to physical and chemical changes, food

also deteriorates due to the activity of specific microbiota, thereby accelerating the breakdown of nutrients (Pothakos, Devlieghere, Villani, BjOrkroth, & Ercolini, 2015). The main purpose of monitoring food is to minimize the risk of human disease due to the foodborne microbiota and its toxins and chemical contaminants.

Microbial pollution has a serious impact on the food industry, drinking water and other environments, and is a major hazard to human health. The need for microbial load assessment of these samples is a prerequisite for avoiding microbial pollution. Methods through traditional cultures or PCR-based methods are either time-consuming and laborious or involve expensive chemicals. Detecting ATP, an important molecule present in an organism, is currently one of the most used rapid alternative techniques. ATP is present in all living cells and can be used to detect the presence of microbial pollution in all contaminated environments. Currently, through bioluminescence analysis, ATP has been used for rapid microbial analysis of food, clinical, pharmaceutical samples and the environment (such as air quality and equipment surface cleanliness) (Mempin, et al., 2013). ATP detection is one of the important means of microbial contamination detection (Manikandan, Hasan, & Wu, 2012).

The direct relationship between intercellular ATP and microbial biomass can be used to quantify microbes in food samples. The variety of bioluminescent-based ATP assay kits are currently available for in situ analysis. ATP levels directly represent metabolically active cells, and studies have found a linear relationship between the total number of ATP molecules present and the total number of colony-forming units, especially in yeast and bacteria (Hyun, et al., 2018). But the content of ATP in cells varies with the type and growth status of the microorganism. For example, the extracellular ATP present for 6 hours *Pseudomonas aeruginosa* was 25.5 ± 1.1

nM/OD and *Acinetobacter junii* was 255.2±56.8 nM/OD (Mempin, et al., 2013).

The level of ATP has been regarded as a great indicator for microbial contents in food safety. The content of microbial and the corresponding ATP will progressively accumulate during the decay process of aquatic products, and the freshness changes of aquatic products could be evaluated by determining the amount of ATP (Wei, et al., 2022). ATP assay is a rapid and sensitive alternative to the traditional plate count techniques in routine microbiological analysis of food and beverages (Bottari, Santarelli, & Neviani, 2015).

In addition, ATP levels can be used to deduce the number of microorganisms found in a food sample or abiotic surface, and several microbial load monitoring assays employ the indirect determination of ATP levels in cells (Michela, et al., 2019). However, these methods have a crucial limitation related to the fact that ATP is present not only in microbial cells but in all viable cells. Many food matrices intrinsically contain large amounts of ATP of nonmicrobial origin, which must be enzymatically degraded to enable the determination of ATP molecules originating exclusively from target microbial cells (Mandal & Biswas, 2020; Vanne, Karwoski, Karppinen, & SjOberg, 1996). Siragusa et al. stated that the major challenge in using microbial ATP to determine total microbial populations in food samples is distinguishing nonmicrobial ATP from microbial ATP (Bottari, et al., 2015; Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010; SHINOZAKI, et al., 2013; Siragusa, W J Dorsa, C N Cutter, & L J Kooh-maraie, 1996). To achieve this, a rapid-microbial ATP assay was proposed in which a filtration device was incorporated to enable extraction of somatic ATP, with a subsequent step of bacterial ATP extraction and quantification (Oshita, et al., 2011; Ratphitagsanti, Park, Lee, Amos Wu, & Lee, 2012). Consequently, measuring ATP is likely the most appropriate

method to estimate microbial contamination on abiotic surfaces with equipment and machinery associated with food processing and preparation (Carrascosa, et al., 2012). However, when considering ham products, ham is a meat-based product, which has undergone post-mortem decay, there is no living cell ATP effect, and ATP levels can be used to deduce the number of microorganisms found in ham.

ATP is typical contaminants containing in milk, beer, corn (Yan, et al., 2021), meat (Mora, et al., 2010; Oshita, et al., 2011) and royal jelly (Xue, Zhou, Wu, Fu, & Zhao, 2009). Therefore, it is necessary to develop facile and effective methods for detecting ATP in foods.

#### 1.4.3.3. Methods for detection of ATP

Taking into account of the different properties of ATP, wide attentions have been attracted for detection of this important target in so many areas. It has garnered a wide interest in analytical chemistry, especially in the methods used to accurately and reproducibly determine its concentration (Ng, et al., 2016). Various analytical techniques including bioluminescence (Ng, et al., 2016), high-performance liquid chromatography (HPLC) (Coolen, et al., 2008), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Kim, et al., 2017), matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) (Manikandan, et al., 2012), electrochemical method (Wang, et al., 2018) and colorimetric method (Huo, et al., 2016) have been applied to the detection of ATP.

**(1) ATP bioluminescence method**

A bioluminescence method measures the light emitted under the enzymatic reactions mediated by luciferase (Ng, et al., 2016). The ATP-dependent bioluminescence method is the most studied and most sensitive. In this ATP-dependent reaction, ATP is required as the energy source for the bioluminescence

to occur. The reaction is sensitive and highly specific to ATP. During this process, the enzyme luciferase, magnesium ion, and molecular oxygen are required. Light is emitted as a result of this reaction with its intensity directly correlates to the concentration of ATP. This method has shown its usefulness in the detection of ATP in most biological samples but not without limitations. Despite the ability of achieving a detection limit as low as pmol/mg (Ugarova, et al., 2016), the enzyme luciferase has shown to be unstable and hence unlikely to be used for the detection of ATP under conditions that are drastically different from physiological conditions. In addition, some biological substances such as plasma and ATPase degrade ATP readily. Also, there may be the possibility of overestimation of ATP concentration with possible contributions from red blood cells and platelets present in biological samples. With these limitations, there have been continuous efforts to develop other ATP detection methods hoping to overcome some of the limitations.

**(2) High-performance liquid chromatography**

Chromatographic method for soluble ATP is generally performed with the High Performance Liquid Chromatography (HPLC), which separates organic compounds flowing through a packed column with a buffer solution as a carrier mobile phase. HPLC method of biochemical quantification has the advantages of high sensitivity, high efficiency and its results are reproducible (Mora, et al., 2010; Xue, et al., 2009). This method provided good chromatographic resolution and peak shape for all adenine nucleotides within a 19 min run time. The baseline was clean, the lower limit of quantification was below 0.3 μmol/L for all adenine nucleotides and the method demonstrated good linearity. Within-day precision ranged from 0.7 to 5.9% and between-days from 2.6 to 15.3%. Simplicity and simultaneous detection of ATP and its metabolites make this method suitable for clinical pharmacokinetic studies (Mora,

et al., 2010; Xue, et al., 2009).

**(3) Liquid chromatography coupled with tandem mass spectrometry**

Kim et al present an LC-MS/MS method for the reliable quantification of 2,3-DPG and ATP from EDTA-$K_2$ human whole blood (WB) simultaneously. Whole blood samples were spiked with stable isotope labeled internal standards, processed by protein precipitation extraction, and analyzed using zwitterionic ion chromatography-hydrophilic interaction chromatography (ZIC-HILIC) coupled with tandem mass spectrometry. The linear analytical range of the assay was 50-3000 μg/mL (Kim, et al., 2017).

**(4) Matrix assisted laser desorption ionization-mass spectrometry**

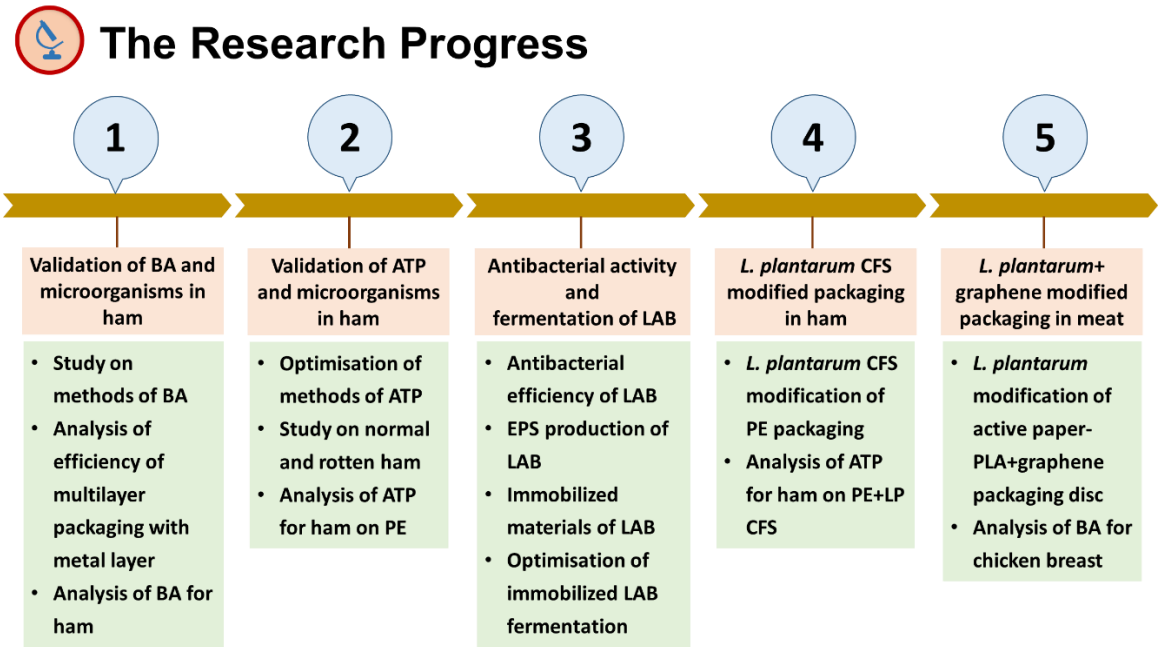
Matrix assisted laser desorption ionization (MALDI) is an ideal soft ionization technique for biomolecular analysis. This technique relies on low molecular weight lasers to absorb organic molecules (called matrices) to ionize cocrystallized analytes. MALDI-mass spectrometry (MS) and matrix-free laser desorption/ionization (LDI) -MS techniques have great potential for sensitive analysis of small molecules. For the preconcentration of analytes in MALDI-MS analysis, surface modified or functionalized MALDI target plates were used in order to enrich the analytes, such as phosphopeptides, peptides, biomolecules and nucleic acids etc (Eriksson, et al., 2010). The report demonstrates that rutile-type titanium dioxide chips are used to capture ATP from the external environment and detected using direct on-chip MALDI-MS. The affinity of rutile titania with ATP molecules has been confirmed, which has led to their successful attomolar detection (Manikandan, et al., 2012).

Although these methods have advanced the detection of ATP, the sensitivity of them needs to be improved. Determination of ATP as the main energy currency of the cells is a pressing need in advanced biological detection. Recently, aptamer-based

biosensors are introduced as a new direct strategy in which the aptamers (Apts) directly bind to the different targets and detect them on the basis of conformational changes and physical interactions. They can also be conjugated to optical and electronic probes such as quantum dot (QD) nanomaterials and provide unique QD aptasensing platforms. Currently, these Apt-based biosensors with excellent recognition features have attracted extensive attention due to the high specificity, rapid response and facile construction (Khojastehnezhad, et al., 2021).

## 1.5. OVERALL OBJECTIVE

The overall objective of this project was to evaluate the effectiveness of the developed new antibacterial packaging materials by monitoring the shelf life markers of meat and meat products, which can effectively prolong the shelf life of meat and meat products.



## 1.6. OUTLINE OF THE THESIS

The specific objectives of this project were:

1) to characterize the preservation efficiency - biogenic amines and microbial load- of commercial available packaging materials (multilayer packaging with metal layer) in 8 different ways on raw and cooked ham;

2) to study the correlation between biogenic amines and microorganisms, ATP and microorganisms in different foods; to select the appropriate shelf life markers of raw chicken meat and ham;

3) to select probiotic bacteria with potential food application and optimize fermentation conditions;

4) to obtain the effectiveness of ATP as a shelf life marker in modified packaging, and to examine *L. plantarum* cell-free supernatant to be used as part of antibacterial packaging for extending ham shelf-life;

5) to prepare and evaluate different prototypes PLA/paper packages with addition of graphene-based composite, probiotic bacteria – *L. plantarum* IMC 509 - on raw chicken meat.

The **general introduction** (**Chapter 1**) provides a general background in concept of antibacterial packaging and shelf life, the important role of packaging and biological indicators. The feasibility of Lactic acid bacteria and graphene as bacteriostatic agents for improved packaging were highlighted, as well as the application background of BA and ATP as shelf life markers. **Chapter 2 –** formation of biogenic amines and growth of spoilage-related microorganisms in the refrigerated ham with different commercial packages; **Chapter 3 –** determination of ATP-related compounds in dry-cured ham by HPLC; **Chapter 4 –** study on the antibacterial

activity and fermentation of Lactic acid bacteria and metabolites; **Chapter 5 –** determination of ATP-related compounds by HPLC to study the effect of cell-free supernatants of *Lactiplantibacillus plantarum* on the shelf life of sliced dry-cured ham; **Chapter 6 –** assessment of shelf-life of chicken breast meat stored in the novel composite graphene and probiotic paper-PLA packaging; **Chapter 7 –** general discussion and summary**.**

## 1.7. References


Agteresch, H. J., Dagnelie, P. C., van den Berg, J. W. O., & Wilson, J. H. P. (1999). Adenosine Triphosphate. *Drugs*, *58*(2), 211-232.

Ahangari, H., Kurbanoglu, S., Ehsani, A., & Uslu, B. (2021). Latest trends for biogenic amines detection in foods: Enzymatic biosensors and nanozymes applications. *Trends in Food Science & Technology*, *112*, 75-87.

Alahi, M. E. E., & Mukhopadhyay, S. C. (2017). Detection Methodologies for Pathogen and Toxins: A Review., *Sensors*, vol. 17.

Alam, A., & Moussa, M. (2020). Preparation of graphene/poly(vinyl alcohol) composite hydrogel films with enhanced electrical and mechanical properties. *Polymer Composites*, *41*(3), 809-816.

Al-Jumaili, A., Alancherry, S., Bazaka, K., & Jacob, M. V. (2017). Review on the Antimicrobial Properties of Carbon Nanostructures., *Materials*, vol. 10.

Allahbakhsh, A. (2017). High barrier graphene/polymer nanocomposite films. (pp. 699-737): Elsevier.

Alvarez, M. A., & Moreno-Arribas, M. V. (2014). The problem of biogenic amines in fermented foods and the use of potential biogenic amine-degrading microorganisms as a solution. *Trends in Food Science & Technology*, *39*(2), 146-155.

Arriagada, P., Palza, H., Palma, P., Flores, M., & Caviedes, P. (2018). Poly (lactic acid) composites based on graphene oxide particles with antibacterial behavior enhanced by electrical stimulus and biocompatibility. *Journal of Biomedical Materials Research Part a*, *106*(4), 1051-1060.

Arrioja-Breton, D., Mani-Lopez, E., Palou, E., & Lopez-Malo, A. (2020). Antimicrobial activity and storage stability of cell-free supernatants from lactic acid bacteria and their applications with fresh beef. *Food Control*, *115*, 107286.

Ashaolu, T. J., Khalifa, I., Mesak, M. A., Lorenzo, J. M., & Farag, M. A. (2021). A comprehensive review of the role of microorganisms on texture change, flavor and biogenic amines formation in fermented meat with their action mechanisms and safety. *Critical Reviews in Food Science and Nutrition*, 1-18.

Azizi-Lalabadi, M., & Jafari, S. M. (2021). Bio-nanocomposites of graphene with biopolymers; fabrication, properties, and applications. *Advances in Colloid and Interface Science*, *292*, 102416.

Balcazar, J. L., Vendrell, D., Blas, I. D., Ruiz-Zarzuela, I., & Muzquiz, J. L. (2004). Probiotics: a tool for the future of fish and shellfish health management. *Journal of Aquaculture in the Tropics*.

Barzegari, A., Kheyrolahzadeh, K., Khatibi, S., Sharifi, S., & Vahed, S. Z. (2020). The Battle of Probiotics and Their Derivatives Against Biofilms. *Infection and Drug Resistance*, *Volume 13*, 659-672.

Bhuyan, M. S. A., Uddin, M. N., Islam, M. M., Bipasha, F. A., & Hossain, S. S. (2016). Synthesis of graphene. *International Nano Letters*, *6*(2), 65-83.

Bintsis, T. (2018). Lactic acid bacteria: their applications in foods.

Bottari, B., Santarelli, M., & Neviani, E. (2015). Determination of microbial load for different beverages and foodstuff by assessment of intracellular ATP. *Trends in Food Science & Technology*, *44*(1), 36-48.

Carrascosa, C., Saavedra, P., Millan, R., Jaber, J. R., Perez, E., Grau, R., Raposo, A., Mauricio, C., & Sanjuan, E. (2012). Monitoring of cleanliness and disinfection in dairies: Comparison of traditional microbiological and ATP bioluminescence methods. *Food Control*, *28*(2), 368-373.

Casaburi, A., Piombino, P., Nychas, G., Villani, F., & Ercolini, D. (2015). Bacterial populations and the volatilome associated to meat spoilage. *Food Microbiology*, *45*, 83-102.

Cavera, V. L., Arthur, T. D., Kashtanov, D., & Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *International Journal of Antimicrobial Agents*, *46*(5), 494-501.

Chieng, B. W., Ibrahim, N. A., Yunus, W. M. Z. W., Hussein, M. Z., Then, Y. Y., & Loo, Y. Y. (2015). Reinforcement of graphene nanoplatelets on plasticized poly (lactic acid) nanocomposites: Mechanical, thermal, morphology, and antibacterial properties. *Journal of Applied Polymer Science*, *132*(11).

Chmiel, M., Roszko, M., Hac-Szymanczuk, E., Cegielka, A., Adamczak, L., Florowski, T., Pietrzak, D., Bryla, M., & Swider, O. (2022). Changes in the microbiological quality and content of biogenic amines in chicken fillets packed using various techniques and stored under different conditions. *Food Microbiology*, *102*, 103920.

Cocolin, L., Dolci, P., & Rantsiou, K. (2011). Biodiversity and dynamics of meat fermentations: the contribution of molecular methods for a better comprehension of a complex ecosystem. *Meat Science*, *89*(3), 296-302.

Coma, V. (2008). Bioactive packaging technologies for extended shelf life of meat-based products. *Meat Science*, *78*(1), 90-103.

Cook, S. P., Vulchanova, L., Hargreaves, K. M., Elde, R., & McCleskey, E. W. (1997). Distinct ATP receptors on pain-sensing and stretch-sensing neurons. *Nature*, *387*(6632), 505-508.

Coolen, E. J. C. M., Arts, I. C. W., Swennen, E. L. R., Bast, A., Stuart, M. A. C., & Dagnelie, P. C. (2008). Simultaneous determination of adenosine triphosphate and its metabolites in human whole blood by RP-HPLC and UV-detection. *Journal of Chromatography B*, *864*(1), 43-51.

Corradini, M. G. (2018). Shelf life of food products: from open labeling to real-time measurements. *Annual Review of Food Science and Technology*, *9*, 251-269.

Dalie, D. K. D., Deschamps, A. M., & Richard-Forget, F. (2010). Lactic acid bacteria - Potential for control of mould growth and mycotoxins: A review. *Food Control*, *21*(4), 370-380.

De Mey, E., De Klerck, K., De Maere, H., Dewulf, L., Derdelinckx, G., Peeters, M., Fraeye, I., Vander Heyden, Y., & Paelinck, H. (2014). The occurrence of N-nitrosamines, residual nitrite and biogenic amines in commercial dry fermented sausages and evaluation of their occasional relation. *Meat Science*, *96*(2, Part A), 821-828.

Del Rio, B., Redruello, B., Linares, D. M., Ladero, V., Fernandez, M., Martin, M. C., Ruas-Madiedo, P., & Alvarez, M. A. (2017). The dietary biogenic amines tyramine and histamine show synergistic toxicity towards intestinal cells in culture. *Food Chemistry*, *218*, 249-255.

Doeun, D., Davaatseren, M., & Chung, M. (2017). Biogenic amines in foods. *Food Science and Biotechnology*, *26*(6), 1463-1474.

Dreyer, D. R., Park, S., Bielawski, C. W., & Ruoff, R. S. (2010). The chemistry of graphene oxide. *Chemical Society Reviews*, *39*(1), 228-240.

Duan, L., Wang, Y., Zhang, Y., & Liu, J. (2015). Graphene immobilized enzyme/polyethersulfone mixed matrix membrane: Enhanced antibacterial, permeable and mechanical properties. *Applied Surface Science*, *355*, 436-445.

Ekici, K., & Omer, A. K. (2018). The determination of some biogenic amines in Turkish fermented sausages consumed in Van. *Toxicology reports*, *5*, 639-643.

Erdag, D., Merhan, O., & Yildiz, B. (2018). Biochemical and pharmacological properties of biogenic amines. *Biogenic amines*, *8*, 1-14.

Eriksson, A., Bergquist, J., Edwards, K., Hagfeldt, A., MalmstrOm, D., & Agmo Hernandez, V. (2010). Optimized Protocol for On-Target Phosphopeptide Enrichment Prior to Matrix-Assisted Laser Desorption − Ionization Mass Spectrometry Using Mesoporous Titanium Dioxide. *Analytical Chemistry*, *82*(11), 4577-4583.

Erkmen, O., & Bozoglu, T. F. (2016). Spoilage of meat and meat products. *Food microbiology: principles into practice. Chichester: John Wiley & Sons*, 279-295.

Feddern, V., Mazzuco, H., Fonseca, F. N., & De Lima, G. (2019). A review on biogenic amines in food and feed: toxicological aspects, impact on health and control measures. *Animal Production Science*, *59*(4), 608-618.

Franti, E. B., Ludmila, Z. A., R, F., & V, P. (2012). Biogenic amines content in selected commercial fermented products of animal origin. *Journal of Microbiology Biotechnology & Food Sciences*, *2*(1), 209-218.

Fu, Y., Zhou, Z., Li, Y., Lu, X., Zhao, C., & Xu, G. (2016). High-sensitivity detection of biogenic amines with multiple reaction monitoring in fish based on benzoyl chloride derivatization. *Journal of Chromatography a*, *1465*, 30-37.

George-Okafor, U., Ozoani, U., Tasie, F., & Mba-Omeje, K. (2020). The efficacy of cell-free supernatants from Lactobacillus plantarum Cs and Lactobacillus acidophilus ATCC 314 for the preservation of home-processed tomato-paste. *Scientific African*, *8*, 395.

Gharsallaoui, A., Oulahal, N., Joly, C., & Degraeve, P. (2016). Nisin as a food preservative: part 1: physicochemical properties, antimicrobial activity, and main uses. *Critical Reviews in Food Science and Nutrition*, *56*(8), 1262-1274.

Goldbeck, O., Desef, D. N., Ovchinnikov, K. V., Perez-Garcia, F., Christmann, J., Sinner, P., Crauwels, P., Weixler, D., Cao, P., Becker, J., Kohlstedt, M., Kager, J., Eikmanns, B. J., Seibold, G. M., Herwig, C., Wittmann, C., Bar, N. S., Diep, D. B., & Riedel, C. U. (2021). Establishing recombinant production of pediocin PA-1 in Corynebacterium glutamicum. *Metabolic Engineering*, *68*, 34-45.

Gopal, P. K. (2022). Bacteria, Beneficial: Probiotic Lactic Acid Bacteria: An Overview. In P. L. H. McSweeney, & J. P. McNamara (Eds.), *Encyclopedia of Dairy Sciences (Third Edition)* (pp. 32-33). Oxford: Academic Press.

Guo, Y. Y., Yang, Y. P., Peng, Q., & Han, Y. (2015). Biogenic amines in wine: A review.

*International Journal of Food Science & Technology*, *50*(7), 1523-1532.

Gurunlu, B., Tasdelen Yucedag, C., Bayramoglu, M. R., & Munroe, P. (2020). Green Synthesis of Graphene from Graphite in Molten Salt Medium. *Journal of Nanomaterials*, *2020*, 7029601.

Hardiansyah, A., Tanadi, H., Yang, M., & Liu, T. (2015). Electrospinning and antibacterial activity of chitosan-blended poly (lactic acid) nanofibers. *Journal of Polymer Research*, *22*(4), 1-10.

Hazards, E. (2011). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal*, *9*(10).

He, L., Ren, J., Shi, Z., & Xu, Z. (2016). Separation of Key Biogenic Amines by Capillary Electrophoresis and Determination of Possible Indicators of Sport Fatigue in Athlete's Urine. *Journal of Chromatographic Science*, *54*(8), 1428-1434.

Honarvar, Z., Hadian, Z., & Mashayekh, M. (2016). Nanocomposites in food packaging applications and their risk assessment for health. *Electronic physician*, *8*(6), 2531.

Hossain, M. I., Sadekuzzaman, M., & Ha, S. (2017). Probiotics as potential alternative biocontrol agents in the agriculture and food industries: A review. *Food Research International*, *100*, 63-73.

Huang, X., Nzekoue, A., Silvi, S., Sagratini, G., & Verdenelli, C. (2020). Lactobacillus Strains Treatment on Commercial Packaging Paper as Preliminary Study for Extending the Shelf-Life of Chicken Meat.

Huo, Y., Qi, L., Lv, X., Lai, T., Zhang, J., & Zhang, Z. (2016). A sensitive aptasensor for colorimetric detection of adenosine triphosphate based on the protective effect of ATP-aptamer complexes on unmodified gold nanoparticles. *Biosensors and Bioelectronics*, *78*, 315-320.

Hyun, B., Cha, H., Lee, N., Yum, S., Baek, S. H., & Shin, K. (2018). Development of an ATP assay for rapid onboard testing to detect living microorganisms in ballast water. *Journal of Sea Research*, *133*, 73-80.

Ishimaru, M., Muto, Y., Nakayama, A., Hatate, H., & Tanaka, R. (2019). Determination of Biogenic Amines in Fish Meat and Fermented Foods Using Column-Switching High-Performance Liquid Chromatography with Fluorescence Detection. *Food Analytical Methods*, *12*(1), 166-175.

Jaguey-Hernandez, Y., Aguilar-Arteaga, K., Ojeda-Ramirez, D., Anorve-Morga, J., Gonzalez-Olivares, L. G., & Castaneda-Ovando, A. (2021). Biogenic amines levels in food processing: Efforts for their control in foodstuffs. *Food Research International*, *144*, 110341.

Jairath, G., Singh, P. K., Dabur, R. S., Rani, M., & Chaudhari, M. (2015). Biogenic amines in meat and meat products and its public health significance: a review. *Journal of Food Science and Technology*, *52*(11), 6835-6846.

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A., & Uyttendaele, M. (2010). Alternative microbial methods: An overview and selection criteria. *Food Microbiology*, *27*(6), 710-730.

Jayan, H., Pu, H., & Sun, D. (2020). Recent development in rapid detection techniques for microorganism activities in food matrices using bio-recognition: A review. *Trends in Food Science & Technology*, *95*, 233-246.

Kandasamy, M., Selvaraj, M., Kumarappan, C., & Murugesan, S. (2022). Plasmonic Ag nanoparticles anchored ethylene diamine modified $TiO_2$ nanowires graphene oxide composites for

dye-sensitized solar cell. *Journal of Alloys and Compounds*, *902*, 163743.

Kanmani, P., Satish Kumar, R., Yuvaraj, N., Paari, K. A., Pattukumar, V., & Arul, V. (2013). Probiotics and Its Functionally Valuable Products-A Review. *Critical Reviews in Food Science and Nutrition*, *53*(6), 641-658.

Khandelwal, G., Kumar, R., & Kumar, V. (2019). Antimicrobial activities of graphene-polymer nanocomposites. (pp. 429-445): Elsevier.

Khojastehnezhad, A., Taghavi, F., Yaghoobi, E., Ramezani, M., Alibolandi, M., Abnous, K., & Taghdisi, S. M. (2021). Recent achievements and advances in optical and electrochemical aptasensing detection of ATP based on quantum dots. *Talanta*, *235*, 122753.

Kim, H., Kosinski, P., Kung, C., Dang, L., Chen, Y., Yang, H., Chen, Y., Kramer, J., & Liu, G. (2017). A fit-for-purpose LC-MS/MS method for the simultaneous quantitation of ATP and 2,3-DPG in human K2EDTA whole blood. *Journal of Chromatography B*, *1061-1062*, 89-96.

Kuley, E., Yazgan, H., Ozogul, Y., Ucar, Y., Durmus, M., Ozyurt, G., & Ayas, D. (2021). Effectiveness of Lactobacilli cell-free supernatant and propolis extract microcapsules on oxidation and microbiological growth in sardine burger. *Food Bioscience*, *44*, 101417.

Kuorwel, K. K., Cran, M. J., Sonneveld, K., Miltz, J., & Bigger, S. W. (2011). Antimicrobial Activity of Biodegradable Polysaccharide and Protein-Based Films Containing Active Agents. *Journal of Food Science*, *76*(3), R90-R102.

Latorre-Moratalla, M. L., Comas-Baste, O., Bover-Cid, S., & Vidal-Carou, M. C. (2017). Tyramine and histamine risk assessment related to consumption of dry fermented sausages by the Spanish population. *Food and Chemical Toxicology*, *99*, 78-85.

Lee, S. J., Kim, J. H., Jung, Y. W., Park, S. Y., & Kim, G. W. (2011). Composition of Organic Acids and Physiological Functionality of Commercial Makgeolli. *Korean Journal of Food Science & Technology*, *43*(2), 206-212.

Lee, Y., Kung, H., Wu, C., Hsu, H., Chen, H., Huang, T., & Tsai, Y. (2016). Determination of histamine in milkfish stick implicated in food-borne poisoning. *Journal of Food and Drug Analysis*, *24*(1), 63-71.

Li, S., Johansson, M., Vidanarachchi, J. K., Pickova, J., & Zamaratskaia, G. (2016). Determination of biogenic amines in aerobically stored beef using high-performance thin-layer chromatography densitometry. *Acta Agriculturae Scandinavica, Section A—Animal Science*, *66*(4), 199-205.

Li, Y., Yu, Z., Zhu, Y., & Cao, Z. (2020). Selection of nitrite-degrading and biogenic amine-degrading strains and its involved genes. *Food Quality and Safety*, *4*(4), 225-235.

Linares, D. M., Del Rio, B., Redruello, B., Ladero, V., Martin, M. C., Fernandez, M., Ruas-Madiedo, P., & Alvarez, M. A. (2016). Comparative analysis of the in vitro cytotoxicity of the dietary biogenic amines tyramine and histamine. *Food Chemistry*, *197*, 658-663.

Liu, L., Jin, T. Z., Coffin, D. R., & Hicks, K. B. (2009). Preparation of Antimicrobial Membranes: Coextrusion of Poly(lactic acid) and Nisaplin in the Presence of Plasticizers. *Journal of Agricultural and Food Chemistry*.

Liu, Q., Wu, J., Lim, Z. Y., Aggarwal, A., Yang, H., & Wang, S. (2017). Evaluation of the metabolic

response of Escherichia coli to electrolysed water by 1H NMR spectroscopy. *Lwt-Food Science and Technology*, *79*, 428-436.

Liu, S., Xu, J., Ma, C., & Guo, C. (2018). A comparative analysis of derivatization strategies for the determination of biogenic amines in sausage and cheese by HPLC. *Food Chemistry*, *266*, 275-283.

Liu, Y., Han, F., Liu, Y., & Wang, W. (2020). Determination of Biogenic Amines in Wine Using Modified Liquid-Liquid Extraction with High Performance Liquid Chromatography-Fluorescence Detector. *Food Analytical Methods*, *13*(4), 911-922.

Liu, Y., Sameen, D. E., Ahmed, S., Dai, J., & Qin, W. (2021). Antimicrobial peptides and their application in food packaging. *Trends in Food Science & Technology*, *112*, 471-483.

Lorenzo, J. M., Munekata, P. E. S., & Dominguez, R. (2017). Role of autochthonous starter cultures in the reduction of biogenic amines in traditional meat products. *Current Opinion in Food Science*, *14*, 61-65.

Malaczewska, J., & Kaczorek-Lukowska, E. (2021). Nisin−A lantibiotic with immunomodulatory properties: A review. *Peptides*, *137*, 170479.

Mandal, P. K., & Biswas, A. K. (2020). Chapter 16 - Modern techniques for rapid detection of meatborne pathogens. In A. K. Biswas, & P. K. Mandal (Eds.), *Meat Quality Analysis* (pp. 287-303): Academic Press.

Manetta, A. C., Di Giuseppe, L., Tofalo, R., Martuscelli, M., Schirone, M., Giammarco, M., & Suzzi, G. (2016). Evaluation of biogenic amines in wine: Determination by an improved HPLC-PDA method. *Food Control*, *62*, 351-356.

Manikandan, M., Hasan, N., & Wu, H. (2012). Rapid and direct detection of attomole adenosine triphosphate (ATP) by MALDI-MS using rutile titania chips. *Analyst*, *137*(21), 5128-5134.

Mariutti, L. R. B., & Bragagnolo, N. (2017). Influence of salt on lipid oxidation in meat and seafood products: A review. *Food Research International*, *94*, 90-100.

Martins, I. M., Rodrigues, S. N., Barreiro, F., & Rodrigues, A. E. (2009). Microencapsulation of thyme oil by coacervation. *Journal of Microencapsulation*, *26*(8), 667-675.

Mehdizadeh, T., Tajik, H., Langroodi, A. M., Molaei, R., & Mahmoudian, A. (2020). Chitosan-starch film containing pomegranate peel extract and Thymus kotschyanus essential oil can prolong the shelf life of beef. *Meat Science*, *163*, 108073.

Mejia-Gomez, C. E., & Balczar, N. (2020). Isolation, characterisation and continuous culture of Lactobacillus spp. and its potential use for lactic acid production from whey. *Ciencia e Tecnologia de Alimentos*(6).

Melnikov, M., Rogovskii, V., Boyko, A., & Pashenkov, M. (2018). The influence of biogenic amines on Th17-mediated immune response in multiple sclerosis. *Multiple Sclerosis and Related Disorders*, *21*, 19-23.

Mempin, R., Tran, H., Chen, C., Gong, H., Kim Ho, K., & Lu, S. (2013). Release of extracellular ATP by bacteria during growth. *Bmc Microbiology*, *13*(1), 301.

Michela, Menegollo, Isabella, Tessari, Luigi, Bubacco, Gyorgy, & Szabadkai. (2019). Determination of ATP, ADP, and AMP Levels by Reversed-Phase High-Performance Liquid Chromatography in

Cultured Cells. *Methods in molecular biology (Clifton, N.J.)*, *1925*, 223-232.

Mohammed, H., Kumar, A., Bekyarova, E., Al-Hadeethi, Y., Zhang, X., Chen, M., Ansari, M. S., Cochis, A., & Rimondini, L. (2020). Antimicrobial mechanisms and effectiveness of graphene and graphene-functionalized biomaterials. A scope review. *Frontiers in Bioengineering and Biotechnology*, *8*, 465.

Mora, L., Hernandez-Cazares, A. S., Aristoy, M., & Toldra, F. (2010). Hydrophilic interaction chromatographic determination of adenosine triphosphate and its metabolites. *Food Chemistry*, *123*(4), 1282-1288.

Moradi, M., Kousheh, S., Almasi, H., Alizadeh, A., & Lotfi, A. (2020). Postbiotics produced by lactic acid bacteria: The next frontier in food safety. *Comprehensive Reviews in Food Science and Food Safety*(11).

Mora-Villalobos, J. A., Montero-Zamora, J., Barboza, N., Rojas-Garbanzo, C., & JP, L. (2020). Multi-Product Lactic Acid Bacteria Fermentations: A Review. *Fermentation*, *6*(23), 23.

Motaghifar, A., Akbari-Adergani, B., Rokney, N., & Mottalebi, A. (2020). Evaluating red meat putrefaction in long term storage in freezing condition based on co-variation of major biogenic amines and Total Volatile Nitrogen. *Food Science and Technology*, *41*, 123-128.

Mukurumbira, A. R., Shellie, R. A., Keast, R., Palombo, E. A., & Jadhav, S. R. (2022). Encapsulation of essential oils and their application in antimicrobial active packaging. *Food Control*, *136*, 108883.

Munir, M. A., Inayatullah, A., & Badrul, H. A. (2021). Fish analysis containing biogenic amines using gas chromatography equipped with flame ionization and mass spectrometer detectors. *Science and Technology Indonesia*, *6*(1), 1-7.

Neofotistos, A., Tsagkaris, A. S., Danezis, G. P., & Proestos, C. (2019). *Emerging Trends in Biogenic Amines Analysis*.: Biogenic Amines.

Ng, S., Lim, H. S., Ma, Q., & Gao, Z. (2016). Optical Aptasensors for Adenosine Triphosphate. *Theranostics*, *6*(10), 1683-1702.

Oshita, S., Al-Haq, M. I., Kawagishi, S., Makino, Y., Kawagoe, Y., Ye, X., Shinozaki, S., & Hiruma, N. (2011). Monitoring of ATP and viable cells on meat surface by UV – Vis reflectance spectrum analysis. *Journal of Food Engineering*, *107*(2), 262-267.

Ozogul, F., Kacar, C., & Hamed, I. (2015). Inhibition effects of carvacrol on biogenic amines formation by common food-borne pathogens in histidine decarboxylase broth. *Lwt-Food Science and Technology*, *64*(1), 50-55.

Ozogul, Y., & Ozogul, F. (2019). Biogenic amines formation, toxicity, regulations in food.

Palecek, E., Billova, S., Havran, L., Kizek, R., Miculkova, A., & Jelen, F. (2002). DNA hybridization at microbeads with cathodic stripping voltammetric detection. *Talanta*, *56*(5), 919-930.

Pandey, S., Sharma, K., & Gundabala, V. (2022). Antimicrobial bio-inspired active packaging materials for shelf life and safety development: A review. *Food Bioscience*, *48*, 101730.

Papageorgiou, M., Lambropoulou, D., Morrison, C., Klodzinska, E., Namieśnik, J., & Plotka-Wasylka, J. (2018). Literature update of analytical methods for biogenic amines determination in food and

beverages. *TrAC Trends in Analytical Chemistry*, *98*, 128-142.

Papageorgiou, M., Lambropoulou, D., Morrison, C., Namieśnik, J., & Plotka-Wasylka, J. (2018). Direct solid phase microextraction combined with gas chromatography – Mass spectrometry for the determination of biogenic amines in wine. *Talanta*, *183*, 276-282.

Park, J., Seo, J., Lee, J., & Kwon, H. (2015). Distribution of Seven N-Nitrosamines in Food. *Toxicological Research*, *31*(3), 279-288.

Paul, M. M. (2017). Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review. *Molecules*, *22*(8).

Piergiovanni, L., & Limbo, S. (2019). Food shelf-life models. (pp. 49-60): Elsevier.

Pinto, F., Carotenuto, G., & Meo, M. (2015). Preparation and thermomechanical characterisation of graphene nanoplatelets/low-density polyethylene composites. *Journal of Thermoplastic Composite Materials*, *28*(6), 745-761.

Pinto, L., Bonifacio, M. A., De Giglio, E., Santovito, E., Cometa, S., Bevilacqua, A., & Baruzzi, F. (2021). Biopolymer hybrid materials: Development, characterization, and food packaging applications. *Food Packaging and Shelf Life*, *28*, 100676.

Pisoschi, A. M., Pop, A., Georgescu, C., Turcus, V., Olah, N. K., & Mathe, E. (2018). An overview of natural antimicrobials role in food. *European Journal of Medicinal Chemistry*, *143*, 922-935.

Pothakos, V., Devlieghere, F., Villani, F., Bjorkroth, J., & Ercolini, D. (2015). Lactic acid bacteria and their controversial role in fresh meat spoilage. *Meat Science*, *109*, 66-74.

Punia Bangar, S., Chaudhary, V., Singh, T. P., & Ozogul, F. (2022). Retrospecting the concept and industrial significance of LAB bacteriocins. *Food Bioscience*, *46*, 101607.

Ratphitagsanti, W., Park, E., Lee, C. S., Amos Wu, R. Y., & Lee, J. (2012). High-throughput detection of spore contamination in food packages and food powders using tiered approach of ATP bioluminescence and real-time PCR. *LWT - Food Science and Technology*, *46*(1), 341-348.

Roselino, M. N., Maciel, L. F., Sirocchi, V., Caviglia, M., Sagratini, G., Vittori, S., Taranto, M. P., & Cavallini, D. C. U. (2020). Analysis of biogenic amines in probiotic and commercial salamis. *Journal of Food Composition and Analysis*, *94*, 103649.

Rosinska, D., & Lehotay, J. (2014). Influence of temperature on production of biogenic amines in pork, beef, and poultry and their hplc determination after postcolumn derivatization. *Journal of Liquid Chromatography & Related Technologies*, *37*(5), 609-619.

Ruiz-Capillas, C., & Herrero, A. M. (2019). Impact of biogenic amines on food quality and safety. *Foods*, *8*(2), 62.

Sadaka, F., Nguimjeu, C., Brachais, C., Vroman, I., Tighzert, L., & Couvercelle, J. (2014). WITHDRAWN: Review on antimicrobial packaging containing essential oils and their active biomolecules. *Innovative Food Science & Emerging Technologies*.

Sagratini, G., Fernandez-Franzon, M., Berardinis, F. D., Font, G., Vittori, S., & Ma Es, J. (2012). Simultaneous determination of eight underivatised biogenic amines in fish by solid phase extraction and liquid chromatography – tandem mass spectrometry. *Food Chemistry*, *132*(1), 537-543.

Salomoni, R., Leo, P., Montemor, A. F., Rinaldi, B. G., & Rodrigues, M. (2017). Antibacterial effect of silver nanoparticles in Pseudomonas aeruginosa. *Nanotechnology, science and applications*, *10*, 115.

Sang, X., Ma, X., Hao, H., Bi, J., Zhang, G., & Hou, H. (2020). Evaluation of biogenic amines and microbial composition in the Chinese traditional fermented food grasshopper sub shrimp paste. *LWT*, *134*, 109979.

Santangelo, M. F., Libertino, S., Turner, A. P. F., Filippini, D., & Mak, W. C. (2018). Integrating printed microfluidics with silicon photomultipliers for miniaturised and highly sensitive ATP bioluminescence detection. *Biosensors and Bioelectronics*, *99*, 464-470.

Scaffaro, R., Botta, L., Maio, A., Mistretta, M. C., & La Mantia, F. P. (2016). Effect of graphene nanoplatelets on the physical and antimicrobial properties of biopolymer-based nanocomposites. *Materials*, *9*(5), 351.

Schirone, M., Esposito, L., D Onofrio, F., Visciano, P., Martuscelli, M., Mastrocola, D., & Paparella, A. (2022). Biogenic Amines in Meat and Meat Products: A Review of the Science and Future Perspectives., *Foods*, vol. 11.

Sherma, J., & Rabel, F. (2018). A review of thin layer chromatography methods for determination of authenticity of foods and dietary supplements. *Journal of Liquid Chromatography & Related Technologies*, *41*(10), 645-657.

Shinozaki, Y., Sato, J., Igarashi, T., Suzuki, S., Nishimoto, K., & Harada, Y. (2013). Evaluation of an Improved Bioluminescence Assay for the Detection of Bacteria in Soy Milk. *Biocontrol Science*, *18*(1), 1-7.

Silva, C., Silva, S., & Ribeiro, S. C. (2018). Application of Bacteriocins and Protective Cultures in Dairy Food Preservation. *Frontiers in Microbiology*, *9*, 594.

Singh, N., Naveen Kumar, S. K., Geethika, M., & Mugesh, G. (2021). A Cerium Vanadate Nanozyme with Specific Superoxide Dismutase Activity Regulates Mitochondrial Function and ATP Synthesis in Neuronal Cells. *Angewandte Chemie International Edition*, 60(6), 3121-3130.

Siragusa, G. R., Dorsa W. J., Cutter C. N., & Kooh-maraie L. J. (1996). Use of a newly developed rapid microbial ATP bioluminescence assay to detect microbial contamination on poultry carcasses. *Journal of Bioluminescence & Chemiluminescence*, *11*(11), 297-301.

Siripongpreda, T., Siralertmukul, K., & Rodthongkum, N. (2020). Colorimetric sensor and LDI-MS detection of biogenic amines in food spoilage based on porous PLA and graphene oxide. *Food Chemistry*, *329*, 127165.

Slavov, S., Alusta, P., Buzatu, D. A., & Wilkes, J. G. (2017). Feature selection from mass spectra of bacteria for serotyping Salmonella. *Journal of Analytical and Applied Pyrolysis*, *124*, 393-402.

Smith, C. J., & Osborn, A. M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *Fems Microbiology Ecology*, *67*(1), 6-20.

Soro, A. B., Noore, S., Hannon, S., Whyte, P., Bolton, D. J., O Donnell, C., & Tiwari, B. K. (2021). Current sustainable solutions for extending the shelf life of meat and marine products in the packaging process. *Food Packaging and Shelf Life*, *29*, 100722.

Sottofattori, E., Anzaldi, M., & Ottonello, L. (2001). HPLC determination of adenosine in human synovial fluid. *Journal of Pharmaceutical and Biomedical Analysis*, *24*(5-6), 1143-1146.

Spinler, J. K., Taweechotipatr, M., Rognerud, C. L., Ou, C. N., Tumwasorn, S., & Versalovic, J. (2008). Human-derived probiotic Lactobacillus reuteri demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. *Anaerobe*, *14*(3), 166-171.

Srivastava, A. K., Dwivedi, N., Dhand, C., Khan, R., Sathish, N., Gupta, M. K., Kumar, R., & Kumar, S. (2020). Potential of graphene-based materials to combat COVID-19: properties, perspectives, and prospects. *Materials Today Chemistry*, *18*, 100385.

Suppakul, P., Miltz, J., Sonneveld, K., & Bigger, S. W. (2003). Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications. *Journal of Food Science*, *68*(2), 408-420.

Takahashi, M. (2018). Aptamers targeting cell surface proteins. *Biochimie*, *145*, 63-72.

Tao, J., Huang, X., Ling, F., Yu, B., Zhou, X., Shen, Q., & Sagratini, G. (2022). Immobilization of Lactic acid bacteria for production of extracellular polysaccharides. *Food Science and Technology*, *42*.

Thummar, V. D., & Ramani, V. M. (2016). Recent application of lactic acid bacteria as source of industrially important compounds.

Tittarelli, F., Perpetuini, G., Di Gianvito, P., & Tofalo, R. (2019). Biogenic amines producing and degrading bacteria: A snapshot from raw ewes' cheese. *LWT*, *101*, 1-9.

Triki, M., Herrero, A. M., Jimenez-Colmenero, F., & Ruiz-Capillas, C. (2018). Quality Assessment of Fresh Meat from Several Species Based on Free Amino Acid and Biogenic Amine Contents during Chilled Storage., *Foods*, vol. 7.

Ugarova, N. N., Lomakina, G. Y., Modestova, Y., Chernikov, S. V., Vinokurova, N. V., O trashevskaya, E. V., & Gorbachev, V. Y. (2016). A simplified ATP method for the rapid control of cell viability in a freeze-dried BCG vaccine. *Journal of Microbiological Methods*, *130*, 48-53.

Umesha, S., & Manukumar, H. M. (2018). Advanced molecular diagnostic techniques for detection of food-borne pathogens: Current applications and future challenges. *Critical Reviews in Food Science and Nutrition*, *58*(1), 84-104.

van Heel, A. J., Montalban-Lopez, M., & Kuipers, O. P. (2011). Evaluating the feasibility of lantibiotics as an alternative therapy against bacterial infections in humans. *Expert Opinion On Drug Metabolism & Toxicology*, *7*(6), 675-680.

Vanne, L., Karwoski, M., Karppinen, S., & Sjoberg, A. M. (1996). HACCP-based food quality control and rapid detection methods for microorganisms. *Food Control*, *7*(6), 263-276.

Vasconcelos, H., de Almeida, J. M. M. M., Matias, A., Saraiva, C., Jorge, P. A. S., & Coelho, L. C. C. (2021). Detection of biogenic amines in several foods with different sample treatments: An overview. *Trends in Food Science & Technology*, *113*, 86-96.

Verma, N., Hooda, V., Gahlaut, A., Gothwal, A., & Hooda, V. (2020). Enzymatic biosensors for the quantification of biogenic amines: a literature update. *Critical Reviews in Biotechnology*, *40*(1), 1-14.

Vilela, C., Kurek, M., Hayouka, Z., Rocker, B., Yildirim, S., Antunes, M. D. C., Nilsen-Nygaard, J., Pettersen, M. K., & Freire, C. S. R. (2018). A concise guide to active agents for active food packaging. *Trends in Food Science & Technology*, *80*, 212-222.

Villalobos-Delgado, L. H., Nevarez-Moorillon, G. V., Caro, I., Quinto, E. J., & Mateo, J. (2019). Natural antimicrobial agents to improve foods shelf life. (pp. 125-157): Elsevier.

Visciano, P., Schirone, M., & Paparella, A. (2020). An Overview of Histamine and Other Biogenic Amines in Fish and Fish Products., *Foods*, vol. 9.

Wang, K., Sun, D., Pu, H., & Wei, Q. (2019). Surface-enhanced Raman scattering of core-shell Au@ Ag nanoparticles aggregates for rapid detection of difenoconazole in grapes. *Talanta*, *191*, 449-456.

Wang, L., Hu, C., & Shao, L. (2017). The antimicrobial activity of nanoparticles: present situation and prospects for the future. *International Journal of Nanomedicine*, *12*, 1227.

Wang, P., Cheng, Z., Chen, Q., Qu, L., Miao, X., & Feng, Q. (2018). Construction of a paper-based electrochemical biosensing platform for rapid and accurate detection of adenosine triphosphate (ATP). *Sensors and Actuators B: Chemical*, *256*, 931-937.

Webster, T. T. (2010). Bactericidal effect of iron oxide nanoparticles on Staphylococcus aureus. *International Journal of Nanomedicine*.

Wei, J., Li, Y., Si, Q., Xiao, Q., Chen, Q., Jiao, T., Chen, Q., & Chen, X. (2022). Hemin/G-quadruplex based electrochemical sensor for highly sensitive detection of ATP in fish. *Journal of Electroanalytical Chemistry*, 116374.

Wei, Q., Wang, X., Sun, D., & Pu, H. (2019). Rapid detection and control of psychrotrophic microorganisms in cold storage foods: A review. *Trends in Food Science & Technology*, *86*, 453-464.

Wojnowski, W., Kalinowska, K., Majchrzak, T., Plotka-Wasylka, J., & Namieśnik, J. (2019). Prediction of the Biogenic Amines Index of Poultry Meat Using an Electronic Nose., *Sensors*, vol. 19.

Xia, M., Xie, Y., Yu, C., Chen, G., Li, Y., Zhang, T., & Peng, Q. (2019). Graphene-based nanomaterials: the promising active agents for antibiotics-independent antibacterial applications. *Journal of Controlled Release*, *307*, 16-31.

Xie, J., Ming, Z., Li, H., Yang, H., Yu, B., Wu, R., Liu, X., Bai, Y., & Yang, S. (2016). Toxicity of graphene oxide to white rot fungus Phanerochaete chrysosporium. *Chemosphere*, *151*, 324-331.

Xue, X. F., Zhou, J. H., Wu, L. M., Fu, L. H., & Zhao, J. (2009). HPLC determination of adenosine in royal jelly. *Food Chemistry*, *115*(2), 715-719.

Yan, X., Ji, Y., Xiao, Y., Xue, X., Liu, J., Li, S., Ai, F., & Zheng, X. (2021). One-pot label-free dual-aptasensor as a chemiluminescent tool kit simultaneously detect adenosine triphosphate and chloramphenicol in foods. *Talanta*, *229*, 122226.

Yong, H. P., Hamidon, F., Rajangan, C., Soh, K. P., & Min, T. L. (2016). Application of Probiotics for the Production of Safe and High-quality Poultry Meat. *Korean Journal for Food Science of Animal Resources*, *36*(5), 567-576.

Yoon, H., Park, J. H., Choi, A., Hwang, H., & Mah, J. (2015). Validation of an HPLC analytical method for determination of biogenic amines in agricultural products and monitoring of biogenic amines in Korean fermented agricultural products. *Toxicological research*, *31*(3), 299-305.

Youssef, A. M., EL-Sayed, S. M., EL-Sayed, H. S., Salama, H. H., & Dufresne, A. (2016). Enhancement of Egyptian soft white cheese shelf life using a novel chitosan/carboxymethyl cellulose/zinc oxide bionanocomposite film. *Carbohydrate Polymers*, *151*, 9-19.

Yuan, B., Zhu, T., Zhang, Z., Jiang, Z., & Ma, Y. (2011). Self-assembly of multilayered functional films based on graphene oxide sheets for controlled release. *Journal of Materials Chemistry*, *21*(10), 3471-3476.

Zannini, E., Waters, D. M., Coffey, A., & Arendt, E. K. (2016). Production, properties, and industrial food application of lactic acid bacteria-derived exopolysaccharides. *Applied Microbiology & Biotechnology*, *100*(3), 1121-1135.

Zhang, Y., Yu, J., Lai, S., Song, J., Wu, X., Wang, D., Pang, L., & Chai, T. (2021). Rapid determination of histamine level in seafood using read-out strips based on high-performance thin layer chromatography modified with self-visualization nanomaterials. *Food Control*, *122*, 107816.

Zhang, Y., Zhang, Y., Zhou, Y., Li, G., Yang, W., & Feng, X. (2019). A review of pretreatment and analytical methods of biogenic amines in food and biological samples since 2010. *Journal of Chromatography a*, *1605*, 360361.

Zhao, W., Liu, Y., Kwok, L., Cai, T., & Zhang, W. (2020). The immune regulatory role of Lactobacillus acidophilus: An updated meta-analysis of randomized controlled trials. *Food Bioscience*, *36*, 100656.

Zhao, Y., An, J., Su, H., Li, B., Liang, D., & Huang, C. (2022). Antimicrobial food packaging integrating polysaccharide-based substrates with green antimicrobial agents: A sustainable path. *Food Research International*, *155*, 111096.

# CHAPTER II

# FORMATION OF BIOGENIC AMINES AND GROWTH OF SPOILAGE-RELATED MICROORGANISMS IN THE REFRIGERATED HAM WITH DIFFERENT COMMERCIAL PACKAGES

## 2.1. ABSTRACT

The current chapter focuses on the preservation of ham, since our first food model of the project was ham, in order to better understand the interactions between packaging and ham.

Ham is one of the traditional fermented products. Some of the compounds formed during the hydrolysis and oxidation of proteins and fats during ham processing form a distinctive ham flavor. Many microbes are involved in this process and biochemical reactions. Ham is fresh, tender, nutritious and contains no preservatives. It is very popular among consumers. However, sterilization is not complete, microbial spoilage can easily occur and shelf life is short, which is the bottleneck in the production and preservation of ham products. Spoilage and pathogenic microorganisms are the most important factors affecting food safety and quality, and food packaging is the most important technical link to inhibit spoilage and pathogenic microorganisms in food transportation. The aim of this study was to investigate the development of BAs and spoilage-causing microorganisms in ham stored at 4°C under different commodity packaging.

## 2.2. INTRODUCTION

Hams are generally cuts of pork that come from the hind leg and can be cooked and served fresh, although most of them are cured in some way, i.e., dry cured and smoked or wet cured and then cooked or smoked (Valous, Mendoza, & Sun, 2010). Italian ham called "Prosciutto" is one of the most popular and famous Italian food in the world. Among them, 3 cooked hams (Cotto, Scelto, Alta Qualità) and 5 raw hams (Modena, Nazionale, Parma, San Daniele, Speck) are the most widely used and internationally known (Table 2-1) (Lucarini, et al., 2013). Raw ham and cooked ham are two important ready-to-eat cooked meat products that have found favor among many peoples. The production process may vary slightly according to the tradition of each production area. Cooked ham is made from boned pork legs that are seasoned with a special flavoring mixture (salt, pepper, juniper, and laurel) and then steam-cooked at about 70 °C. Raw ham is obtained by salting and then seasoning the leg (hind limb) of the pig. The preparation combines two preservation methods: salting with a special aromatic mixture (salt, pepper, juniper, rosemary and bay leaf) and a mild smoking phase according to traditional family recipes. Dry aging allows to improve and enhance the flavor and sensory characteristics. The addition of nitrates and nitrites is allowed in both raw and cooked hams (Lucarini, et al., 2013).

The production of cooked meat ham involves several steps that regulate the composition of microorganisms and their concentration, which affects the shelf life of the product (Blanco Lizarazo, Sierra-Cadavid, Montoya R, & Ospina-E, 2022). The raw materials for cooked ham contain a variety of microorganisms, mainly from the genus LAB, Pseudomonas and Brochothrix. Studies have shown that Leuconostoc spp. is the most important genus in spoiled cooked ham (Blanco Lizarazo, Sierra-Cadavid,

Montoya R, & Ospina-E, 2022). The proportion and composition of the microbiota varies depending on processing, storage, and batch. Nevertheless, cooking reduces the concentration and diversity of spoilage and pathogenic bacteria. However, due to cross-contamination during slicing, cooling, and packaging, the likelihood of recontamination by microorganisms increases (Cauchie, et al., 2020).

**Table 2-1.** Moisture, ash, protein, lipid and energy content in traditional Italian ham (fresh weight)

| Ham | Moisture (g/100g) | Ash (g/100g) | Protein (g/100g) | Lipid (g/100g) | Energy kcal/portion (50g) |
|---|---|---|---|---|---|
| **Cooked ham** | | | | | |
| Cotto | 72.2 ± 1.1 | 2.8 ± 0.2 | 15.7 ± 1.0 | 7.6 ± 1.1 | 69 |
| Scelto | 70.0 ± 1.2 | 2.7 ± 0.2 | 17.5 ± 1.1 | 9.2 ± 1.6 | 77 |
| Alta Qualità | 66.8 ± 2.3 | 2.6 ± 0.2 | 18.0 ± 1.5 | 11.9 ± 2.6 | 91 |
| **Raw ham** | | | | | |
| Modena | 45.6 ± 3.0 | 5.8 ± 0.6 | 25.6 ± 1.6 | 22.9 ± 3.5 | 154 |
| Nazionale | 50.5 ± 3.2 | 6.6 ± 1.4 | 27.8 ± 2.5 | 13.7 ± 5.1 | 117 |
| Parma | 50.3 ± 2.0 | 5.5 ± 0.4 | 25.9 ± 1.5 | 18.3 ± 2.8 | 134 |
| San Daniele | 50.2 ± 2.1 | 5.3 ± 0.5 | 25.7 ± 1.4 | 18.6 ± 2.8 | 135 |
| Speck | 43.6 ± 3.9 | 5.3 ± 1.3 | 30.7 ± 2.8 | 19.1 ± 5.1 | 150 |

Values are the M ± SD of three analyses.

Ham fermentation depends on the environment and indigenous microorganisms to form a rich microbiota that is critical for flavor and aroma development. High microbial populations have been found on the surface of dry-cured ham, which can influence the aging process. Molds and yeasts are present in large numbers on the surface of dry-cured ham and influence the formation of volatile compounds and the development of flavor characteristics. Low microbial counts inside dry-cured ham are

usually below 6 log CFU/g. However, in exceptional cases, an abundant microbiota was found with total aerobic counts approaching 8 log CFU/g, with LAB being the predominant microbial group (Zhu, et al., 2021). Gram-positive, catalase-positive cocci are the most important microorganisms in dry-cured ham. Their proteolytic, lipolytic, catalase and nitrate reductase activities may contribute to the sensory characteristics of the product (Picon & Nunez, 2022).

Although the curing process can extend the shelf life of foods, studies have shown that due to the microbial diversity of dry-cured meats, microbial hazards and potentially toxic metabolic compounds may eventually occur, with BAs being particularly prominent (Mutz, et al., 2019). BAs are present in numerous dry-cured meats due to the growth of fermentative bacteria such as LAB and spoilage bacteria such as Enterobacteriaceae and *Pseudomonas* spp. (Rosario, Rodrigues, Bernardes, & Conte-Junior, 2021; Sang, et al., 2020). Among them, TYR, PUT, HIS, CAD and SPD are the most common (Vasconcelos, et al., 2021).

However, there are currently no data on the changes of BAs in different ham packages. In order to monitor and control the quality of ham, it is necessary to detect the quality changes and common indicators of ham in different packages, provide a theoretical basis for the objective representation of ham spoilage, and provide technical support for the safety control and monitoring system of ham. In this study, the development of BAs and spoilage-causing microorganisms in ham under different conditions was investigated by principal component analysis (PCA).

# 2.3. MATERIALS AND METHODS

## 2.3.1. Packaging materials

8 Tested packages kindly provided by ESSEOQUATTRO s.p.a. industry (Carmignano di Brenta, Italy), an Italian manufacturer of packaging for fresh food. For over 40 years it has been offering innovative products, tested to protect the health of the consumer, technologically advanced to facilitate the consumption of fresh and environmentally-friendly foods. Esseoquatro offers several lines of food packaging.

The packaging samples “multilayer” protected by European Patent EP 1584464 A1. The base packaging was a sheet comprising a first layer formed by a virgin kraft of pure cellulose with very low weights and coupled to a second layer made of high density (HD) polyethylene (PE) with a third metallic layer (silver colour given by the presence of an aluminum powder equal to 0.005 gr/sqm) fixed between them (Figure 2-1). The polycoupled sheet was composed of 100% pure long-fibber cellulose; The paper bag was in kraft with the characteristic havana colour. Ovtene is an eco-friendly food packaging, 100% recyclable material composed mainly of calcium carbonate and other trace minerals. This reduces polyethylene use by 60% while conserving resources and reducing waste.

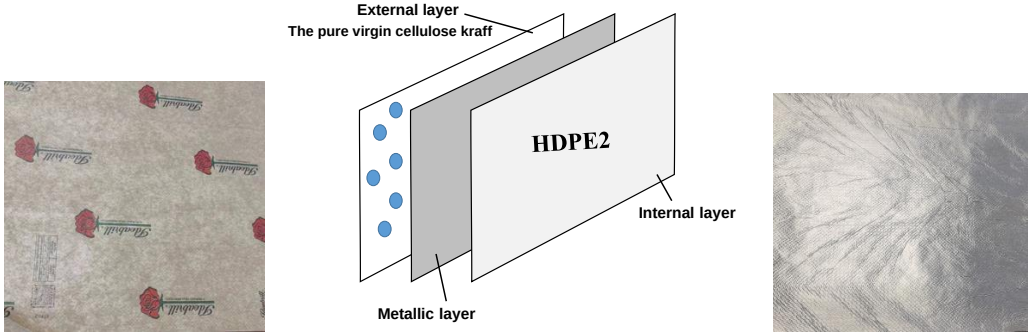



**Figure 2-1.** Technical structure of the used “multilayer” packaging

In our study, we evaluated principally 8 types of packaging assembled in 8 different ways for their ability to conserved raw and cooked ham (Figure 2-2). The packagings were:

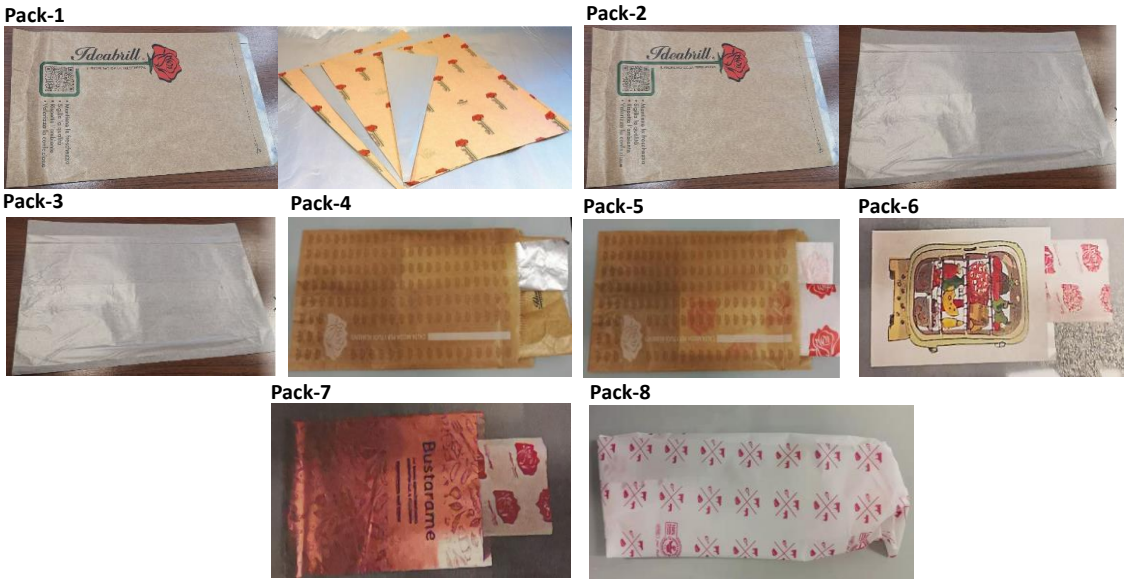



**Figure 2-2.** 8 types of packaging combinations.

Pack 1: multilayer sheet + multilayer bag

Pack 2: polycoupled sheet with wings + multilayer bag

Pack 3: polycoupled sheet with wings

Pack 4: multilayer sheet + kraft paper bag

Pack 5: polycoupled sheet + kraft paper bag

Pack 6: polycoupled sheet + metallized bag

Pack 7: polycoupled sheet + copper bag

Pack 8: Ovtene

## 2.3.2. Sample collection


**Figure 2-3.** Raw ham and cooked ham.

Raw ham (Prosciutto crudo 100 g) and cooked ham (Prosciutto cotto 150 g) were supplied by a local supermarket in Camerino. Each packaging is realized with around 3 slices (10-12 g/slice) of ham for the analysis at T2, T5 and T7 for both the raw and the cooked ham, and samples were maintained at 4 °C to simulate the storage conditions of the consumer. A part (around 40 g) of the ham was reserved and analysed the same day as the control (T0) for each part. As 3 packages of the sample was used to perform the test each day. During the day of the analysis, the sample was separated: 20 g for the microbiological analysis, 15 g for the chemical analysis and 5 g for pH measurement. All analyses were performed in triplicate.

**Figure 2-4.** Raw ham in the polycoupled bag.

**Figure 2-5.** Cooked ham in multilayer sheet, with under the kraft paper bag.

### 2.3.3. Chemicals and Reagents

Tryptamine hydrochloride (TRY, $C_{10}H_{12}N_2 \cdot HCl$, >99%, CAS No. 343-94-2), 2-phenylethylamine hydrochloride (PHE, $C_8H_{11}N \cdot HCl$, >98%, CAS No. 156-28-5), putrescine dihydrochloride (PUT, $C_4H_{12}N_2 \cdot 2HCl$, >98%, CAS No. 333-93-7), cadaverine dihydrochloride (CAD, $C_5H_{14}N_2 \cdot 2HCl$, >98%, CAS No. 1476-39-7), histamine dihydrochloride (HIS, $C_5H_9N_3 \cdot 2HCl$, >99%, CAS No. 56-92-8), tyramine hydrochloride (TYR, $C_8H_{11}NO \cdot HCl$, >98%, CAS No. 60-19-5), spermidine trihydrochloride (SPD, $C_7H_{17}N_3 \cdot 3HCl$, >98%, CAS No. 334-50-9) and spermine tetrahydrochloride (SPM, $C_{10}H_{26}N_4 \cdot 4HCl$, >98%, CAS No. 306-67-2) for standard solutions preparation were provided by Sigma-Aldrich (Milano, Italy). 1,7-diaminoheptane (98%, CAS No. 646-126 19-5) as the internal standard was provided by Sigma-Aldrich (Milano, Italy). Trichloroacetic acid (TCA, ≥99.0%, CAS No. 76-03-9), acetone (≥99.5% CAS No. 67-64-1), hydrochloric acid (HCl, 37%, CAS No. 7647-01-0), sodium hydroxide anhydrous (NaOH, ≥98%, CAS No.1310-73-2), acetonitrile ($CH_3CN$, HPLC gradient grade, ≥99.9%, CAS No. 75-05-8), sodium carbonate anhydrous ($Na_2CO_3$, ≥99.5%, CAS No. 497-19-8), dansyl chloride

($C_{12}H_{12}ClNO_2S$, 98% CAS No. 605-65-2) and methanol ($CH_3OH$, HPLC gradient grade, ≥99.9%, CAS No. 67-56-1) for extraction and derivatization were from Sigma-Aldrich (Milano, Italy).

A stock solution of BAs was prepared by dissolving 10 mg of each compound in 10 mL HCl 0.1 M (Merck Darmstadt, Germany) and was stored in a glass stopper bottle at 4 °C. Standard working solutions of different concentrations, prepared daily with stock solutions of different aliquots appropriately diluted with deionized water (resistivity <8 MΩ×cm), from the Milli-Q SP Reagent Water System (Millipore, 139 Bedford, MA, USA). The derivatization solution was prepared by dansyl chloride in acetone (10%).

### 2.3.4. Analysis of BAs

Chemical methods are based on the monitoring of molecules that allow to determine the degree of deterioration of the food. The BAs analytical procedure is based on previously published methods with slight modifications (Sirocchi, et al., 2013). Each slice of ham was cut into thin strips and ground with a blender, then 5 g was extracted in a centrifuge tube with 5% TCA by Ultra-Turrax S 18N-10G homogenizer (IKA-Werke Gmbh & Co., Germany). Added 0.2 mL of a 10 mg/L 1,7-diaminoheptane solution as internal standard, 0.3 mL of $Na_2CO_3$ saturated solution and 50 µL of NaOH 2N to 1 mL of isolated supernatant. For the derivatization, used 2 mL of dansyl chloride solution and place the sample at 45 °C for 45 min. Then, eliminated excess dansyl chloride by adding 100 µL of $NH_4OH$ 28%. SPE STRATA X 33 µ Cartridges, 200 mg/6 mL (Phenomenex, Bologna, Italy) were conditioned with 5 mL of $CH_3CN$ followed by 5 mL of Milli-Q water. Samples

were purified by the cartridge and elution with 4 mL $CH_3CN$. Samples were stored at 4 ℃ and filtered on a 0.45 µm PTFE filter (Supelco Bellefonte, Pennsylvania, USA) prior to analysis. BAs separation was achieved using the Gemini C18 analysis column (250×4.6 mm I.D., particle size 4 µm) from Phenomenex (Torrance, CA, USA). The column was constant at 25 ℃. The mobile phases analyzed by HPLC were Milli-Q water (A) and $CH_3OH/CH_3CN$ 70:30 *v*/*v* solution (B) at a flow rate of 0.5 mL/min. The gradient program was: 0 min 60% B, 10 min 70% B, 20 min 90% B, 26 min 100% B, 29 min 100% B, and 32 min 60% B up to 40 min. The injection volume was 20 µL. The HPLC system was coupled to a Diode Array Detector (DAD). Measured the peak response at 254 nm.

In this study, the amines monitored as shelf-life markers were 8: tryptamine (TRY), 2-phenylethylamine (2-PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), tyramine (TYR), spermidine (SPD), spermine (SPM).

In addition, specific indexes were determined as freshness markers: Biogenic Amine Index (BAI), Chemical Quality Index (CQI), Spermidine/Spermine ratio (SPD/SPM) and the Total of the monitored Biogenic Amines (Total BAs). These indexes were obtained according to the following formula:

BAI = putrescine + cadaverine + histamine + tyramine

CQI = (putrescine+cadaverine+histamine)/(spermine+spermidine+1)

SPD/SPM = spermidine/spermine

Total BAs = putrescine + cadaverine + histamine + tyramine +spermine +spermidine + phenylethylamine.

The CQI was proposed by(MIETZ & KARMAS, 1977) to evaluate the quality of fishand seafood. The B.A.I was created by Veciana- (Veciana-Nogues, Marine-Font, & Vidal-Carou, 1997) to improve the C.Q.I. The SPD/SPM ratio was proposed by

(Silva & Gloria, 2002) and is considered suitable to assess the chicken meat quality (Sirocchi, et al., 2013). The Total BAs was used to have more ample vision on the BAs evolution in the different type of samples.

### 2.3.5. Microbiological analysis

Microbiological analyses are an important tool for assessing the level of food safety and hygiene. In this study reliable microbiological parameters were taken into account to define the hygienic conditions of cooked ham and raw ham, found at the time of deposition of the products in the different types of packaging in the studio, and to monitor them during the shelf-life period of the products lasting 7 days.

10 g of ham from every packaging were homogenized in 90 ml of peptone solution (0.1%) in a Stomacher - Easy MIX (AES Laboratory, Bruz, France). A series of ten-fold dilutions ($10^{-2}$ to $10^{-10}$) was prepared and a given amount of each dilution was spread on several specific media: Plate Count Agar (PCA, Oxoid), Violet Red Bile Glucose Agar (VRBGA, Oxoid), Agar Base with selective supplements CFC, (PAB, Oxoid) and Streptomycin Thallous Acetate Actidione Agar (STAA, Oxoid), Tryptose sulphite cycloserine Agar TSC supplemented with streptomycin sulphate and thallous acetate for counting of mesophilics bacteria, Enterobacteriaceae, *Pseudomonas* spp. and *Brochotrix thermosphacta*, and *Clostridium perfringens* respectively.

The bacterial counts were made after 24 - 48 h of aerobic incubation at 25 °C both for *Pseudomonas* spp. and *B. thermosphacta*, after 24 - 48 h of aerobic incubation at 37 °C both for mesophilic bacteria and for Enterobacteriaceae. The C. *perfringens* was incubated and count in anaerobic. Gram stain, morphological and biochemical

analysis were performed on selected colonies isolated from each sample in order to confirm the strain identity.

Standard pathogenic strains - *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* DSM 1103 - were purchased from ATCC and DSM. Stock strains stored at -20 °C in 15% (w/w) glycerol were re-activated in Tryptic soy broth (TSB) (Oxoid) at 37 °C for 24-48 h. After incubation, single colonies of each pathogenic strain were isolated on Tryptic soy agar (TSA, Oxoid). All selected bacteria were cultured in corresponding broth for 24-48 h at 37 °C under aerobic conditions.

### 2.3.6. pH measurement

5 g of ham sample were chopped and subsequently transferred into a sterile stomacher bag. The pH of each sample at every time points were measured by an electronic pH meter (Mettler Toledo, Columbus, UK) equipped with a probe for solids.

### 2.3.7. Statistical analysis

Each monitored BA was detected by comparing its specific retention time and UV spectrum with relative standard. The concentration of the analytes was determined not directly from their peak area but from the response factor (R. F = analyte peak area/I.S peak area) to avoid any operator bias. The calibration curves used for analyte quantification were generated by plotting the response factors of BAs standard versus the concentrations.

Analyses were performed in triplicate and data were expressed as mean ± standard deviation (SD). The relative standard deviation (%RSD = 100×S.D/mean) was

calculated to control the precision of the results obtained. T-test Student was applied to assess whether the results differences between the compared packaging were statistically significant. Probability level ($p$<0.05) was considered statistically significant.

Every measurement was repeated at least 3 times. Data were processed by analysis of variance (ANOVA). Principal components analysis (PCA) was also applied to the data of indicators in each packaging. All statistical procedures were computed using SPSS 25 and Origin 2021.

## 2.4. RESULTS AND DISCUSSION

### 2.4.1. Linearity of the analytical method

Because the method was already validated previously, we only established the linearity of the analytical method itself. The linearity of the analytical method was evaluated using HPLC-DAD and the internal standard 1,7-diaminoheptane. In fact, a standard mixture containing the 8 main BAs (TRY, PHE, PUT, CAD, HIS, TYR, SPD, SPM) was injected progressively and at increasing concentrations in HPLC-DAD (0.5, 1, 5, 10, 25 ppm) in the presence of a known concentration of the internal standard (1,7-diaminoheptane) in table 2-2.

Table 2-3 reports the respective values of the correlation coefficients $R^2$ of the analyzed molecules are reported, all the coefficients confirm the linearity of the method ($R^2 \geq 0.994$).

The analytical methods were previously validated in accordance with the standards of the European Regulation on Quantitative Confirmation Methods (EC 2002/657). HPLC-DAD chromatogram of the 25 mg/L BAs mixture and

1,7-diaminoheptane used as an internal standard is shown in Figure 2-6. Calculate the calibration curve for each BA was using the response factor (ratio of the BA peak area to the inner standard peak area). Under these HPLC conditions, each BA and internal standard was clearly resolved, indicating that the method can be used for the quantitative determination of BAs in food samples.

**Table 2-2.** Response factors to various concentrations.

| BAs | 1 ppm | 5 ppm | 10 ppm | 25 ppm |
|---|---|---|---|---|
| **TRY** | 0.872 | 2.714 | 4.904 | 11.027 |
| **PHE** | 0.770 | 2.632 | 5.057 | 11.262 |
| **PUT** | 0.482 | 3.265 | 7.222 | 15.077 |
| **CAD** | 0.725 | 3.345 | 7.036 | 14.214 |
| **HIS** | 0.067 | 0.300 | 0.686 | 2.293 |
| **TYR** | 1.492 | 4.834 | 8.930 | 22.441 |
| **SPD** | 0.133 | 1.738 | 4.469 | 10.321 |
| **SPM** | 0.061 | 1.207 | 3.477 | 8.230 |

**Table 2-3.** Linearity coefficient of the 8 BAs selected.

| Biogenic Amines | $R^2$ |
|---|---|
| Tryptamine (TRY) | 0.996 |
| Phenylalanine (PHE) | 0.997 |
| Putrescine (PUT) | 0.995 |
| Cadaverine (CAD) | 0.994 |
| Histamine (HIS) | 0.995 |
| Tyramine (TYR) | 0.996 |
| Spermidine (SPD) | 0.996 |
| Spermine (SPM) | 0.999 |

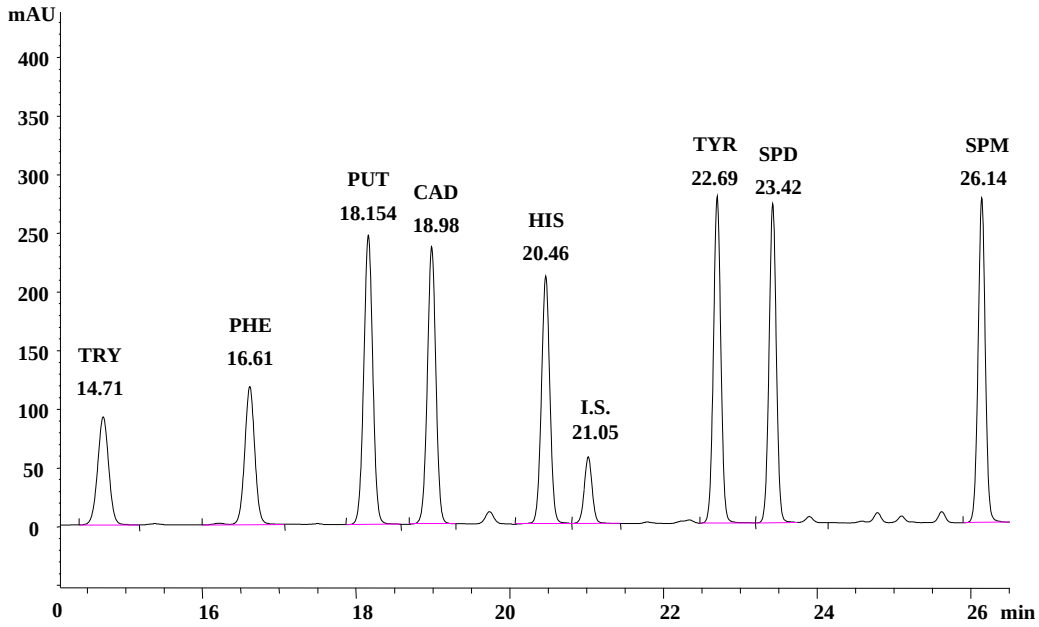



**Figure 2-6.** HPLC-DAD chromatogram of the 25 mg/L standard solution of all analysed BAs and Internal Standard. Legend: Tryptamine (TRY), Phenylethylamine (PHE), Putrescine (PUT), Cadaverine (CAD), Histamine (HIS), 1,7- Diaminoheptane Internal Standard (I.S.), Tyramine (TYR), Spermidine (SPD), Spermine (SPM)

## 2.4.2. Comparative study on packaging of cooked ham

The aim of the present study was to evaluate and compare the efficacy of 8 types of packaging materials on the preservation of cooked ham by monitoring biogenic amines, pH and microbiological parameters (CeIRSA, 2017). Microbial strains included total aerobic mesophiles, *Escherichia coli* (β-glucoronidase positive), Enterobacteriaceae, *Staphylococcus* spp. (coagulase positive), *Bacillus cereus* (presumed), Sulfite-Reducing Anaerobic bacteria, *Clostridium perfringens.* The counts of Sulfite-Reducing Anaerobic bacteria, *Clostridium perfringens* and *E. coli* (β-glucoronidase positive) were under detection limits in ham during the whole study period.

### 2.4.2.1. Research packaging 1, 2, 3

Changes in biogenic amines of cooked ham during 7-day storage in Pack 1, 2, and

3 in Figure 2-7. Looking at the global BA values, the efficiency of package 1 tends to be higher than that of Pack 3 in T2 and Pack 2 in T5. However, the differences are not statistically significant.

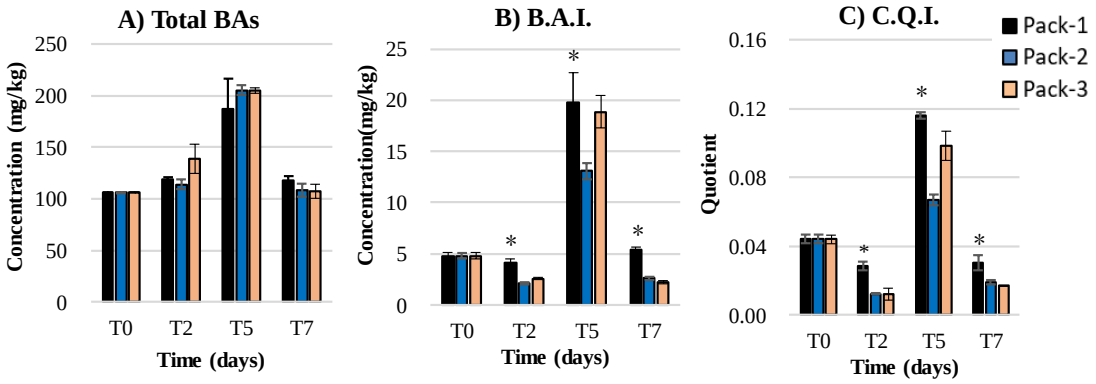



**Figure 2-7.** Changes of biogenic amines of cooked ham during 7 days of storage inside of Pack 1, 2 and 3. A) The total of the monitored biogenic amines, B) Biogenic amines index, C) Chemical quality index. **Statistically significant different ($p<0.05$, Student's t test)*

The pH values of the 3 packaged ham samples continued to increase during storage, with the largest difference at T5, the highest at Pack 2, and the lowest at Pack 3 (Figure 2-8).

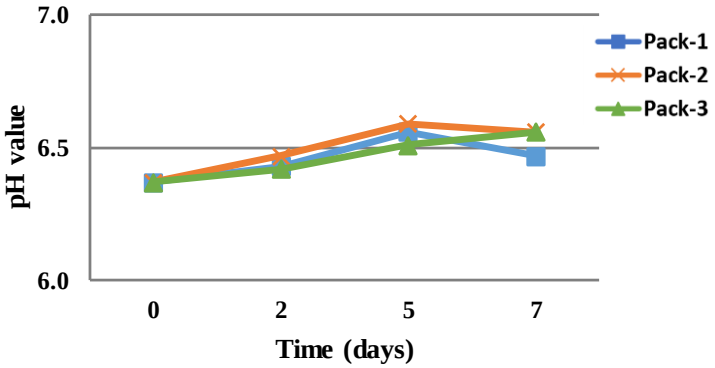



**Figure 2-8.** Changes of pH value of cooked ham during 7 days of storage inside of Pack 1, 2 and 3.

After 5 and 7 days of storage, the levels of total aerobic mesophiles, total Enterobacteriaceae, and *Bacillus cereus* detected on the ham in Pack 2 and Pack 3 were significantly lower than in Pack 1 (Figure 2-9A, B, and D). After 5 and 7 days of

storage, the levels of total *Staphylococcus* spp. (coagulase-positive) were also significantly lower in Pack 2 and Pack 3 than in Pack 1. In addition, the levels detected on ham in Pack 3 were significantly lower than those in Pack 2 (Figure 2-9C). In general, Pack 2 and Pack 3 showed better preservation of microbiological quality of cooked ham by limiting the levels of aerobic mesophiles, Enterobacteriaceae, *B. cereus*, *Staphylococcus* spp. (coagulase positive) up to 7 days of storage compared to Pack 1 (a significant difference was observed especially after 2 days of storage). In addition, Pack 2 was more efficient in limiting *Staphylococcus* spp. than Pack 3. Concluding, Pack 2 and Pack 3 were more efficient in maintaining the microbiological quality than Pack 1, with values also consistent with the acceptance of the guidelines for microbiological analysis of food (CeIRSA, 2017).

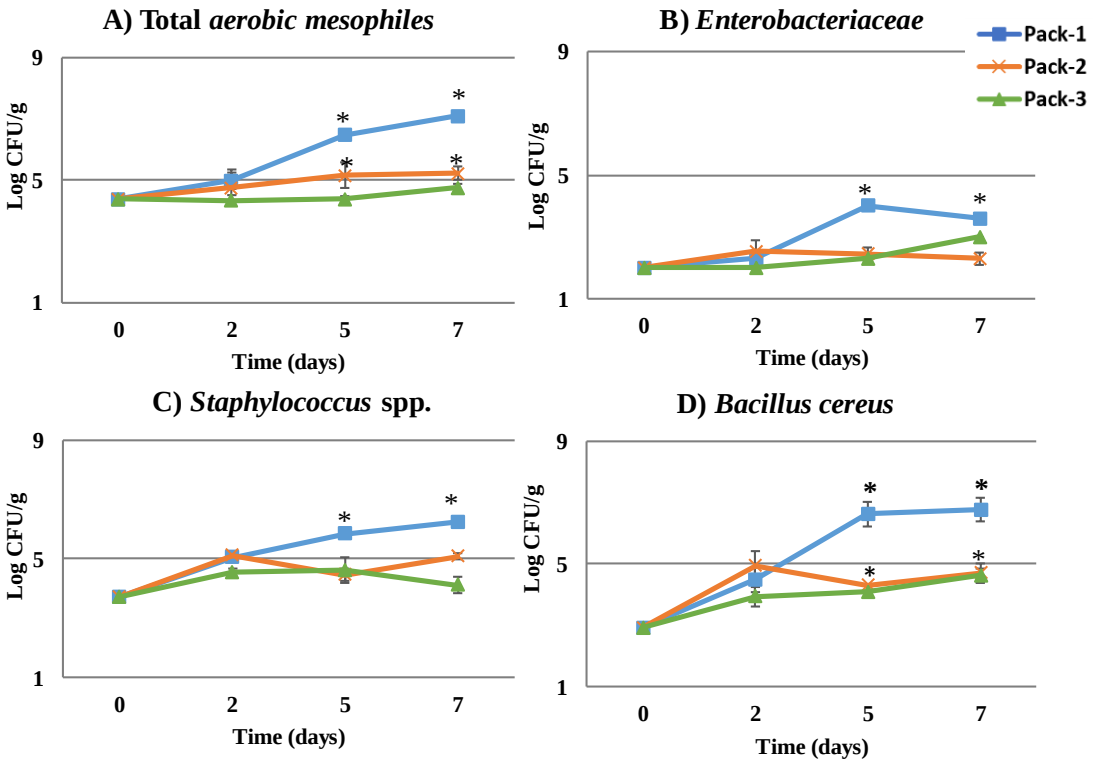



**Figure 2-9.** Changes of microorganism of cooked ham during 7 days of storage inside of Pack 1, 2 and 3. A) Total aerobic mesophiles, B) Enterobacteriaceae, C) *Staphylococcus* spp., D) *Bacillus cereus*. **Statistically significant different ($p<0.05$, Student's t test)*

### 2.4.2.2. Research packaging 1, 4, 5

The aim of the present study was to evaluate and compare the efficiency of 3 packaging materials (Pack 1, 4 and 5) in preserving cooked ham by monitoring biogenic amines, pH and microbiological parameters. In relation to the studied indices, Pack 1 shows a higher efficiency than Pack 4 and 5. In fact, the total concentrations of biogenic amines are lower in the samples of cooked ham stored in Pack 1. These differences are significant at T2 and T7 (Figure 2-10).

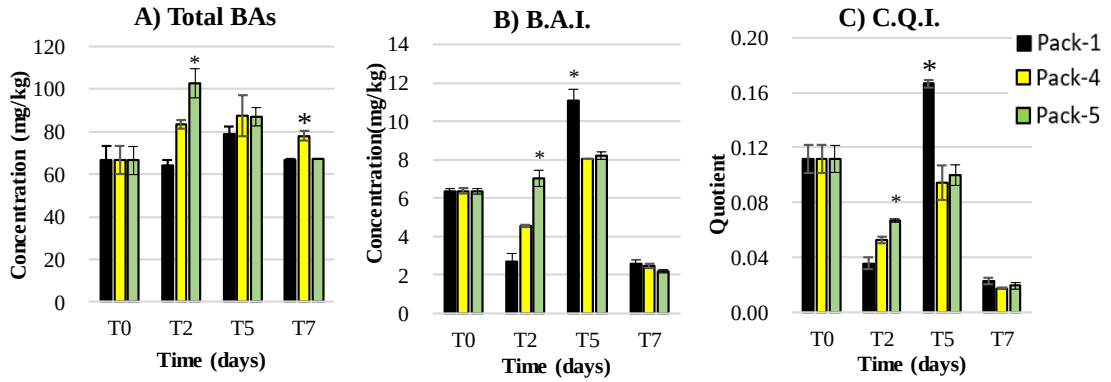



**Figure 2-10.** Changes of biogenic amines of cooked ham during 7 days of storage inside of Pack 1, 4 and 5. A) The total of the monitored biogenic amines, B) Biogenic amines index, C) Chemical quality index. **Statistically significant different ($p<0.05$, Student's t test)*

The pH values of the 3 packaged ham samples hardly changed 5 days before storage, and the Pack 4 Pack 5 decreased on day 7 (Figure 2-11).

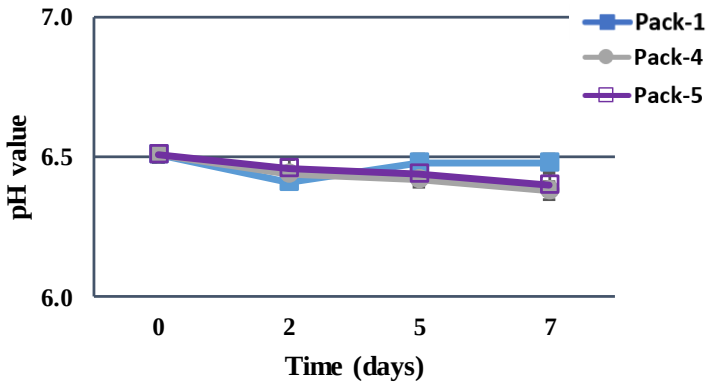



**Figure 2-11.** Changes of pH value of cooked ham during 7 days of storage inside of Pack 1, 4 and 5.

As Figure 2-12A shows, Pack 5 was the most efficient in limiting the growth of aerobic mesophiles in the samples of cooked ham for 5 days in the refrigerator compared to the other two packaging bags. The number of mesophiles found in the samples inside Pack 5 was significantly lower than those inside Pack 4 after 2 days and lower than Pack 1 after 5 days of storage. All packaging systems were efficient in maintaining low levels of Enterobacteriaceae in the ham during 7 days of storage. There was no significant difference between them (Figure 2-12B).

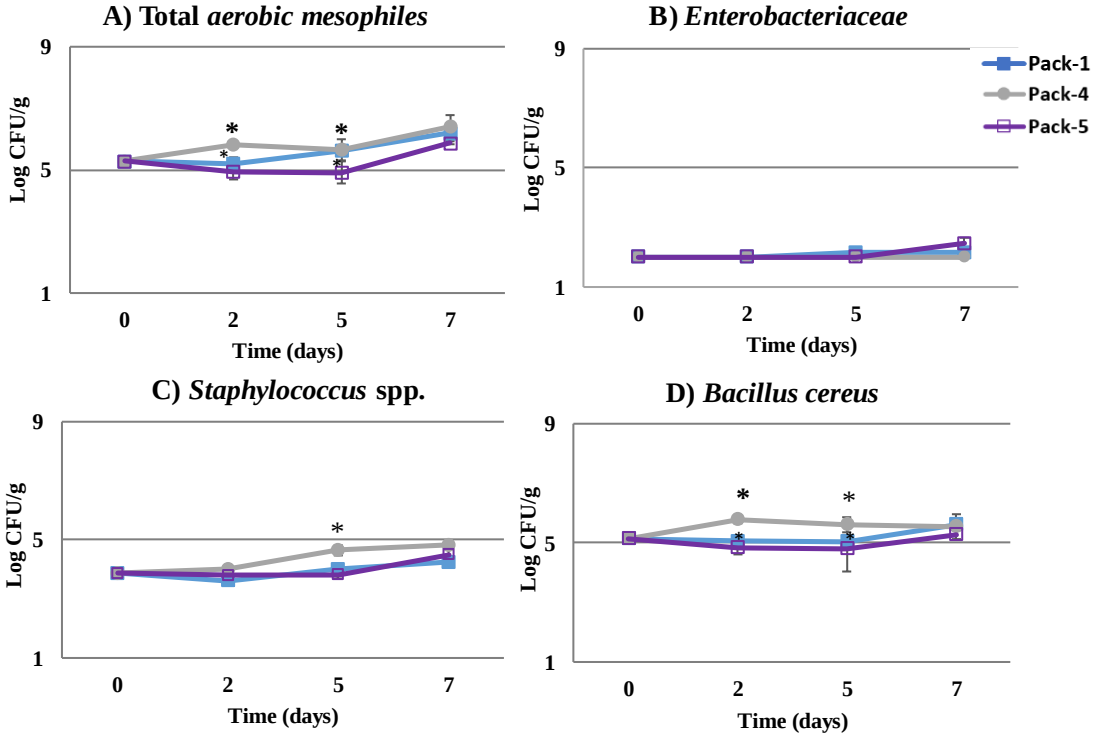



**Figure 2-12.** Changes of microorganism of cooked ham during 7 days of storage inside of Pack 1, 4 and 5. A) Total aerobic mesophiles, B) Enterobacteriaceae, C) *Staphylococcus* spp., D) *Bacillus cereus*. **Statistically significant different ($p<0.05$, Student's t test)*

Pack 1 and Pack 5 performed better than Pack 4 in controlling the number of *Staphylococcus* spp. (coagulase positive) throughout the study period, while Pack 1 and Pack 5 showed excellent abilities in preserving cooked ham for at least 5 days in cold storage (Figure 2-12C). Pack 1 was significantly better than Pack 4 at controlling

the amount of *B. cereus* over 5 days, while the ham in Pack 5 had significantly less *B. cereus* than Pack 4 after 2 days. In summary, Pack 5 was the most efficient compared to the other two packaging bags (Figure 2-12D). According to the guidelines for microbiological analysis of food (CeIRSA, 2017), cooked ham is still considered acceptable when the amount of aerobic mesophiles is below 6 log CFU/g, so in this case, Pack 1 and Pack 5 were more efficient throughout the study period, with values always below 6 log CFU/g. The amount of Enterobacteriaceae was below 3 Log CFU/g, which is still acceptable, as expected. Thus, the study showed that all packages were able to maintain ham quality for up to 7 days. *Staphylococcus* spp. counts (coagulase positive) should be less than 4 log CFU/g according to the guidelines (CeIRSA, 2017), so Pack 1 and Pack 5 were effective for up to 4 days. *B. cereus* already had values of 5 log CFU/g at the beginning of the study, even if this is not acceptable from the point of view of efficiency, the value of Pack 1 and Pack 5 was kept stable without the number increasing over time.

#### 2.4.2.3. Research packaging 1, 6, 7

The aim of the present study was to evaluate and compare the efficiency of 3 types of packing materials (Pack 1, 6 and 7) in the preservation of cooked ham by monitoring biogenic amines, pH and microbiological parameters. The total BA of slight differences during the shelf life. However, the three packages (Pack 1, 6 and 7) showed exceptionally high efficiency compared to the other packages studied. In fact, the samples stored in these packages showed no significant increase in BA during the study (Figure 2-13).

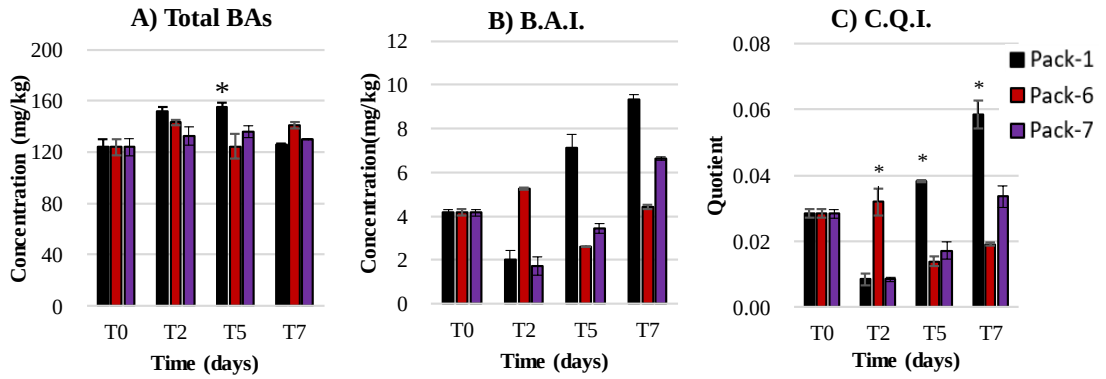



**Figure 2-13.** Changes of biogenic amines of cooked ham during 7 days of storage inside of Pack 1, 6 and 7. A) The total of the monitored biogenic amines, B) Biogenic amines index, C) Chemical quality index. **Statistically significant different ($p<0.05$, Student's t test)*

The pH values of all ham samples decreased steadily during the storage period, with around 0.5 in samples stored in Pack 6 and 7 While, around 0.75 in Pack 1 stored ham (Figure 2-14).

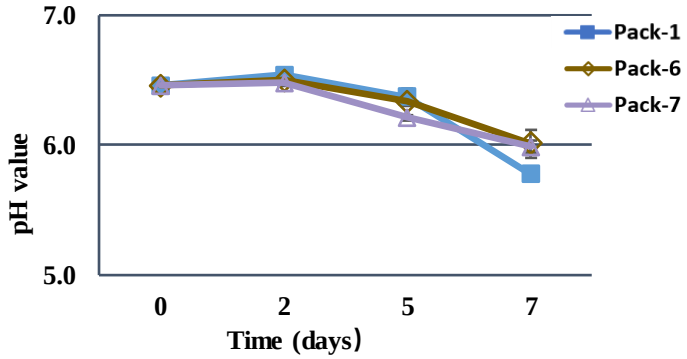



**Figure 2-14.** Changes of pH value of cooked ham during 7 days of storage inside of Pack 1, 6 and 7.

For total aerobic mesophiles, Pack 6 maintained its value below 6 log for 5 days, with a slight increase after 7 days. In contrast, the sample from Pack 8 contained mesophiles below 6 Log CFU/g for up to 2 days (Figure 2-15A). With respect to Enterobactriaceae, all samples showed an increase after 2 days of storage, and this amount was maintained after 5 days, followed by a further increase after 7 days. Pack 1 had a relatively lower amount of Enterobactriaceae than the other two samples

(Figure 2-15B). Pack 6 and Pack 7 were able to maintain relatively low levels of *Staphylococcus* spp. at T2 storage, with this low growth trend noted only for Pack 6 during the following period (Figure 2-15C). Figure 2-15D shows that all samples showed a similar growth trend over time, while Pack 6 showed better limitation of numbers compared to the other samples. In general, Pack 6 showed better preservation of the microbiological quality of the cooked ham by limiting the amounts of total aerobic mesophiles, *B. cereus* and *Staphylococcus* spp. until T7 storage compared to the other two packagings (significant differences were observed mainly after 2, 5 and 7 days). In addition, Pack 1 was efficient in limiting the amounts of Enterobacteriaceae up to 5 days. In conclusion, Pack 6 best preserves the microbiological quality of the cooked ham, maintaining values compatible with the acceptance criteria established in the guidelines for microbiological analysis of foods.

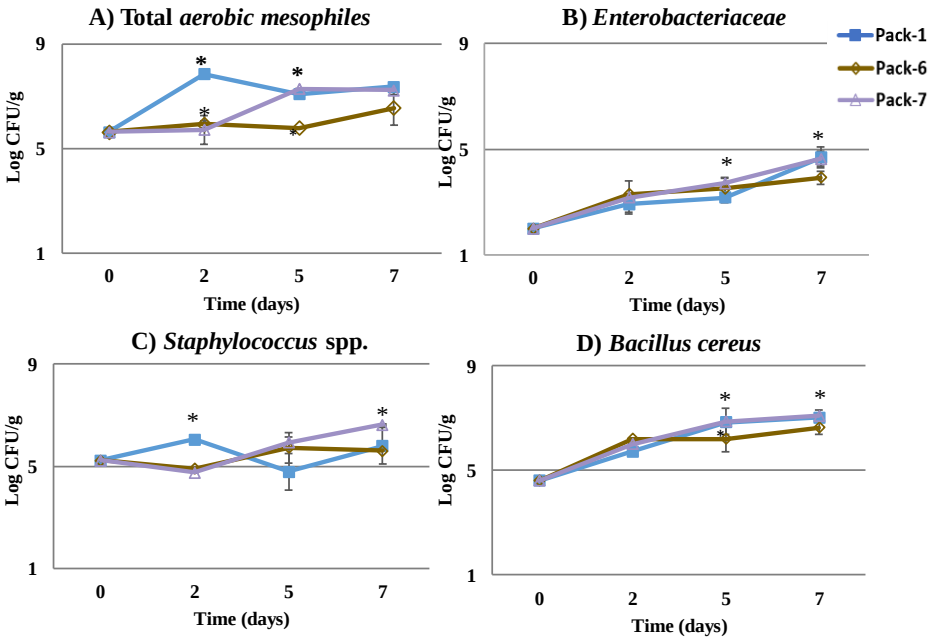



**Figure 2-15.** Changes of microorganism of cooked ham during 7 days of storage inside of Pack 1, 6 and 7. A) Total aerobic mesophiles, B) Enterobacteriaceae, C) *Staphylococcus* spp., D) *Bacillus cereus*. **Statistically significant different ($p<0.05$, Student's t test)*

### 2.4.2.4. Research packaging 1, 8

The aim of the present study was to evaluate and compare the efficiency of 2 packaging materials ( Pack 1 and 8) in the preservation of cooked ham by monitoring biogenic amines, pH and microbiological parameters. The evolution of the studied indices during the shelf life of cooked ham shows a trend until T5, in which the samples stored in Pack 1 have lower BA levels than those stored in Pack 8. However, these differences are not statistically significant (Figure 2-16).

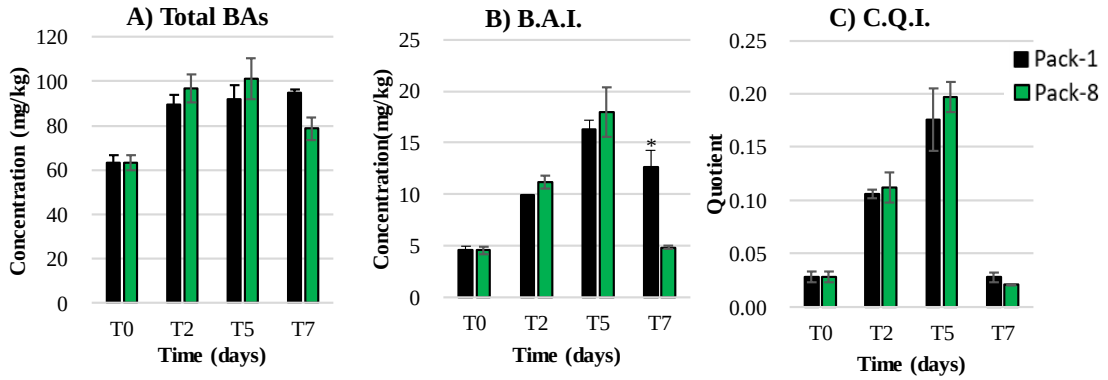



**Figure 2-16.** Changes of biogenic amines of cooked ham during 7 days of storage inside of Pack 1 and 8. A) The total of the monitored biogenic amines, B) Biogenic amines index, C) Chemical quality index. **Statistically significant different ($p<0.05$, Student's t test)*

The pH values of 2 packaged ham samples remained unchanged in the 7 days prior to storage (Figure 2-17).

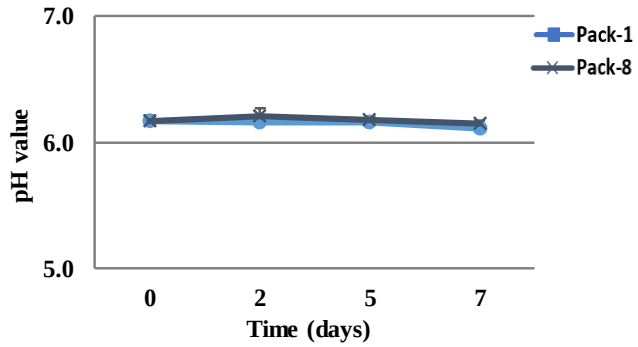



**Figure 2-17.** Changes of pH value of cooked ham during 7 days of storage inside of Pack 1 and 8.

In Pack 1 and Pack 8, the product has the same values for all considered microbial

counts: *aerobic mesophilic* microorganisms, Enterobacteriaceae, presumably *B. cereus*, coagulase-positive *Staphylococci* (Figure 2-18). The cooked ham behaved the same in Pack 1 and Pack 8.

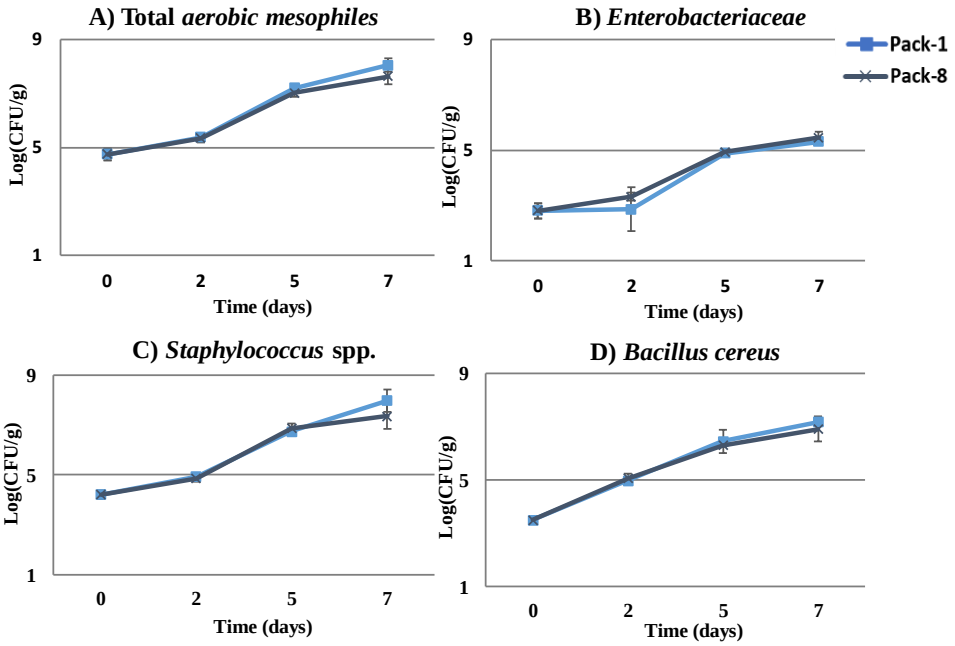



**Figure 2-18.** Changes of microorganism of cooked ham during 7 days of storage inside of Pack 1and 8. A) Total aerobic mesophiles, B) Enterobacteriaceae, C) *Staphylococcus* spp., C) *Bacillus cereus. *Statistically significant different (p<0.05, Student's t test)*

## 2.4.3. Comparative study on packaging of raw ham

The aim of the present study was to evaluate and compare the efficiency of 8 types of packaging materials in the preservation of cured ham by monitoring biogenic amines, pH, and microbiological parameters (CeIRSA, 2017). Microbial strains include *E. coli* (β-glucoronidase positive), Enterobacteriaceae, *Staphylococcus* spp. (coagulase positive), Sulfite-Reducing Anaerobic bacteria, *Clostridium perfringens*.

### 2.4.3.1. Research packaging 1, 2, 3

The aim of the present study was to evaluate and compare the efficacy of 3 types of packing materials (Pack 1, 2 and 3) on the preservation of raw ham by monitoring

biogenic amines, pH and microbiological parameters. Considering the global BA values, Pack 1, 2 and 3 shows the same efficiency in preserving raw ham up to T7.

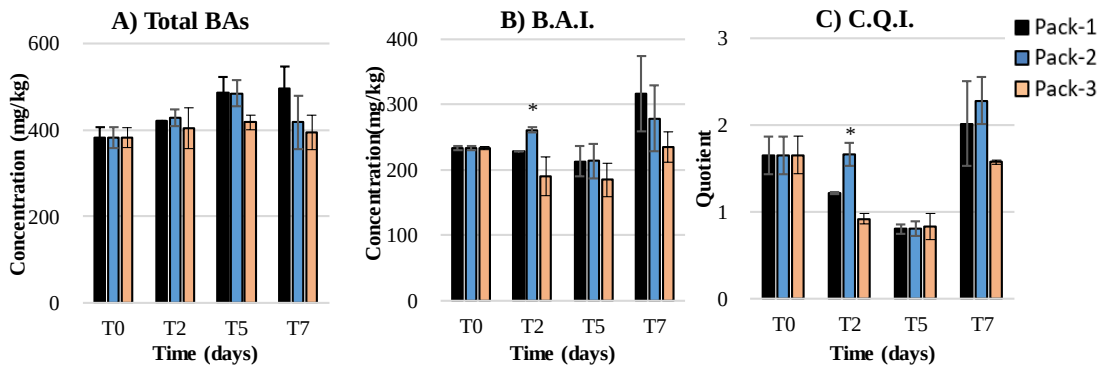



**Figure 2-19.** Changes of biogenic amines of raw ham during 7 days of storage inside of Pack 1, 2 and 3. A) The total of the monitored biogenic amines, B) Biogenic amines index, C) Chemical quality index. **Statistically significant different ($p<0.05$, Student's t test)*

The pH values of the 3 packaged ham samples remained unchanged during the 7 days before storage (Figures 2-20).

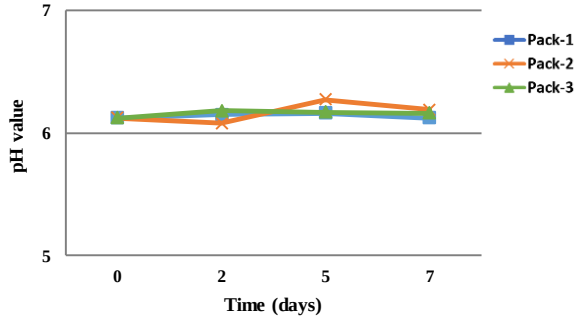



**Figure 2-20.** Changes of pH value of cooked ham during 7 days of storage inside of Pack 1, 2 and 3.

Although there was no significant difference between the different packages, the number of *Staphylococcus* spp. (coagulase positive) detected in the ham inside Pack 1 was lower than in the other two packaging materials. All three packages were effective in controlling the growth of Enterobacteriaceae, *E. coli (*β-glucoronidase positive), Sulfite-Reducing Anaerobic bacteria, *Clostridium perfringens*. The respective bacterial counts were all below the detection limit during the study period.

All three packages effectively preserved the microbiological quality of the raw ham. The only detected presence of *Staphylococcus* spp. (coagulase-positive) was below 5 log (CFU/g), which is considered acceptable according to the levels recommended in the Guidelines for Microbiological Food Analysis (CeIRSA, 2017).

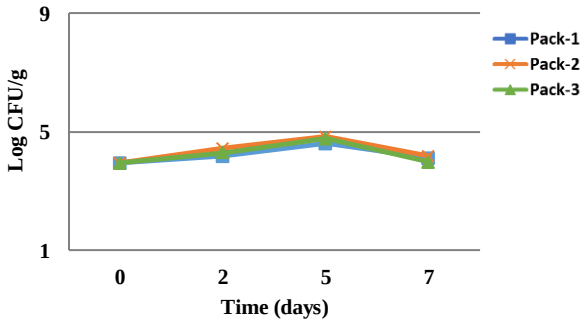



**Figure 2-21.** *Staphylococcus aureus* (coagulase positive) count from raw ham samples during 7 days of storage inside of Pack 1, 2 and 3.

### 2.4.3.2. Research packaging 1, 4, 5

The aim of the present study was to evaluate and compare the efficiency of 3 types of packing materials (Pack 1, 4 and 5) in the preservation of raw ham by monitoring biogenic amines, pH and microbiological parameters. Considering the values of the total BAs, it can be observed that Pack 1 preserves raw ham better than Pack 4 and 5 on T5, although there is no statistically significant difference.

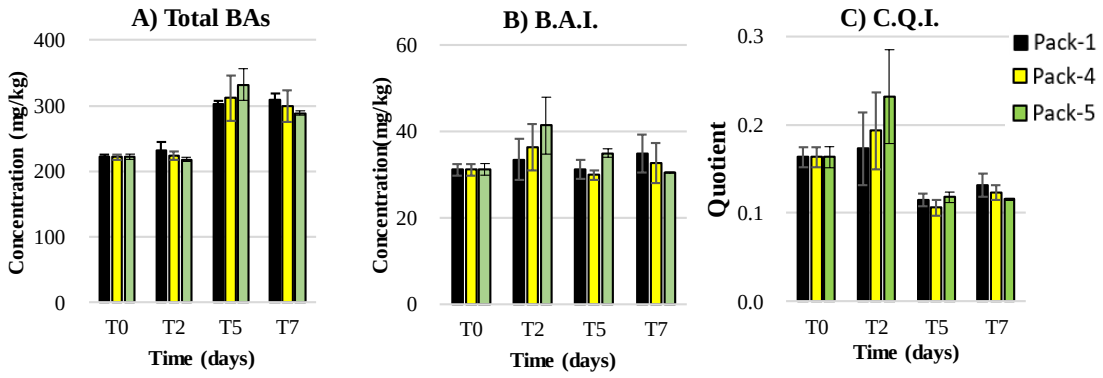



**Figure 2-22.** Changes of biogenic amines of raw ham during 7 days of storage inside of Pack 1, 4 and 5. A) The total of the monitored biogenic amines, B) Biogenic amines index, C) Chemical quality index.

There was a sudden increase in Enterobacteriaceae counts after 2 days in both Pack 1 and Pack 4 packaging systems, although the increase was not statistically significant. However, from day 5 to day 7, all packaging bags showed similar effects when controlling for Enterobacteriaceae levels (Figure 2-23A). While there was no significant difference between the different packages, the number of *Staphylococcus* spp. (coagulase positive) in ham stored in Pack 5 showed no change during the 7-day storage period (Figure 2-23B). Pack 4 promoted the growth of *Staphylococcus* spp. after 2 days, then the levels decreased in the following days, reaching the lowest level on the last day of storage compared to the other two packaging systems. While Pack 1 exerted an inhibitory effect on ham only after 5 days of storage, with a slight increase after another two days. The counts of β-glucuronidase positive *E. coli*, Sulfite-Reducing Anaerobic bacteria, *Clostridium perfringens* were below the detection limit in the samples in the three packages during the 7-day study. In conclusion, Pack 5 exhibited good preservative properties by limiting all microbial counts during the 7-day storage in raw ham. Pack 1 was able to inhibit *Staphylococcus* spp. counts (coagulase positive) only up to 5 days of storage.

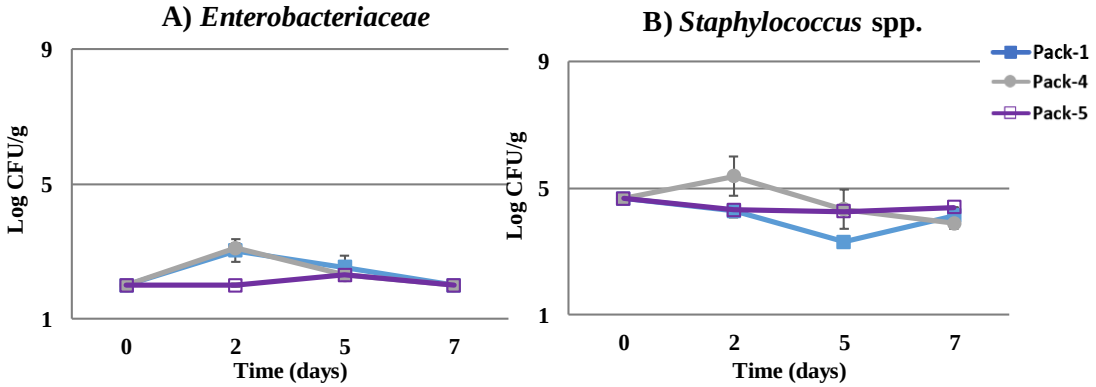



**Figure 2-23.** Changes of microorganism of raw ham during 7 days of storage inside of Pack 1, 4 and 5. A) Enterobacteriaceae, B) *Staphylococcus* spp.

### 2.4.3.3. Research packaging 1, 6, 7

The aim of the present study was to evaluate and compare the efficacy of 3 types of packing materials (Pack 1, 6 and 7) in preserving raw ham by monitoring biogenic amines, pH and microbiological parameters. The total BA of slight differences during the shelf life. However, the three packages (Pack 1, 6 and 7) showed exceptionally high efficiency compared to the other packages studied. In fact, the samples stored in these packages did not show a significant increase of BA during the study.

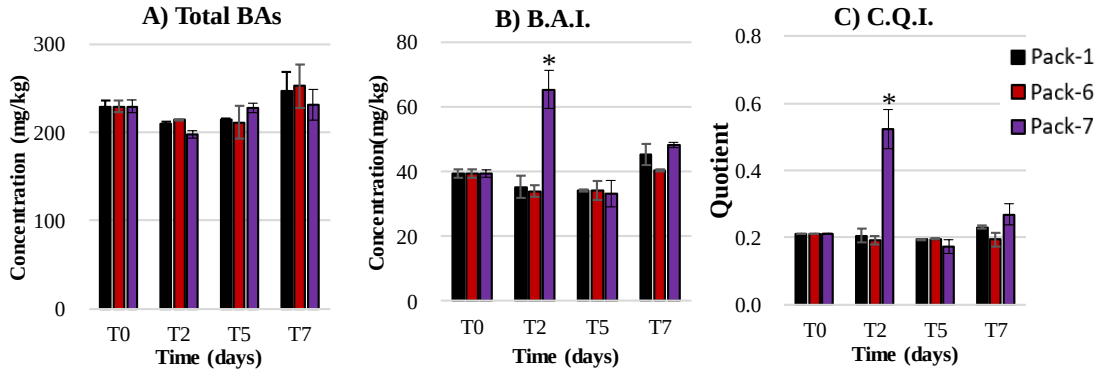



**Figure 2-24.** Changes of biogenic amines of raw ham during 7 days of storage inside of Pack 1, 6 and 7. A) The total of the monitored biogenic amines, B) Biogenic amines index, C) Chemical quality index. **Statistically significant different ($p<0.05$, Student's t test)*

All three packaging systems were shown to be efficient in maintaining the acidity of the raw ham during the 7-day preservation period (Figure 2-25).

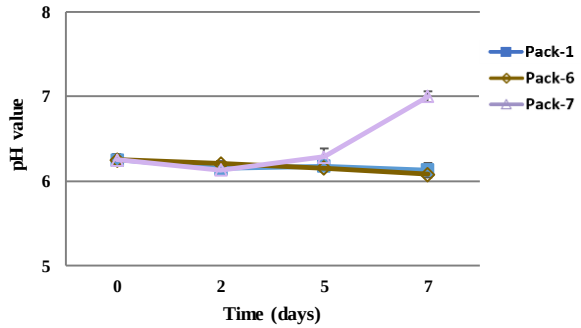



**Figure 2-25.** Changes of pH value of cooked ham during 7 days of storage inside of Pack 1, 6 and 7.

Figure 2-26A shows that all three packages were able to limit the number of

Enterobacteriaceae less than 3 log CFU/g for 7 days in package 6, 6 days for Pack 7, and about 4 days for Pack 1. Pack 6 was able to maintain low concentrations of Staphylococcus spp. with little variation up to 7 days. While Pack 1 showed better preservation property for 5 days compared to Pack 7 (Figure 2-26B). The number of β-glucuronidase-positive *E. coli, Clostridium perfringens*, Sulfite-Reducing Anaerobic bacteria were below the detection limit during the 7-day study. In general, Pack 6 showed better preservation characteristics for raw ham microbiological quality by limiting the levels of Enterobacteriaceae, *Staphylococcus* spp. (coagulase-positive) up to 7 days of storage compared to Pack 1 (a significant difference was observed especially after 7 days of storage), although a slight increase was observed after 2 days of storage. In addition, Pack 1 was effective in limiting bacterial growth for a short storage period (2 days). In conclusion, Pack 6 was more efficient than the other two packs in maintaining the microbiological quality of the raw ham, with values also consistent with the acceptance of the guidelines for microbiological analysis of food (CeIRSA, 2017).

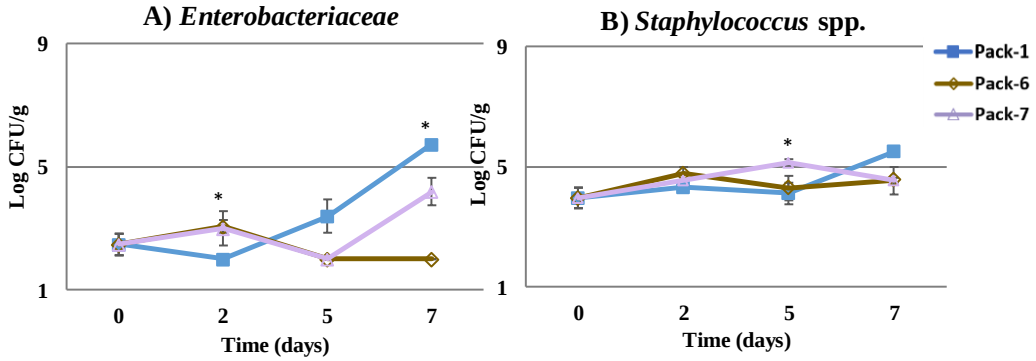



**Figure 2-26.** Changes of microorganism of raw ham during 7 days of storage inside of Pack 1, 6 and 7. A) Enterobacteriaceae, B) *Staphylococcus* spp. **Statistically significant different ($p<0.05$, Student's t test)*

### 2.4.3.4. Research packaging 1, 8

The aim of the present study was to assess and compare the efficiency of 2 kinds

of packaging materials (Pack 1 and 8) on the preservation of raw ham monitoring biogenic amines, pH and the microbiological parameters. Considering the global BA levels, Pack 8 performs slightly better than Pack 1 in preserving raw ham (Figure 2-27).

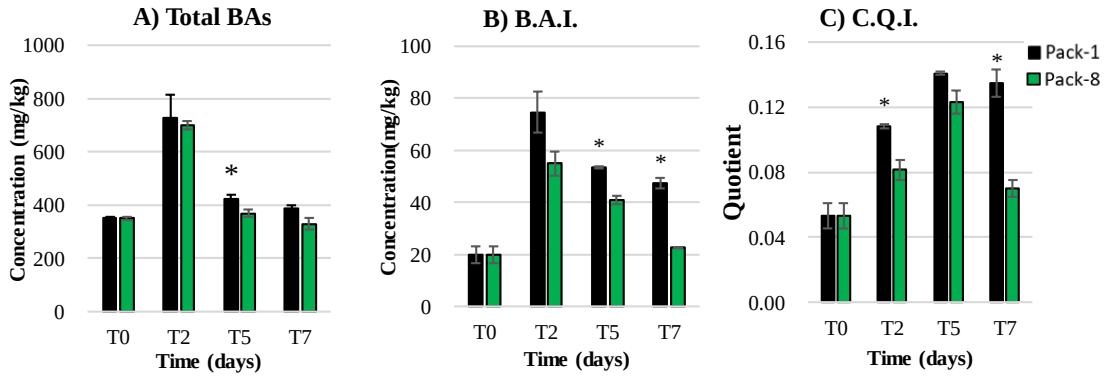



**Figure 2-27.** Changes of biogenic amines of raw ham during 7 days of storage inside of Pack 1 and 8. A) The total of the monitored biogenic amines, B) Biogenic amines index, C) Chemical quality index. **Statistically significant different ($p<0.05$, Student's t test)*

Within Pack 1 and Pack 8 the product has the same bacterial count values as coagulase positive *Staphylococci* (Figure 2-28). The counts for Enterobacteriaceae, positive *E. coli* β-glucuronidase, anaerobic sulphite reducers and *Cl. perfringens* in both packaging are below the test detection threshold for the entire period. Raw ham has the same behavior in Pack 1 and Pack 8.

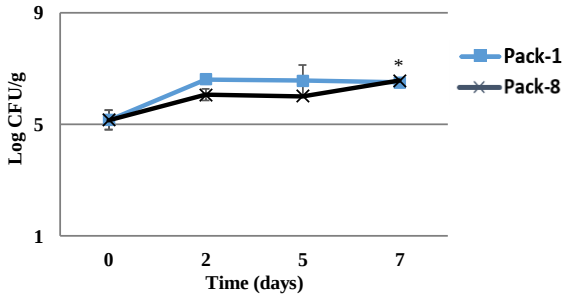



**Figure 2-28.** *Staphylococcus aureus* (coagulase positive) count from raw ham samples during 7 days of storage inside of Pack 1and 8. **Statistically significant different ($p<0.05$, Student's t test)*

All samples studied have CQI lower than 3. Pack 2 and 3 demonstrate levels of

efficiency not statistically different from Pack 1 in ham preservation. Pack 4, 5 showed lower efficiency levels than Pack 1. Pack 1, 6 and 7 demonstrated high levels of efficiency by limiting the increase in BA during ham storage. Pack 1 showed a tendency to preserve cooked ham better than Pack 8.

## 2.4.4. The correlation of indicators of the ham in different packages

### 2.4.4.1. A principal component analysis in cooked ham

Principal component analysis allows a better overview of the relationship between variables. The results of PCA applied to the mean values of the parameters of cooked ham are summarized in Figure 2-29, 30, 31.

For cooked ham packaged in Pack 1, PCA showed that about 88.7% of variability was explained by two first principal components, while two principal components in Pack 6 and Pack 7 explained about 85.0% and 90.8% of variability, respectively. Principal component 1 (PC1) was the most important variable in terms of differences among packing conditions, as it explained 71.7%, 57.8%, and 83.5% of the total variability in Pack 1, Pack 6, and Pack 7, respectively.

PC1 was positively associated with microbial analyses and protein content change (parts of BAs content). Histamine cannot be detected in cooked ham. As can be seen in Figure 2-29, all indicators are on the positive side of PC1 except for putrescine and spermidine, but the levels of Enterobacteriaceae, *Bacillus cereus*, and tyramine are significantly lower than the others. Total *aerobic mesophiles*, *Staphylococcus* spp, cadaverine and tryptamine are not only together but also have high values on the positive side of PC1.

The values of spermidine and spermine are on the negative side of PC1 (Figure 2-30), while the others are on the positive side of PC1.

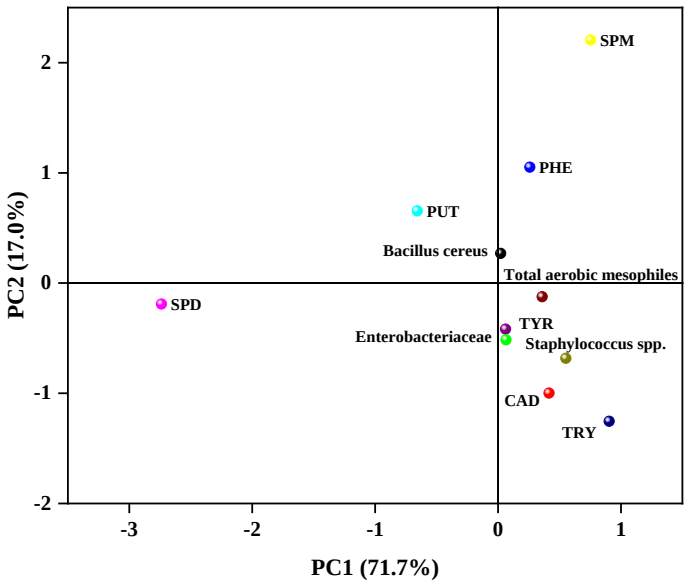



**Figure 2-29.** Relationships among the biogenic amines and microbial counts properties obtained by PCA in Pack 1 packaged cooked ham.

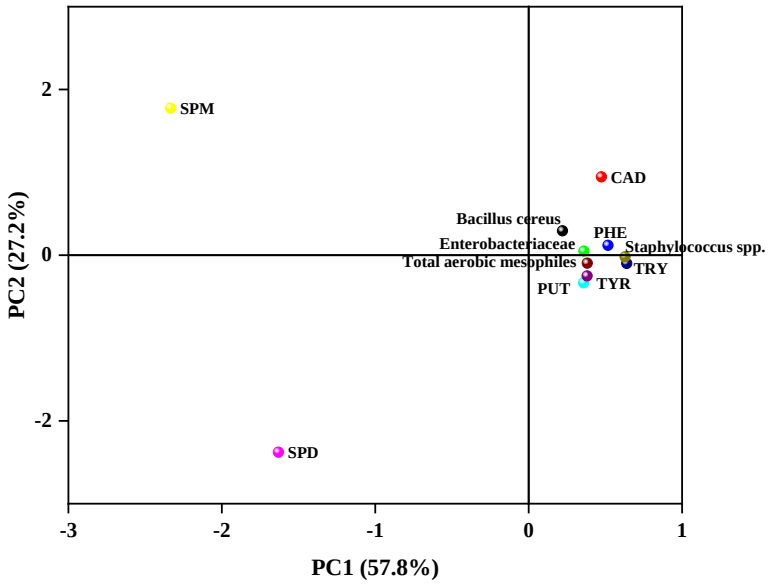



**Figure 2-30.** Relationships among the biogenic amines and microbial counts properties obtained by PCA in Pack 6 packaged cooked ham.

In Figure 2-31, all indicators are on the positive side of PC1 except putrescine and spermidine. In particular, tyramine, phenylethylamine, and microbial indicators have a

lower value on the positive side of PC1. Spermine, cadaverine and tryptamine have high values on the positive side of PC1.

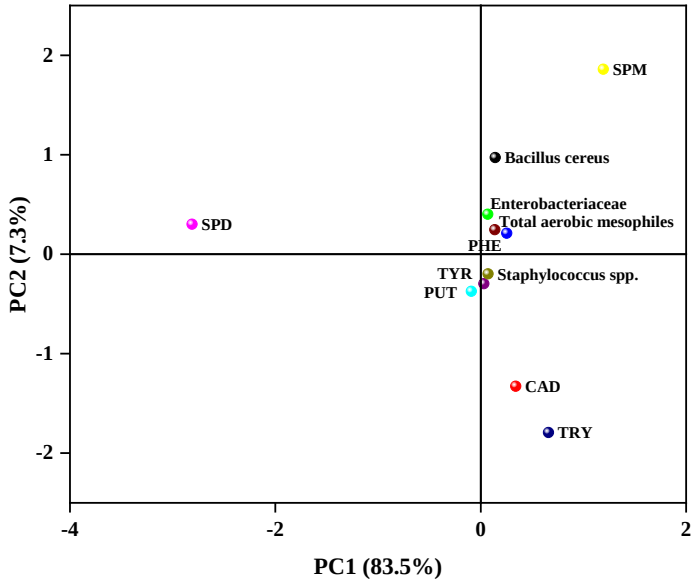



**Figure 2-31.** Relationships among the biogenic amines and microbial counts properties obtained by PCA in Pack 7 packaged cooked ham.

In conclusion, PC1 can distinguish the indicators from the packaging conditions. The indicators of cooked ham packaged under Pack 1 were related to total *aerobic mesophiles*, *Staphylococcus* spp, cadaverine, tryptamine, phenylethylamine and spermine; other biogenic amines could not reflect shelf life. The indicators of cooked ham packaged under Pack 6 were related to biogenic amines and microbial analysis have high values on the positive side of PC1, except spermidine and spermine. The indicators for the samples from package 7 were spermine, cadaverine and tryptamine, which could contribute to reduce the shelf life of cooked ham, while other biogenic amines and microbial indicators did not reflect the shelf life.

### 2.4.4.2. A principal component analysis in raw ham

The results of PCA applied to the mean values of raw ham parameters are summarized in Figure 2-32, 33, 34. In raw ham packaged in Pack 1, PCA shows that

about 83.2% of variability could be explained by two first principal components, while two principal components in Pack 6 and Pack 7 explained about 81.9% and 93.9% of variability, respectively. Principal component 1 (PC1) was the most important variable in terms of differences among packing conditions, as it explained 62.9%, 56.6%, and 68.2% of the total variability in Pack 1, Pack 6, and Pack 7, respectively.

PC1 was positively related to microbial analysis and protein change (portions of BAs content). As shown in Figure 2-32, tyramine and putrescine have high values on the positive side of PC1. *Staphylococcus spp*, cadaverine, tryptamine, phenylethylamine and spermine not only come together but also on the negative side of PC1. The values of Enterobacteriaceae, spermidine and histamine are on the negative side of PC1.

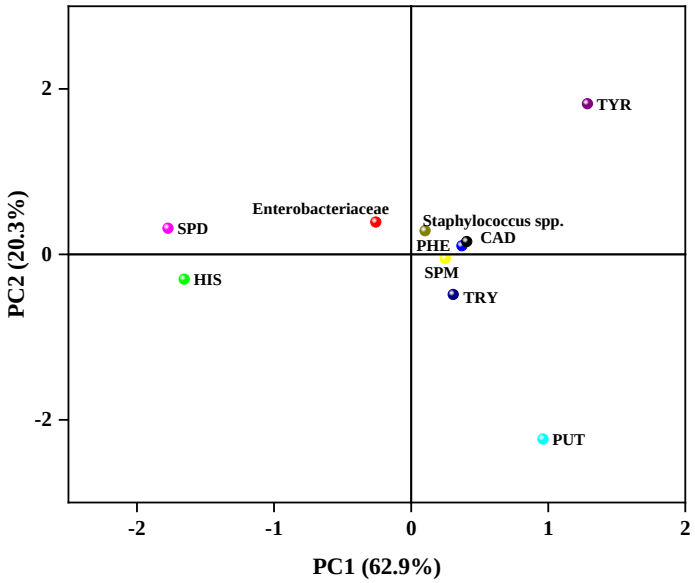



**Figure 2-32.** Relationships among the biogenic amines and microbial counts properties obtained by PCA in Pack 1 packaged raw ham.

As we can see from Figure 2-33, the situation of Pack 1 and Pack 6 is essentially similar, but Enterobacteriaceae is on the positive side of PC1 in Pack 6.

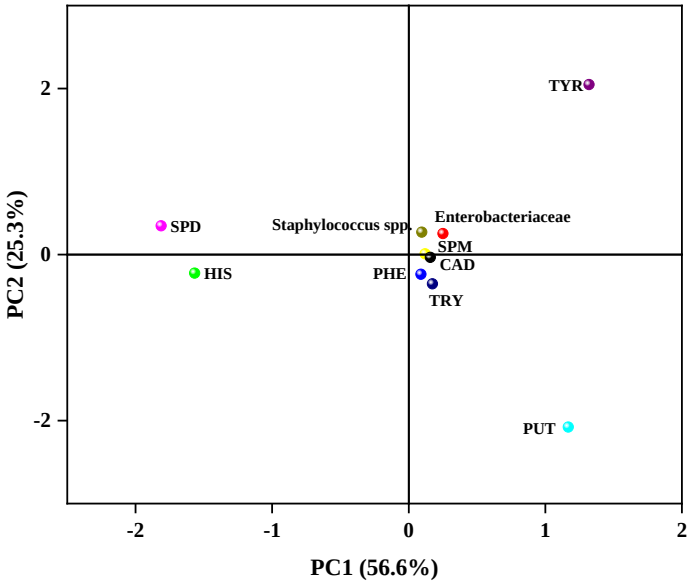



**Figure 2-33.** Relationships among the biogenic amines and microbial counts properties obtained by PCA in Pack 6 packaged raw ham.

In Figure 2-34, histamine has high levels on the positive side of PC1. Enterobacteriaceae, *Staphylococcus* spp, tyramine, spermine, cadaverine, and phenylethylamine not only come together but are also on the positive side of PC1.

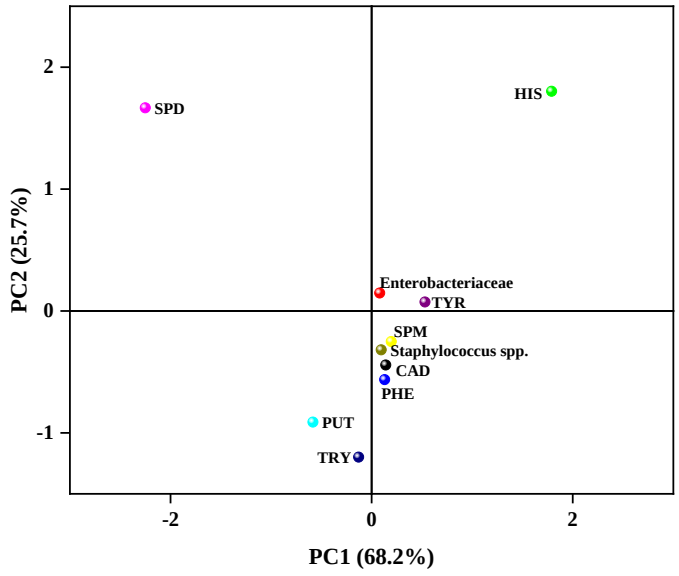



**Figure 2-34.** Relationships among the biogenic amines and microbial counts properties obtained by PCA in Pack 7 packaged raw ham.

In summary, PC1 was able to distinguish the indicators from the packaging

conditions. The indicators of raw ham packaged under Pack 1 were related to tyramine, putrescine, *Staphylococcus* spp, cadaverine, tryptamine, phenylethylamine and spermine; other biogenic amines could not reflect the shelf life. have high values on the positive side of PC1. Pack 6, the situation is similar: tyramine and putrescine have high values on the positive side of PC1. This means that there is a high correlation between tyramine, putrescine and raw ham. Compared to Pack 1 and Pack 6, Pack 7 is quite different. While the indicators for the samples under Pack 7 were histamine, which could contribute to reduce the shelf life of raw ham, other BAs and microbial indicators could not reflect the shelf life.

Similar values were found by different authors. Galgano indicated that tyramine, putrescine, and cadaverine can be used as spoilage indicators for fresh chilled beef packed in aerobic air with biopolymers (Galgano, Favati, Bonadio, Lorusso, & Romano, 2009). Cadaverine was mainly associated with the number of Enterobacteriaceae; *Bacillus cereus* was strongly correlated with tyramine formation and to a lesser extent with putrescine, cadaverine, and histamine. Under different packaging conditions, packaging affected the formation of BAs during storage of sardines (Li, et al., 2014). There are significant differences in tyramine, putrescine and cadaverine levels in the three packages.

## 2.5. CONCLUSIONS

In our study, we mainly analyzed 8 types of packages, which were composed in 8 different ways, for their ability to preserve raw and cooked ham. The analytical method based on extraction, derivatization, purification and determination in HPLC-DAD allows us to quantify the content of biogenic amines in ham contained in

different types of packaging. The results obtained show that all the samples studied have a chemical quality index lower than 3. The total content of BAs showed slight differences during shelf life. Chemical, microbiological and pH analyzes confirmed the equivalence of the different packages. It can be concluded that the packages provide a high level of food safety to protect the consumer.

However, the three packages (Pack1 - multilayer sheet + multilayer bag, Pack 6 - polycoupled sheet + metallized bag and Pack 7 - polycoupled sheet + copper bag) have exceptionally high efficiency compared to the other packages studied. The samples stored in these packages did not show a significant increase in BAs during the study. The packaging system can help distinguish the development and correlation of BAs and spoilage-related microorganisms in the product. The indicators of microorganisms and BAs resulting from packaging conditions could be distinguished from PC1 by PCA analysis. The results of PCA applied to the mean values of the parameters in Pack 1, 6, and 7 showed that tyramine, putrescine, and cadaverine could be used as spoilage indicators for ham. PC1 was the most important variable related to the differences between packaging conditions, as it accounted for 46%, 52.4%, and 53.6% of the total variability in Pack 1, Pack 6, and Pack 7, respectively. PC1 was positively related to microbial analysis and protein change (parts of biogenic amine content).

The changes in biogenic amines, microbial content, and pH during ham preservation are very small compared to raw meat (Alessandroni, et al., 2022), likely due to the reduction in moisture and increased NaCl concentration in the ham. Studies have shown that NaCl concentration has a significant effect on endogenous microbiota (Lactic acid bacteria, *mesophilic aerobic* bacteria, *psychrotrophic* bacteria, *Staphylococcus* spp. and Enterobacteriaceae) and biological amine content (histamine,

tyramine, putrescine, cadaveramine and spermatine) in dry-cured meat. The higher NaCl cluster showed a lower content of BAs. A negative correlation between microbial count and BAs content in the lower NaCl cluster suggests that the higher BA's content in the lower NaCl cluster may be the result of a stress response mechanism. On the other hand, the salt concentration in the higher NaCl cluster had an inhibitory effect on the formation of BA except histamine. The collective results suggest a NaCl threshold that minimizes the formation of BAs in dry cured meats (Mutz, et al., 2022).

## 2.6. References


Alessandroni, L., Caprioli, G., Faiella, F., Fiorini, D., Galli, R., Huang, X., Marinelli, G., Nzekoue, F., Ricciutelli, M., Scortichini, S., Silvi, S., Tao, J., Tramontano, A., Turati, D., & Sagratini, G. (2022). A shelf-life study for the evaluation of a new biopackaging to preserve the quality of organic chicken meat. *Food Chemistry*, *371*, 131134.

Blanco Lizarazo, C. M., Sierra-Cadavid, A., Montoya R, A. M., & Ospina-E, J. C. (2022). Analysis of microbiota structure in cooked ham as influenced by chemical composition and processing treatments: Identification of spoilage bacteria and elucidation on contamination route. *Current Research in Food Science*, *5*, 726-734.

Cauchie, E., Delhalle, L., Taminiau, B., Tahiri, A., Korsak, N., Burteau, S., Fall, P. A., Farnir, F., Bare, G., & Daube, G. (2020). Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packed minced pork meat samples by 16S rDNA metagenetic analysis. *Frontiers in Microbiology*, *10*, 3074.

CeIRSA (2017). Linee Guida per l'analisi del Rischio nel campo della microbiologia degli alimenti (allegato B). In: Protocollo tecnico della Regione Piemonte per l'effettuazione dei controlli microbiologici ufficiali sugli alimenti e l'interpretazione e gestione degli esiti analitici (updated 21.08.2017).

Galgano, F., Favati, F., Bonadio, M., Lorusso, V., & Romano, P. (2009). Role of biogenic amines as index of freshness in beef meat packed with different biopolymeric materials. *Food Research International*, *42*(8), 1147-1152.

Li, M., Tian, L., Zhao, G., Zhang, Q., Gao, X., Huang, X., & Sun, L. (2014). Formation of biogenic amines and growth of spoilage-related microorganisms in pork stored under different packaging conditions applying PCA. *Meat Science*, *96*(2, Part A), 843-848.

Lucarini, M., Saccani, G., D Evoli, L., Tufi, S., Aguzzi, A., Gabrielli, P., Marletta, L., & Lombardi-Boccia, G. (2013). Micronutrients in Italian ham: A survey of traditional products. *Food Chemistry*, *140*(4), 837-842.

Mietz, J. L., & Karmas, E. (1977). Chemical quality index of canned tuna as determined by high-pressure liquid chromatography. *Journal of Food Science*, *42*(1), 155-158.

Mutz, Y. S., Kaic Alves Rosario, D., Alves De Aguiar Bernardo, Y., Paulo Vieira, C., Vilela Pinto Moreira, R., Bernardes, P. C., & Conte-Junior, C. A. (2022). Unravelling the relation between natural microbiota and biogenic amines in Brazilian dry-cured loin: a chemometric approach. *International Journal of Food Science & Technology*, *57*(3), 1621-1629.

Mutz, Y. S., Rosario, D. K. A., Castro, V. S., Bernardes, P. C., Paschoalin, V. M. F., & Conte-Junior, C. A. (2019). Prior Exposure to Dry-Cured Meat Promotes Resistance to Simulated Gastric Fluid in Salmonella Typhimurium., *Foods*, vol. 8.

Picon, A., & Nunez, M. (2022). Volatile compounds in high-pressure-treated dry-cured ham: A review. *Meat Science*, *184*, 108673.

Rosario, D. K. A., Rodrigues, B. L., Bernardes, P. C., & Conte-Junior, C. A. (2021). Principles and applications of non-thermal technologies and alternative chemical compounds in meat and fish. *Critical Reviews in Food Science and Nutrition*, *61*(7), 1163-1183.

Sang, X., Ma, X., Hao, H., Bi, J., Zhang, G., & Hou, H. (2020). Evaluation of biogenic amines and

microbial composition in the Chinese traditional fermented food grasshopper sub shrimp paste. *LWT*, *134*, 109979.

Silva, C. M. G., & Gloria, M. B. A. (2002). Bioactive amines in chicken breast and thigh after slaughter and during storage at 4±1 ℃ and in chicken-based meat products. *Food Chemistry*, *78*(2), 241-248.

Sirocchi, V., Caprioli, G., Cecchini, C., Coman, M. M., Cresci, A., Maggi, F., Papa, F., Ricciutelli, M., Vittori, S., & Sagratini, G. (2013). Biogenic amines as freshness index of meat wrapped in a new active packaging system formulated with essential oils ofRosmarinus officinalis. *International Journal of Food Sciences and Nutrition*, *64*(8), 921-928.

Valous, N. A., Mendoza, F., & Sun, D. (2010). Emerging non-contact imaging, spectroscopic and colorimetric technologies for quality evaluation and control of hams: a review. *Trends in Food Science & Technology*, *21*(1), 26-43.

Vasconcelos, H., de Almeida, J. M. M. M., Matias, A., Saraiva, C., Jorge, P. A. S., & Coelho, L. C. C. (2021). Detection of biogenic amines in several foods with different sample treatments: An overview. *Trends in Food Science & Technology*, *113*, 86-96.

Veciana-Nogues, M. T., Marine-Font, A., & Vidal-Carou, M. C. (1997). Biogenic Amines as Hygienic Quality Indicators of Tuna. Relationships with Microbial Counts, ATP-Related Compounds, Volatile Amines, and Organoleptic Changes. *Journal of Agricultural and Food Chemistry*, *45*(6), 2036-2041.

Zhu, Y., Guo, Y., Yang, F., Zhou, C., Tang, C., & Zhou, G. (2021). Combined application of high-throughput sequencing and UHPLC-Q/TOF-MS-based metabolomics in the evaluation of microorganisms and metabolites of dry-cured ham of different origins. *International Journal of Food Microbiology*, *359*, 109422.

# CHAPTER III

# DETERMINATION OF ATP-RELATED COMPOUNDS IN DRY-CURED HAM BY HPLC

## 3.1. ABSTRACT

Due to the small number of microorganisms, the microbial corruption detection index of fresh meat is not sensitive for ham quality detection, which makes it difficult to detect ham corruption. This research aims to study the evolution of ATP-related compounds in dry-cured ham and to assess their suitability as chemical markers of food contamination. A rapid high-performance liquid chromatography (HPLC) method was developed for the analysis of five ATP-related compounds namely, ATP, ADP, AMP, hypoxanthine, and Ino in sliced dry-cured ham. Moreover, a positive correlation between ATP content and the microbial load was found in ham during storage. Subsequently, the ATP-related compounds in ham were extracted with perchloric acid, and extracts were adjusted to pH 6.0-6.5 with potassium carbonate, then submitted to HPLC using a SunFire$^{TM}$ C18 (4.6 mm×mm, 5 μm) column and 0.05 mol/L $KH_2PO_4$-$K_2HPO_4$ methanol at a ratio of 9:1 (*v/v*) as the mobile phase at a flow rate of 0.6 mL/min. Correlation coefficients of the four ATP-related compounds in ham samples were very high ($R^2 \geq 0.995$). The limits of detection of the four ATP-related compounds were low, and their contents were within the linear range of 0.05-200 μg/mL.

## 3.2. INTRODUCTION

Dry-cured ham is a traditional meat product highly appreciated worldwide, with Spain, France, and Italy as the main European manufacturers (Fernandez, Hospital, Cabellos, & Hierro, 2020). It is manufactured by salting, air-drying, and ripening of fresh pig hind legs using traditional techniques, and the final product is typically sold sliced and packaged, which then must be stored at low temperatures (2-4 °C) (Piras, Fois, Casti, Mazza, & Mazzette, 2016). However, dry-cured ham is prone to microbial spoilage when preservatives are not added and has a short self-life (Rubio, et al., 2006).

Although rapid detection methods for microbial monitoring have been developed, conventional methods are commonly preferred for ham samples. It has been estimated that there are currently in excess of 40 methods to measure and detect bacterial spoilage in meats (Hassoun, Sahar, Lakhal, & A ï-Kaddour, 2019; Jay & James, 2000; Kumar, 2021; Ren, Fang, Yang, & Han, 2022). However, each rapid detection method has unique advantages and the best application range. Many rapid detection methods are food-specific, and their best performance is conditioned to the food matrix (Wei, Wang, Sun, & Pu, 2019). Current rapid enumeration methods are generally based on microscopy, measurement of ATP levels or electrical phenomena (Ellis & Goodacre, 2001; Ramsey & Parikh, 2021).

There are currently many detection methods for ATP-related compounds, including bioluminescence (Mitchell, et al., 2020), nuclear magnetic resonance spectroscopy (NMR), capillary electrophoresis (CE) (Soga & Imaizumi, 2015), radioimmunoassay (Roberts, Morris, & Clifford, 1991), thin-layer chromatography (TLC) (Dingle, Hines, & Fraser, 2006) and enzymatic assays (Lee, et al., 2010). However, high-performance liquid chromatography (HPLC) has become the most used technique for the analysis of nucleotides and nucleosides

in biological samples owing to its versatility, short analysis time, and high resolution (Mora, et al., 2010). Nonetheless, the use of HPLC to determine ATP-related compounds in hams has not yet been proposed.

## 3.3. MATERIALS AND METHODS

### 3.3.1. Materials and reagents

All chemicals and chromatographic reagents were of HPLC grade. Adenosine triphosphate (ATP, $C_{10}H_{16}N_5O_{13}P_3$, ≥ 98%, CAS No. 56-65-5), adenosine diphosphate (ADP, $C_{10}H_{15}N_5O_{10}P_2$, ≥ 95%, CAS No. 58-64-0), adenosine monophosphate (AMP, $C_{10}H_{14}N_5O_7P$, ≥ 98%, CAS No. 61-19-8), inosine (Ino, $C_{10}H_{12}N_4O_5$, ≥ 99%, CAS No. 58-63-9), and hypoxanthine (Hx, $C_5H_4N_4O$, ≥ 98%, CAS No. 68-94-0) were purchased from Anhui Cool Bioengineering Co., Ltd. Potassium dihydrogen phosphate, potassium dihydrogen phosphate, methanol, perchloric acid, and anhydrous potassium carbonate were purchased from Tianjin Yongda Chemical Reagent Co., Ltd. MRS broth was purchased from Beijing aoboxing Biotechnology Co., Ltd.

Individual stock solutions (1 mg/mL) were prepared by dissolving 10 mg of each ATP-related compound (ATP, ADP, AMP, Ino, and Hx) in 10 mL of deionized water (< 18 MΩ×cm) and stored in glass-stopper bottles at 4 °C. Deionized water was obtained with the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). Standard working solutions at various concentrations (200, 50, 5, 0.5, and 0.05 μg/mL) were daily prepared by diluting different aliquots of the stock solutions in water.

## 3.3.2. Ham samples preparation

The samples of ham were sliced Italian Parma dry-cured ham, which were purchased at Maile food store (Nanan, China). *Lactiplantibacillus plantarum* CICC 20022 was obtained at the China Center of Industrial Culture Collection.

Packages of vacuum-sealed sliced ham were opened, further divided, and wrapped in a previously prepared commercial polyethylene (PE) fresh-keeping bag (20×30 cm) kindly provided by a local supermarket (Zhengzhou, China). Prior to packaging, the surface of PE bags was sterilized under an ultraviolet lamp for up to 3 h. Each sample of ham (about 100 g) was stored at 4 ℃. At days 0, 2, 5, 7, 14, 21 and 30 microbiological, chemical and sensorial analysis were conducted.

## 3.3.3. Determination of ATP-related compounds

### 3.3.3.1. Extraction of ATP-related compounds

Briefly, 2 g of ham samples were finely minced and mixed with 12 mL of 5% perchloric acid solution in an aseptic sampling bag for 2 min at 8 times/s to obtain homogenates. Homogenates were left to stand in an ice bath for 30 min, then centrifuged for 10 min at 4 ℃ at 13000 rpm. Then, 2 mL of the supernatant was removed and transferred to 200 μL of potassium carbonate solution (2 mol/L), then manually shaken. The pH of the resulting mixture was adjusted to 6 - 6.5. The mixture was centrifuged at 13,000 rpm at 4 ℃ for 10 min and then filtered using a 0.22-μm membrane filter prior to the analysis.

### 3.3.3.2. HPLC analysis

The analysis of ATP-related compounds was conducted on Waters HPLC instrument equipped with Waters 1525 Binary Pump, Waters 2489 UV/Visible

detector, Sunfire$^{TM}$ C18 chromatographic column (4.6×250 mm, 5 m) at 25 ℃. The mobile phase A was 0.05 mol/L $KH_2PO_4$-$K_2HPO_4$, whereas the mobile phase B was 0.05 mol/L $KH_2PO_4$-$K_2HPO_4$ and methanol at a 9:1 ratio (*v/v*) at pH 6.5 previously filtered with a 0.45-μm membrane filter. The flow rate was 0.6 mL/min, injection volume was 10 μL, and detection wavelength was 254 nm.

#### 3.3.3.3. Assay validation of linearity

Linearity of the method has been assessed using three calibration curves per analyte constructed correlating the corresponding peak areas (mAU*s) against the concentration of the analyte. Each calibration curve was calculated using peak areas at six standard concentrations which range was selected based on the expected concentration of analyte in the dry-cured ham samples.

#### 3.3.3.4. Recovery

Recovery data of all five compounds were determined by adding with 50 μg/mL high concentration and 5 μg/mL low concentration of standards to ham samples. At each level, analyses were performed in duplicate and recoveries were calculated by comparison with rotten ham sample.

### 3.3.4. Analysis of volatile organic compounds

5 g of sliced Parma Italian ham was finely minced and mixed with 25 mL saturated salt water, and beaten for 2 min with a beating aseptic homogenizer at 8 times/s to prepare homogenate Standby. Place the homogenized sample in a 50 mL headspace vials and seal it tightly with an aluminum cover, then place it in a constant temperature shaker at 40 ℃ for 2 h. Manual injection, 400 μL samples per injection.

An Agilent GC/MS (7890B/5977A, Santa Clara, California, USA) and HP-5MS

(30m×250μm×0.25μm) were used for volatile flavor compounds study. The determination condition was set as follows: firstly, the GC oven was set at 50 ℃ and kept for 5 min. Then, the temperature was increased to 200 ℃ at a rate 5 ℃/min and increased to 250 ℃ at a rate 20 ℃/min and kept at 250 ℃ for 10minutes. MS detection was conducted in EI mode with electron energy of 70 eV, and then in full-scan mode with m/z of 50−450.

### 3.3.5. Microbiological analysis

1 g of ham samples was obtained and placed in sterile sampling bags containing 9 mL of 0.85% NaCl and homogenized for 2 min in an aseptic homogenizer at 8 times/s to make 1: 10 sample dilutions. Similarly, the sample dilutions were continuously used in sterile isotonic saline to prepare serial decimal dilutions. Three suitable dilutions of the sample were selected, and 1 mL of each dilution of the sample was added into the sterile petri dish. The 15 mL of plate count agar medium (Qingdao Haibo Bio-Technology Co., Ltd., Shandong, China) cooled to 46 ± 1 ℃ was poured into the sterile petri dish, and the sterile petri dish was rotated to make it thoroughly mixed. The petri dish was inverted after the agar solidified and incubated at 37 ℃ ± 1 for 24 h. The total bacterial count (TBC) was counted manually. The results were expressed as colony-forming units (CFU)/g.

### 3.3.6. Sensory evaluation

The flavor of ham was evaluated at 25 ℃ room temperature and bright natural light. The evaluation indexes included acceptability, color, flavor, odor and texture of ham. Select 5 sensory evaluators and give oral training to the team members before

evaluating the products. Sensory evaluators need to score the samples on a 10 point (Clariana, et al., 2011). Finally, the overall sensory acceptability of the product was evaluated according to the weight of acceptability 20%, tissue state 25%, color15%, odor 25%, and crumbliness 15%.

### 3.3.7. Statistical analysis

The results of the test were expressed as mean ± standard deviation. The statistical difference was calculated using Student's *t* test. $P < 0.05$ was regarded as significant. All tests were performed in duplicate. LEFse (LDA Effective Size) analysis of volatile flavor compounds was carried out on Galaxy website.

## 3.4. RESULTS AND DISCUSSION

### 3.4.1. Optimization of the proposed chromatographic method

#### 3.4.1.1. Condition optimization

To establish optimal conditions for the separation of five ATP-related compounds (i.e., ATP, ADP, AMP, Ino, and Hx) by HPLC, the impact of different mobile phase compositions, concentrations, and flow rates was examined. Considering that all five ATP-related compounds are polar, small variations in pH values may alter the retention and selectivity of the target analyte, thus affecting chromatographic separation. Therefore, a phosphate buffer solution was used as the mobile phase to ensure mobile phase pH stability. 0.04 mol/L $KH_2PO_4$-$K_2HPO_4$ buffer was tested in the mobile phase as reported in previous papers (Hwang, Chen, Shiau, & Jeng, 2000; Zur Nedden, Eason, Doney, & Frenguelli, 2009). In addition, several flow rates were

tested to obtain optimal separation of target analytes. The best result was obtained at a flow rate of 0.6 mL/min, but a peak identified as Ino overlapped with the AMP peak (Figure 3-1).

The salt concentration has an important effect on retention and selectivity in chromatography by influencing analyte ionization. Thus, 0.05 mol/L $KH_2PO_4$-$K_2HPO_4$ buffer was tested, increasing its concentration to shorten the chromatogram and improve peak shape (Figure 3-2).

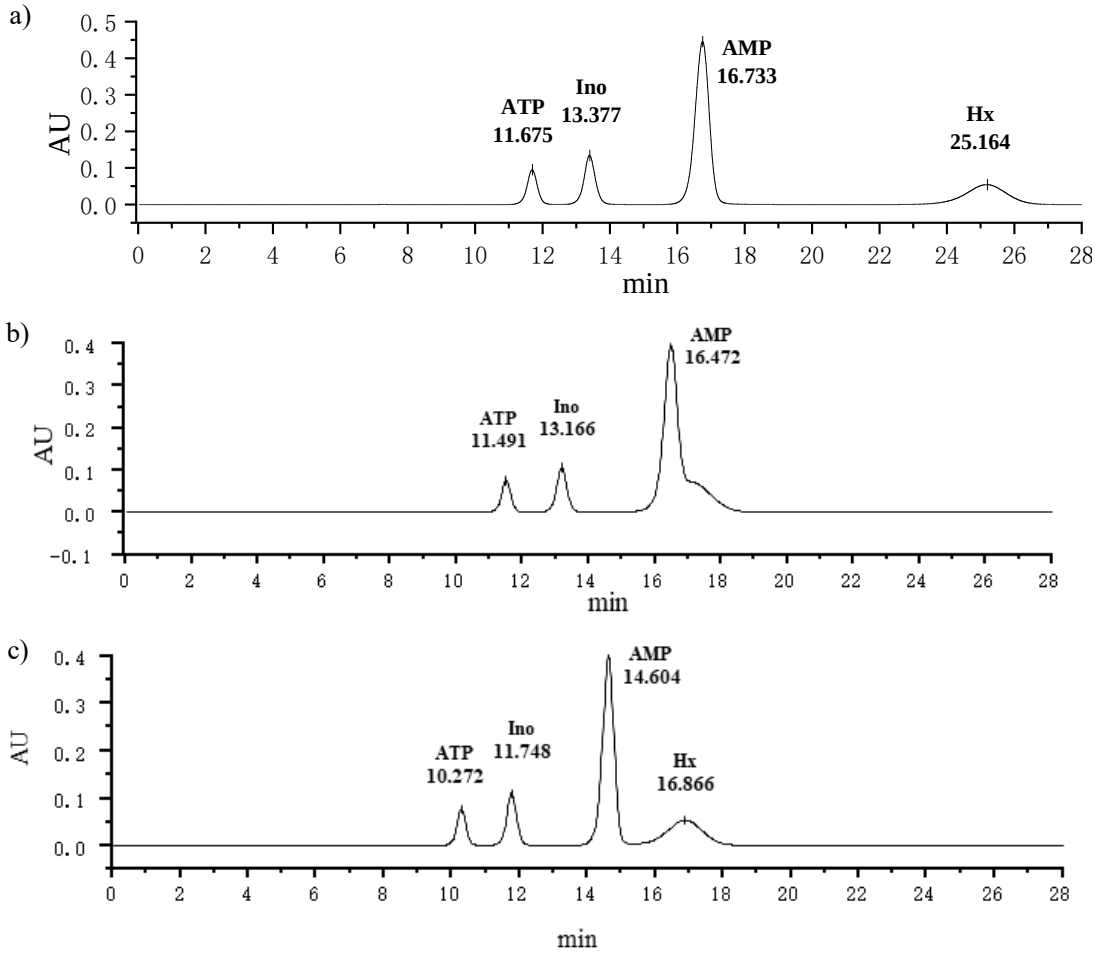



**Figure 3-1.** Chromatograms of ATP-related compounds identified in sliced Italian Parma ham samples using mobile phases at different flow rates: a) 0.6 mL/min; b) 0.7 mL/min; c) 0.8 mL/min.

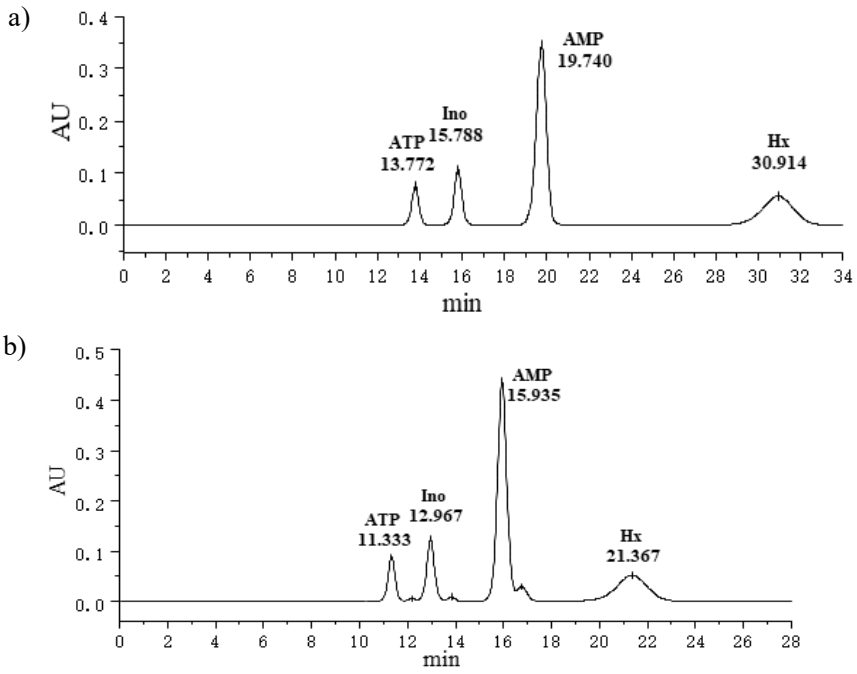



**Figure 3-2.** Chromatograms of ATP-related compounds identified in sliced Italian Parma ham samples using mobile phases at different concentrations: a) 0.04 mol/L; b) 0.05 mol/L.

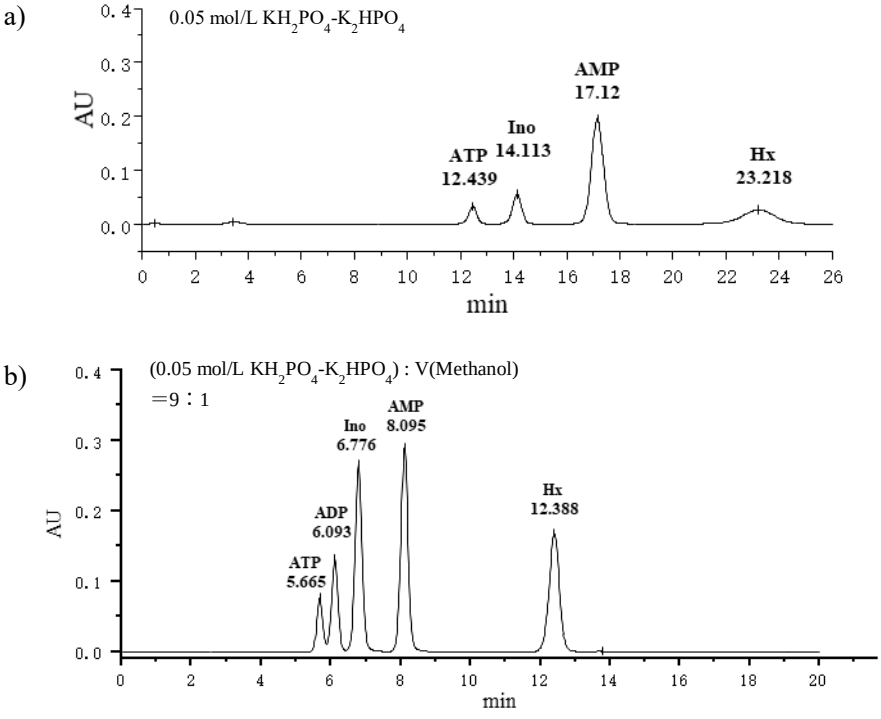



**Figure 3-3.** Chromatograms of ATP-related compounds identified in sliced Italian Parma ham samples with different mobile phases: a) 0.05 mol/L $KH_2PO_4$-$K_2HPO_4$; b) 0.05 mol/L $KH_2PO_4$-$K_2HPO_4$ and methanol at a ratio of 9:1 (*v/v*).

Methanol was tested in the mobile phase as reported in previous reports (Hwang, et al., 2000; Xue, et al., 2009) to improve the separation of analytes and different buffer concentrations were tested to determine the most adequate regarding solvent solubility and its interaction with target compounds. The mobile phase with 0.05 mol/L $KH_2PO_4$-$K_2HPO_4$ (A) and methanol (B) at a ratio of 9:1 (*v/v*) was selected since it provided the best results in terms of peak separation (Figure 3-3). Moreover, the peak shape of analytes was improved and their separation was allowed for 14 min.

### 3.4.1.2. Linearity and calibration standards

The linearity of the method was calculated using 7 concentrations of mixed standard solutions (0.05-200 µg/mL). Linear regression showed good linearity within the range of 0.05-200 µg/mL with a coefficient of determination $R^2 \geq 0.995$. Thus, these findings revealed that the proposed method can be applied in the determination of the five surveyed ATP-related compounds over a wide range of concentrations in Table 3-1 and Figure 3-4.

**Table 3-1.** Peak area of corresponding standard concentration.

| Standard compound | Standard concentration (µg/mL) and peak area (mAU) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.05 | RSD % | 0.5 | RSD % | 5 | RSD % | 50 | RSD % | 100 | RSD % | 200 | RSD % |
| ATP | 25632 | 7.94 | 43929 | 4.24 | 171427 | 7.62 | 976250 | 2.18 | 2315156 | 0.04 | 3902156 | 2.02 |
| ADP | 22172 | 4.85 | 59804 | 7.55 | 337755 | 9.44 | 1794959 | 4.50 | 3662833 | 9.93 | 5948878 | 0.51 |
| Ino | 26038 | 9.52 | 95690 | 4.11 | 761153 | 4.42 | 3943239 | 5.69 | 5573190 | 9.72 | 10656533 | 0.92 |
| AMP | 29879 | 1.62 | 96456 | 4.81 | 741394 | 4.35 | 4340078 | 0.03 | 9845724 | 2.35 | 15691448 | 2.28 |
| Hx | 28427 | 1.01 | 83256 | 8.10 | 738596 | 2.14 | 3505323 | 0.56 | 6965043 | 4.32 | 11375649 | 4.81 |

Legend: adenosine triphosphate (ATP), adenosine diphosphate (ADP), inosine (Ino), adenosine monophosphate (AMP) and hypoxanthine (Hx).

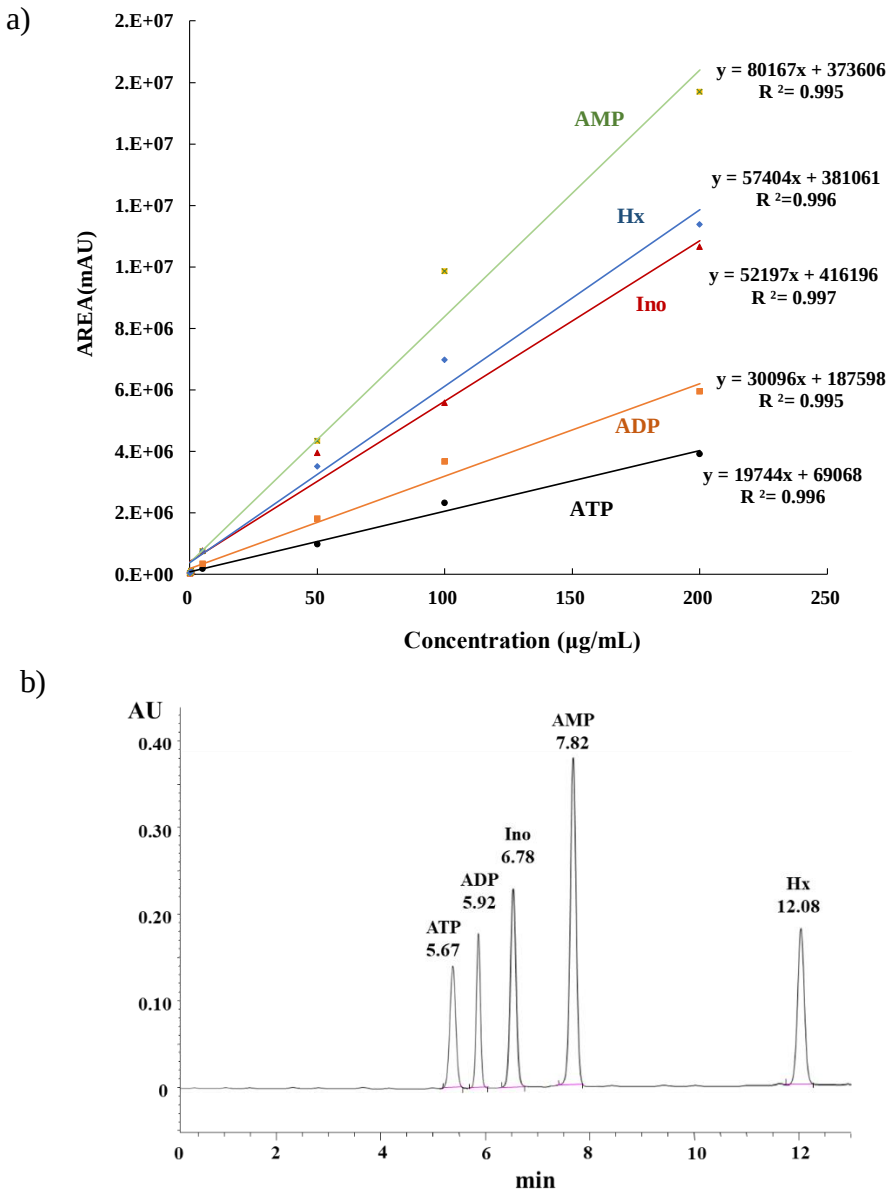



**Figure 3-4.** ATP-related compounds yielded a linear relationship. a) Linear equations; b) HPLC chromatogram of a 200 µg/mL standard solution of all analysed ATP-related compounds and Internal Standard. Legend: adenosine triphosphate (ATP), adenosine diphosphate (ADP), inosine (Ino), adenosine monophosphate (AMP) and hypoxanthine (Hx).

### 3.4.1.3. Method validation

The developed analytical method showed good reproducibility. Indeed, the intraday (run-to-run precision) reproducibility and interday (day-to-day precision) reproducibility of five targeted ATP-related compounds were measured through three replicate determinations

on the same day (intraday) and on three consecutive days (interday). The intraday precision expressed in %RSDs ranged from 0.5 to 4.8% while the interday precision varied between 4.1 to 8.1% confirming the good repeatability of the method.

#### 3.4.1.4. HPLC analysis of ham samples

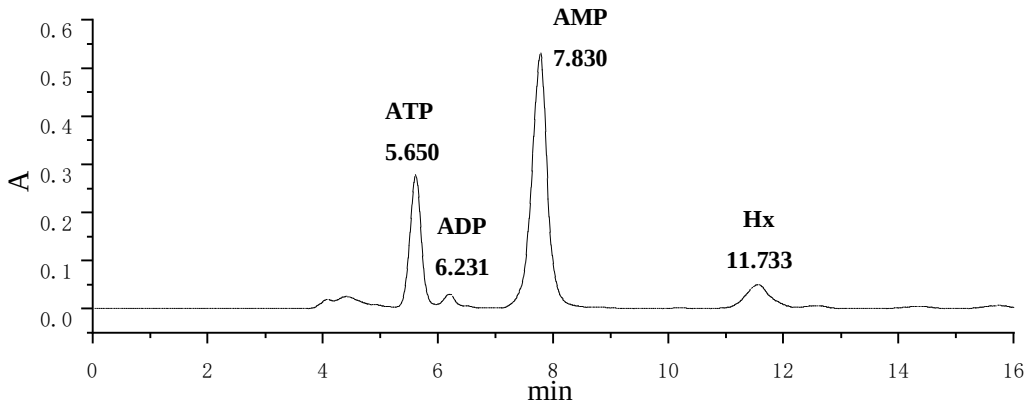



**Figure 3-5.** Typical chromatogram of actual sliced Italian Parma ham samples. Legend: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and hypoxanthine (Hx).

The developed and validated analytical method was applied to ham samples to prove the ability of the method to monitor the levels of ATP-related compounds in ham samples. The retention times of the four peaks (ATP, ADP, AMP, and Hx) of the sample solutions (Figure 3-5) agreed with those of the standard substances. ATP, ADP, AMP, and Hx in the chromatogram of the ham samples were separated in 13 min. However, the peak of Ino does not appear, which may be due to low inosine levels in the sample. Inosine and hypoxanthine are endogenous non-protein components, usually at low concentrations (e.g. 200-400 ng/mL). HPLC method has been used to determine inosine and hypoxanthine in human plasma using an aqueous mobile phase of trifluoroacetic acid and methanol gradient (Farthing, et al., 2007). The extraction solvent is also a very important parameter because it influences the shape of the peak. A high proportion of water in the extraction solvent could result in lower retention,

poorer efficiency, and a worse separation but, on the other hand, nucleotides can not be easily solubilized in highly organic solutions. Adequate solubilization was achieved by using 5% perchloric acid solution as an extraction solvent. Good solubilization of all the studied compounds was also obtained but Ino showed low stability in these solutions.

### 3.4.1.5. Recovery rate of standard substance spiked

Two different concentrations (low and high) of the five surveyed ATP-related compounds were added to samples on days 0 and 30 with known concentration, and the samples were analyzed with the established method. The results are shown in Table 3-2.

**Table 3-2.** Recovery rate of target analytes in ham samples on days 0 and 30 of storage.

| Analyte | Days | 50μg/mL[a] | 5μg/mL[a] | Theoretical value | Recovered[a] | Recovery (%) |
|---|---|---|---|---|---|---|
| ATP | 0 | 134.32 | 93.25 | 45 | 41.07 | 91% |
| | 30 | 256.79 | 212.07 | 45 | 44.72 | 99% |
| ADP | 0 | 40.60 | 4.78 | 45 | 35.82 | 80% |
| | 30 | 44.63 | 5.83 | 45 | 38.80 | 86% |
| Ino | 0 | 1.28 | 0.19 | 45 | 1.09 | 2% |
| | 30 | 3.33 | 1.26 | 45 | 2.06 | 5% |
| AMP | 0 | 90.22 | 48.19 | 45 | 42.02 | 93% |
| | 30 | 151.80 | 109.62 | 45 | 42.18 | 94% |
| Hx | 0 | 45.27 | 8.85 | 45 | 36.41 | 81% |
| | 30 | 62.59 | 23.04 | 45 | 39.55 | 88% |

[a] Each value corresponds to twice replicates at each concentration level.

The recovery rates of ATP, ADP, AMP, and Hx varied between 80-99%, indicating satisfactory repeatability of the proposed method. However, the recovery of Ino below 5% was observed. The low Ino recovery rates may be due to the interference of sample extraction process on Ino and its instability in the extraction conditions. Further studies are needed to understand the impact of the extraction method on the

Ino recovery. However, collectively, these results showed that the proposed method was suitable for the extraction of the main four ATP-related compounds namely, ATP, ADP, AMP, and Hx in ham samples. Moreover, considering the low levels of Ino reported in food samples (ng/g), its low recovery has a reduced impact on the total assessment of ATP-related compounds in ham samples.

## 3.4.2. Study on normal and rotten ham

### 3.4.2.1. Analysis of volatile flavor compounds

One of the most important quality evaluation indexes of dry-cured ham is flavor, which directly affects the sensory quality of products. Gas chromatography-mass spectrometry (GC-MS) was performed to reveal volatile flavor compounds of normal and spoiled hams. Table 3-3 reveals the volatile flavor compounds of normal ham and rotten ham stored in different days.

**Table 3-3.** Identification and determination of relative contents of volatile odor compounds in the control and artificially contaminated sliced Italian Parma ham samples.

| ID | RT | Compound name | Relative content % | |
|---|---|---|---|---|
| | | | Day 0 | Day 30 |
| | **Aldehyde** | | **58.03** | **8.59** |
| 1 | 20.173 | Nonanal | N.D. | 8.59 |
| 2 | 21.67 | Undecanal | 0.27 | N.D. |
| 3 | 26.46 | 7-hexadecenal | 5.53 | N.D. |
| 4 | 31.483 | 15-octadecenal | 1.87 | N.D. |
| 5 | 32.174 | Z-11-pentadecenal | 1.10 | N.D. |
| 6 | 33.506 | Cis-9-hexadecenal | 0.87 | N.D. |
| 7 | 36.007 | 4-(2,2-dimethyl-6-methylenecyclohexyl) butanal | 3.69 | N.D. |
| 8 | 36.152 | 13-octadecenal | 2.91 | N.D. |
| 9 | 36.773 | 9,12-octadecadienal | 4.07 | N.D. |
| 10 | 38.937 | 2，6，10-dodecatrienal | 14.15 | N.D. |

| | | | | |
|---|---|---|---|---|
| 11 | 39.153 | 7,11-hexadecadienal | 15.98 | N.D. |
| 12 | 40.957 | 4,9,13,17-tetramethyl-4,8,12,16-octadeca tetraenal | 7.57 | N.D. |
| | **Ketones** | | **10.47** | **N.D.** |
| 13 | 29.942 | 5,9-undecadien-2-one | 3.18 | N.D. |
| 14 | 27.094 | 1-cyclododecyl-ethanone | 4.10 | N.D. |
| 15 | 29.942 | 6,10-dimethyl-5,9-undecadien-2-one | 3.18 | N.D. |
| | **Alcohols** | | **24.59** | **2.87** |
| 16 | 30.329 | 2-hexadecanol | 0.71 | 2.87 |
| 17 | 32.725 | Trans-farnesol | 2.23 | N.D. |
| 18 | 39.826 | Trans-geranylgeraniol | 12.49 | N.D. |
| 19 | 42.805 | Cholestan-3-ol | 9.16 | N.D. |
| | **Hydrocarbons** | | **4.37** | **71.31** |
| 20 | 16.684 | Octamethyl-cyclotetrasiloxane | N.D. | 1.96 |
| 21 | 32.81 | 1,2-15,16-diepoxyhexadecane | 1.77 | N.D. |
| 22 | 33.071 | Caryophyllene oxide | 2.60 | N.D. |
| 23 | 34.932 | Hexadecane | N.D. | 4.22 |
| 24 | 35.228 | Hexadecamethyl-cyclooctasiloxane | N.D. | 4.51 |
| 25 | 36.873 | 2,6,10-trimethyl-tetradecane, | N.D. | 5.41 |
| 26 | 38.573 | Hexadecamethyl-heptasiloxane | N.D. | 32.87 |
| 27 | 40.425 | Tetradecamethyl-hexasiloxane, | N.D. | 22.35 |
| | **Benzene containing compounds** | | **N.D.** | **10.37** |
| 28 | 43.869 | 2-ethylhexyl ester-3-(4-methoxyphenyl)-2-propenoic acid | N.D. | 7.41 |
| 29 | 47.31 | 2-tert-butyl-6-(3-tert-butyl-2-methoxy-5-methylbenzyl)-4-methylphenol | N.D. | 2.96 |
| | **Acids** | | **2.54** | **4.28** |
| 30 | 26.629 | Geranyl isovalerate | 0.77 | N.D. |
| 31 | 28.11 | [1,1'-bicyclopropyl]-2-octanoic acid | 0.63 | N.D. |
| 32 | 32.286 | 7,10-octadecadienoic acid | 1.13 | N.D. |
| 33 | 39.543 | Oleic acid | N.D. | 4.28 |
| | **Sulfur compounds** | | **N.D.** | **2.58** |
| 34 | 38.297 | Tert-hexadecanethiol | N.D. | 2.58 |

Note: RT - Retention Time, N.D. - not detect.

A total of 34 volatile flavor compounds were detected, including 23 normal hams and 12 spoiled hams. The flavor compounds were classified into aldehydes (12), ketones (3), alcohols (4), hydrocarbons (8), benzene containing compounds (2), acids (4) and sulfur compounds (1). It can be seen from the table that normal ham is rich in aldehydes and alcohols. The levels of hydrocarbons, acids, benzene- and sulfur-containing volatile compounds in spoiled hams were higher than normal hams.

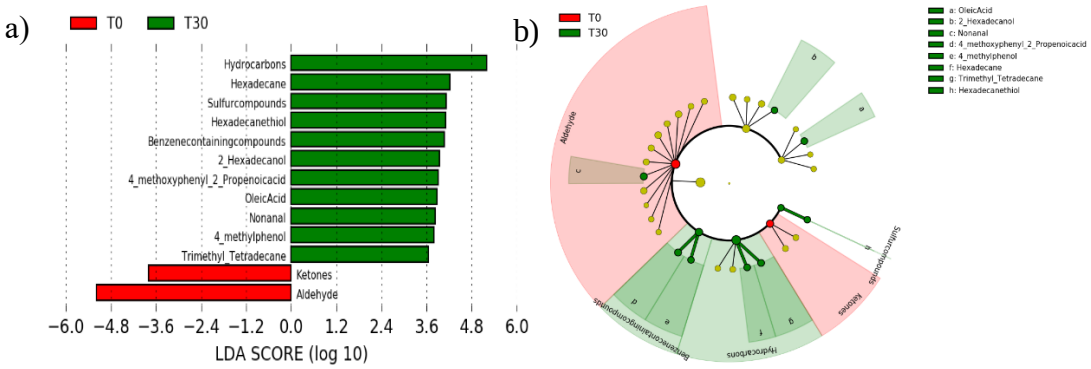



**Figure 3-6.** Characteristic volatile flavor compounds in T0 (red) and T30 (green) sliced Italian Parma dry-cured ham with linear discriminant analysis *(LDA)* under refrigerator conditions. a) Histogram of the distribution of LDA values; b) Evolutionary branch diagram.

In order to further screen, the significant identification factors between normal and spoiled ham, LEFse analysis was performed, and the threshold was set to LDA >3. The results are shown in Figure 3-6. A total of 13 identification factors with significant differences were identified, including 2 kinds of normal ham and 11 kinds of spoiled ham (Figure 3-6a). Except for acids, there were significant differences between normal and spoiled ham. Specifically, normal ham is rich in aldehydes and alcohols; High content of hydrocarbons, sulfur compounds and benzene are the characteristics of spoiled ham, especially hexadecane, hexadecanethiol, hexadecanol (Figure 3-6b). Aldehydes could be produced by lipid oxidation and Strecker reaction of amino acids, which made an important contribution to the overall flavor of

dry-cured ham. In normal ham, 11 aldehydes were detected, accounting for 58.03% of the total area of flavor substances, while only 1 aldehyde was detected in rotten ham, accounting for 8.59% of the total area.

### 3.4.2.2. Changes of total bacterial count in ham during storage

The dry-cured ham slices were sealed in PE fresh-keeping bags and stored at 4℃. On the 0, 2, 7, 14, 21 and 30 days of storage, the changes of total bacterial count (TBC) of ham slices were measured to study changes of spoilage microorganisms in ham slices during storage (Figure 3-7). It can be seen that the total bacteria count is gradually increasing during the storage period of ham. This means the spoilage microorganisms are gradually increasing. Within 0-14 days, the total bacteria number increased obviously, showing logarithmic growth stage. After 14 days, the microbial biomass remained at a relatively stable stage, with no obvious change. The total bacteria count reached the maximum at 21 days. According to the microbiological limit requirements of ready-to-eat food, the limit value of $10^4$ CFU/g is usually acceptable. The results showed that vacuum-packed ham slices safety standards within 10 days after opening. Generally, it is recommended to eat ham slices within three days after being opened.

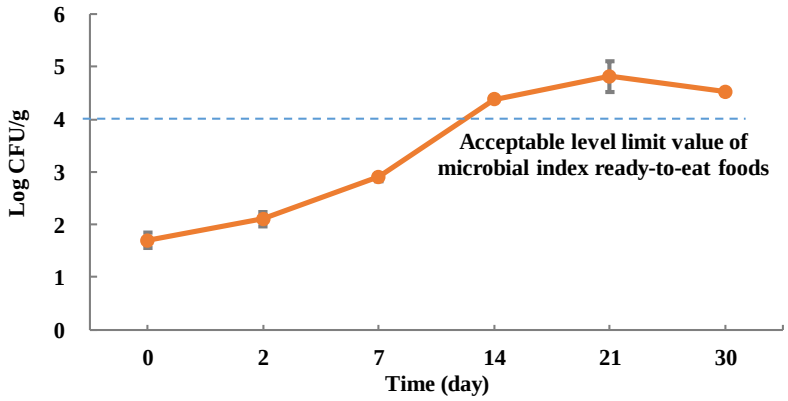



**Figure 3-7.** Variation in the total bacterial count of ham slices for 30 days of storage.

### 3.4.2.3. Sensory evaluation results

To establish an effective method for prediction and reducing spoilage, physicochemical characteristics of normal and spoiled hams were compared. The flavor of ham samples was evaluated under controlled and constant temperature (25 °C) and bright natural light. Evaluation indexes included the acceptability, color, flavor, odor, and texture of ham samples. A sensory panel was formed by five evaluators, and team members were trained before sensorial evaluation panels. Panelists evaluated samples on a 10-point scale. The overall sensory acceptability of ham samples was rated based on the weight of the following parameters: acceptability 20%, tissue state 25%, color15%, odor 25%, and crumbliness 15%. As can be seen from the scoring results and overall acceptability (Figure 3-8), the state of ham changed obviously after 7 days, the color gradually changed from pink to dark red, and the viscosity gradually became viscous. Acceptable within 7 days of storage.

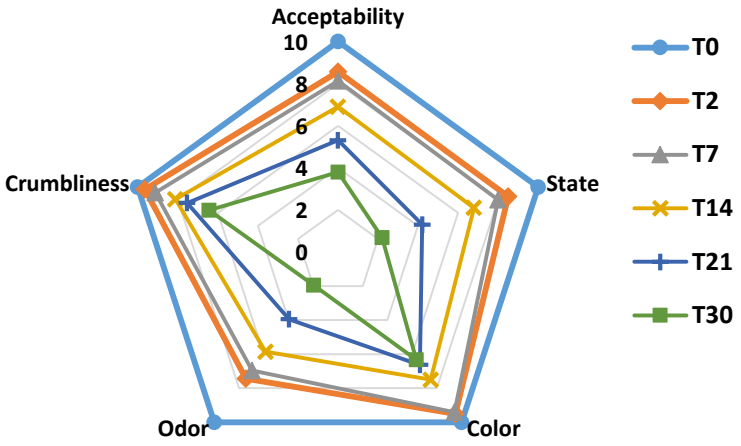



**Figure 3-8.** Changes of five main characters of sliced Italian Parma dry-cured ham during 7 days under refrigerator conditions, including acceptability, state, color, odor and crumbliness. T0, T2, T7, T14, T21, T30.

### 3.4.3. Application of the analytical method on ham samples during storage

Table 3-4 shows a comprehensive comparison of the dynamic changes in the content of ATP-related compounds in ham samples within 7 days of storage. The analytical method allowed the extraction and quantification of four ATP-related compounds (ATP, ADP, AMP, and Hx) as reported in Figure 3-9. Among the four ATP-related compounds, ATP concentration was the highest (>100 mg/kg), followed by AMP (>50 mg/kg). They increased with the storage time and the fastest increase was observed in the first two days. The concentrations of ADP and Hx were low (<10 mg/kg) with reduced variations during the storage time.

**Table 3-4.** The results of the ATP-related compounds and the TBC in ham samples from T0 to T7

| **Concent-ration (mg/kg)** | **Storage time (days)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **T0** | **RSD %** | **T2** | **RSD %** | **T5** | **RSD %** | **T7** | **RSD %** |
| **ATP** | 95.9 ± 5.3 | 5.5 | 152.4 ± 4.5 | 1.4 | 162.5 ± 2.3 | 1.0 | 172.0 ± 1.8 | 2.8 |
| **ADP** | n.d | - | 6.5 ± 0.4 | 6.3 | 6.7 ± 0.6 | 15.0 | 6.4 ± 0.5 | 12.2 |
| **AMP** | 53.9 ± 3.1 | 16.0 | 96.8 ± 1.4 | 1.4 | 106.3 ± 2.8 | 2.6 | 108.4 ± 3.0 | 3.4 |
| **Hx** | 3.9 ± 0.2 | 16.6 | 13.4 ± 0.4 | 2.7 | 10.9 ± 0.2 | 1.6 | 10.9 ± 0.2 | 1.8 |
| Log CFU/g | | | | | | | | |
| **TBC** | 1.7 ± 0.08 | 8.0 | 2.1 ± 0.1 | 14.4 | 2.7 ± 0.08 | 7.7 | 2.9 ± 0.1 | 2.6 |

n.d: non detected (<LOQ)

The contents of ATP-related compounds in samples stored from day 0 to day 7, were determined, and the correlation of these parameters with a total bacterial count in samples was investigated. Figure 3-9 provided a linear relationship between ATP levels from living bacteria in ham samples (mg/kg) and the total number of bacteria estimated from the standard plate count (CFU/g). It is worth exploring the

relationships between ATP-related compounds and bacteria. The contents of ATP, AMP, and TBC values in ham samples increased with increasing storage time and showed a good correlation, thus proving that measured ATP compounds were representative of microbial ATP and that the increase in the content of ATP-related compounds in ham samples during storage was positively correlated with the microbial load in samples.

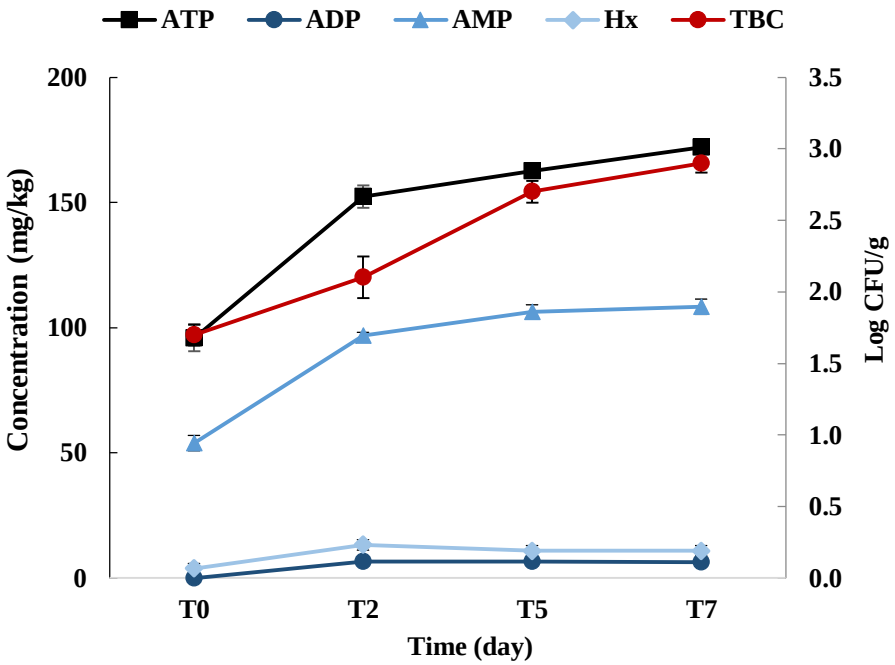



Figure 3-9. Changes in the concentration of ATP-related compounds and total bacterial count in sliced Italian Parma ham samples during storage. Legend: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), hypoxanthine (Hx) and total bacterial count (TBC).

The levels of analytes (ATP, ADP, AMP, and Hx) obtained through HPLC are related to microbial energy metabolism. Indeed, the level of ATP-related compounds is proportional to the amount of bacterial ATP present in the sample, which is used to correlate the number of viable bacteria (Ratphitagsanti, Park, Lee, Amos Wu, & Lee, 2012). As such, the reproduction of bacteria in the process of ham corruption seems to

play an important role in the increase of ATP-related compounds. The advantage of using HPLC to detect ATP-related compounds in sliced dry-cured ham is that the storage on day 2 shows a higher trend and a larger difference earlier than TBC. Indeed, the high increase in ATP content was observed on day 2, while for TBC, the high increase was observed on day 5.

In this study, the total bacterial count in sliced dry-cured ham increased with the increase in storage time. All sliced dry-cured ham after day 2 of storage had counts of microorganisms greater than 2 log CFU/g, whereas on day 7 were close to 3 log CFU/g. These results are consistent with those reported on Iberian dry-cured ham (Martin, et al., 2008). In fresh meat such as chicken, the microbial content is generally higher than 3 log CFU/g at day 0 and higher than 4 log CFU/g after 7 days of storage (Alessandroni, et al., 2022). The lower microbial contents in the ham could be a consequence of the reduction in moisture and the high NaCl concentration. Due to the small number of microorganisms, the microbial corruption detection index of fresh meat is not sensitive for ham quality detection, which makes it difficult to detect ham corruption.

These results showed that the content of ATP-related compounds could be considered as spoilage markers of ham samples during storage together with the intact bacterial cell count. Changes in the content of ATP-related compounds within 7 days of storage have a determining impact on sliced ham quality.

## 3.5. CONCLUSIONS

In the present study, an HPLC-based method for the separation and quantification of ATP-related compounds was proposed. SunFire$^{TM}$ C18 (4.6 mm×mm, 5μm) was

used and the mobile phase was V (0.05mol/L $KH_2PO_4$-$K_2HPO_4$) : V (methanol) = 9:1 buffer at a flow rate of 0.6 mL/min, and was detected by high performance liquid chromatography at 254 nm and quantified by external standard method. The developed HPLC method had high sensitivity as well as good linearity and repeatability. The validated method successfully detected four ATP-related compounds (ATP, ADP, AMP and Hx) in dry ham samples, while Ino was not measurable in ham. Moreover, the recovery of ATP-related compounds in ham samples was in accordance with the application detection range. Moreover, a positive correlation was found between ATP content and microbial load during storage in ham samples. Thus, the method developed here can be generally applied for the determination of four ATP-related compounds in dry ham.

## 3.6. References


Alessandroni, L., Caprioli, G., Faiella, F., Fiorini, D., Galli, R., Huang, X., Marinelli, G., Nzekoue, F., Ricciutelli, M., Scortichini, S., Silvi, S., Tao, J., Tramontano, A., Turati, D., & Sagratini, G. (2022). A shelf-life study for the evaluation of a new biopackaging to preserve the quality of organic chicken meat. *Food Chemistry*, *371*, 131134.

Clariana, M., Guerrero, L., Sarraga, C., Diaz, I., Valero, A., & Garcia-Regueiro, J. A. (2011). Influence of high pressure application on the nutritional, sensory and microbiological characteristics of sliced skin vacuum packed dry-cured ham. Effects along the storage period. *Innovative Food Science & Emerging Technologies*, *12*(4), 456-465.

Dingle, J. R., Hines, J. A., & Fraser, D. I. (2006). Postmortem Degradation of Adenine Nucleotides in Muscle of the Lobster, Homarus americanus. *Journal of Food Science*, *33*(1), 100-103.

Ellis, D. I., & Goodacre, R. (2001). Rapid and quantitative detection of the microbial spoilage of muscle foods: current status and future trends. *Trends in Food Science & Technology*, *12*(11), 414-424.

Farthing, D., Sica, D., Gehr, T., Wilson, B., Fakhry, I., Larus, T., Farthing, C., & Karnes, H. T. (2007). An HPLC method for determination of inosine and hypoxanthine in human plasma from healthy volunteers and patients presenting with potential acute cardiac ischemia. *Journal of Chromatography B*, *854*(1), 158-164.

Fernandez, M., Hospital, X. F., Cabellos, C., & Hierro, E. (2020). Effect of pulsed light treatment on Listeria inactivation, sensory quality and oxidation in two varieties of Spanish dry-cured ham. *Food Chemistry*, *316*, 126294.

Hassoun, A., Sahar, A., Lakhal, L., & Aït-Kaddour, A. (2019). Fluorescence spectroscopy as a rapid and non-destructive method for monitoring quality and authenticity of fish and meat products: Impact of different preservation conditions. *LWT*, *103*, 279-292.

Hwang, D. F., Chen, T. Y., Shiau, C. Y., & Jeng, S. S. (2000). Seasonal variations of free amino acids and nucleotide‐related compounds in the muscle of cultured Taiwanese puffer Takifugu rubripes. *Fisheries Science*, *66*(6), 1123-1129.

Jay, & James, M. (2000). Modern Food Microbiology 6th Ed. (Chapter 7), 113-130.

Kumar, Y. (2021). Isothermal amplification-based methods for assessment of microbiological safety and authenticity of meat and meat products. *Food Control*, *121*, 107679.

Lee, H., Ho, M., Bhuwan, M., Hsu, C., Huang, M., Peng, H., & Chang, H. (2010). Enhancing ATP-based bacteria and biofilm detection by enzymatic pyrophosphate regeneration. *Analytical Biochemistry*, *399*(2), 168-173.

Martin, A., Benito, M. J., Hernandez, A., Perez-Nevado, F., Cordoba, J. J., & Cordoba, M. G. (2008). Characterisation of microbial deep spoilage in Iberian dry-cured ham. *Meat Science*, *78*(4), 475-484.

Mitchell, B. G., McGhie, A., Whiteley, G., Farrington, A., Hall, L., Halton, K., & White, N. M. (2020). Evaluating bio-burden of frequently touched surfaces using Adenosine Triphosphate bioluminescence (ATP): Results from the Researching Effective Approaches to Cleaning in Hospitals (REACH) trial. *Infection, Disease & Health*, *25*(3), 168-174.

Mora, L., Hernandez-Cazares, A. S., Aristoy, M., & Toldra, F. (2010). Hydrophilic interaction

chromatographic determination of adenosine triphosphate and its metabolites. *Food Chemistry*, *123*(4), 1282-1288.

Piras, F., Fois, F., Casti, D., Mazza, R., & Mazzette, R. (2016). Shelf Life of Sliced Dry-Cured Ham Packaged Under Vacuum: Shelf Life of Sliced Dry-Cured Ham. *Journal of Food Processing and Preservation*, *40*(6), 1223-1228.

Ramsey, S., & Parikh, S. (2021). Rapid microbiological testing of biologics products using atp bioluminescence. *Cytotherapy*, *23*(5, Supplement), S201.

Ratphitagsanti, W., Park, E., Lee, C. S., Amos Wu, R. Y., & Lee, J. (2012). High-throughput detection of spore contamination in food packages and food powders using tiered approach of ATP bioluminescence and real-time PCR. *LWT - Food Science and Technology*, *46*(1), 341-348.

Ren, Q., Fang, K., Yang, X., & Han, J. (2022). Ensuring the quality of meat in cold chain logistics: A comprehensive review. *Trends in Food Science & Technology*, *119*, 133-151.

Roberts, B., Morris, B. A., & Clifford, M. N. (1991). Comparison of radioimmunoassay and spectrophotometric analysis for the quantitation of hypoxanthine in fish muscle. *Food Chemistry*, *42*(1), 1-17.

Rubio, B., Martinez, B., Gonzalez-Fernandez, C., Garcia-Cachan, M. D., Rovira, J., & Jaime, I. (2006). Influence of storage period and packaging method on sliced dry cured beef "Cecina de Leon": Effects on microbiological, physicochemical and sensory quality. *Meat Science*, *74*(4), 710-717.

Soga, T., & Imaizumi, M. (2015). Capillary electrophoresis method for the analysis of inorganic anions, organic acids, amino acids, nucleotides, carbohydrates and other anionic compounds. *Electrophoresis*, *22*(16), 3418-3425.

Wei, Q., Wang, X., Sun, D., & Pu, H. (2019). Rapid detection and control of psychrotrophic microorganisms in cold storage foods: A review. *Trends in Food Science & Technology*, *86*, 453-464.

Xue, X. F., Zhou, J. H., Wu, L. M., Fu, L. H., & Zhao, J. (2009). HPLC determination of adenosine in royal jelly. *Food Chemistry*, *115*(2), 715-719.

# CHAPTER IV

# STUDY ON THE ANTIBACTERIAL ACTIVITY AND FERMENTATION OF LACTIC ACID BACTERIA AND METABOLITES

## 4.1. ABSTRACT

Since LAB is widely used in food and has health-promoting properties, they are considered by food experts as a new type of biopreservative to ensure food safety and quality. In this case, our aims are to investigate the application potential of *Lactobacillus* in food preservation. However, before building such a prototype, we need to carefully characterize and select the probiotic strain to achieve the desired results.

The aim is to investigate the antibacterial and probiotic properties of *Lactiplantibacillus plantarum*, which is different from other LAB, to provide a theoretical basis for further application of *L. plantarum*. *L. plantarum* can not only enhance the nutritional value of food, but also produce antibacterial substances and prolong the storage time of food. This study screened 6 Lactobacillus strains. Among them, *L. plantarum* to produce antimicrobial peptides was not the most prominent. However, the highest extracellular polysaccharide (EPS) production was found in *L. plantarum*. EPS can be used as thickeners, emulsifiers, and gels in foods. The immobilization technology can effectively simplify and improve the separation and regeneration of extracellular polysaccharides. In this study, the different immobilized carriers of sodium alginate, agar, gelatin-glutaraldehyde, and carrageenan were

assessed for fermentation. Different diameters of beads were selected, and the optimum culture temperature, carrier concentration, and inoculum volume were evaluated. The results showed that the agar-embedding method was the best immobilization method and the optimum process conditions were as follows: 3% inoculum of *L. plantarum*, 30 g/L of agar as immobilization carrier with a particle diameter of 1 cm, and fermentation at 37°C for 24 h. Through orthogonal experiments, the maximum exopolysaccharide (EPS) yield obtained was 1489.9 mg/L.

**Practical Application:** The immobilization technology can effectively simplify and improve the separation and regeneration of extracellular polysaccharides. The study provides a basis for improving the separation and regeneration performance of EPS and continuous fermentation to produce LAB EPS. Moreover, the immobilized LAB cells can be well maintained and can be used repeatedly, effectively increasing the rate of use of the strain and reducing the production cost.

## 4.2. INTRODUCTION

Depending on the bacteria species and available substrates, LAB fermentation produces various valuable metabolites. As of now, most of the identified antibacterial substances are low molecular weight compounds consisting of EPS, bacteriocins, hydrogen peroxide, organic acids, short-chain fatty acids, biosurfactants, ethanol and bacteriocin-like substances (Barzegari, Kheyrolahzadeh, Khatibi, Sharifi, & Vahed, 2020).

The production of LAB EPS is affected by many environmental factors and intracellular factors. LAB secrete different types of EPS (Miao, et al., 2015). Important factors for the total yield of EPS produced from LAB include the

composition of the medium (carbon and nitrogen sources, vitamins, minerals, etc.), LAB strains, and growth conditions (temperature, agitation, incubation time, pH, and oxygen tension). One of the main disadvantages of the EPS-producing LAB is the small amount of polymer synthesized, which varies from 25-500 mg/L. The highest production levels reported so far were obtained for the mesophilic strains *Lactobacillus rhamnasus 9595M* (1200 mg/L) and *Lactobacillus sakei 0-1* (1375 mg/L) (Mozzi, Giori, & Valdez, 2010). Optimization of the growth environment is critical if maximal EPS production by LAB strains is to be achieved (Patel, Majumder, & Goyal, 2012). To produce EPS using the biological microcapsule, the cells are evenly dispersed in the gel beads, which can maintain the viability and intracellular enzyme activity of the bacteria for a long time, for continuous production, and protect the cells from adverse conditions such as acid, alkali, and harmful ions. Immobilized particles are also conducive to simple strain activation and product extraction process (Miao, et al., 2015). Continuous fermentation of immobilized (Menchavez & Ha, 2019) LAB to produce EPS can simplify and optimize the industrial production process (Thakur, Panesar, & Saini, 2019), which can substantially reduce production costs (Idris & Suzana, 2006). The excessive production cost of LAB EPS also restricts its large-scale industrial production. However, there is no research on the immobilization of lactic acid bacteria to produce exopolysaccharides.

In this study, high-yielding EPS LAB strains were firstly screened, immobilized, and used for shake flask fermentation. The optimized immobilization method, fermentation production temperature, and seed access amount were selected, and orthogonal experiments were carried out to determine the optimized immobilized LAB EPS fermentation process conditions. The study provides a basis for improving the separation and regeneration performance of EPS and continuous fermentation to

produce LAB EPS.

## 4.3. MATERIALS AND METHODS

### 4.3.1. Materials

Six strains of LAB, *Lactobacillus bulgaricus* CGMCC1.6970, *Lactobacillus acidophilus* CGMCC1.1878, *Lactiplantibacillus plantarum* GIMCC1.191, *Streptococcus thermophilus* CGMCC1.2718, *Lactobacillus casei* CGMCC1.3206, and *Lactococcus lactis subsp. latis* CICC6242 LL9 were provided by Laboratory of College of Food and Bioengineering, Zhengzhou University of Light Industry.

Indicator bacteria: *Bacillus subtilis* was provided by Laboratory of College of Food and Bioengineering, Zhengzhou University of Light Industry.

MRS media: peptone 10 g/L, beef extract 10 g/L, yeast extract 5 g/L, ammonium citrate 2 g/L, glucose 20 g/L, Tween 80 1 mL/L, sodium acetate 5 g/L, dipotassium phosphate 2 g/L, magnesium sulfate 0.58 g/L, manganese sulfate 0.25 g/L, agar 20 g/L, distilled water to 1000 mL, adjusted to pH 6.2-6.6.

Test medium: Nutrient agar medium.

The dialysis bag treatment solution consisted of solution A (1.6 g of sodium hydrogen carbonate and 0.296 g of EDTA dissolved together in 800 mL of distilled water) and solution B (0.298 g of EDTA dissolved in 800 mL of distilled water).

### 4.3.2. Strain activation

The bacterial powder was inoculated into MRS liquid medium, cultured at 37 ℃ 24 h, subcultured 2 to 3 times, and stained for microscopic examination. The selected

excellent strains were transferred to MRS slants and stored in a refrigerator at 4 ℃ for use.

### 4.3.3. Bacteriostatic testing

The antibacterial activities of the screened LAB were determined using the Oxford Cup antibacterial method (Ju, et al., 2021). 20 mL of sterilized test medium into the sterile Petri dish and allow it to solidify. The indicator bacteria *Bacillus subtilis* 100 μL with concentration of $10^6$ CFU/mL was then coated to the surface of test medium, until it was fully coated evenly can be put into the sterile Oxford cup, the spacing of each Oxford cup at least 20 mm. Then, 200 μL of LAB CFS was added to the hole of the Oxford cup, allowed to diffuse at room temperature for 2 h and then cultured at 37 ℃ for 24 h, followed by measurement of the bacteriostatic circle diameter.

### 4.3.4. Microbial cell fixation:

#### 4.3.4.1. Calcium alginate method

The logarithmic growth phase fermentation solution was centrifuged, and a cell suspension was prepared with an equal volume of normal saline. Ten milliliters of the bacterial suspension were added into a defined concentration of immobilized carrier solution (alginic acid sodium solution). After mixing, granulation was carried out with a syringe by dropping 2% calcium chloride solution and immobilized for 1.5-2 h.

#### 4.3.4.2. Agar fixation method

The melted agar solution (3%) was mixed with the logarithmic growth phase fermentation broth using a syringe to drop the mixture into a measuring cylinder

containing liquid paraffin in the upper layer and the lower layer was water. The granules were filtered using sterile gauze and rinsed three times with sterile water for further use.

#### 4.3.4.3. Gelatin-glutaraldehyde fixation method

The gelatin solution and the logarithmic growth phase fermentation broth were uniformly mixed to obtain the final gelatin concentration of 10%. To the mixture, glutaraldehyde was directly added, stirred uniformly, and poured into a sterilized culture dish. After coagulation, the gel was cut into small squares of 5 mm × 5 mm × 5 mm and rinsed with sterile water for use.

#### 4.3.4.4. Carrageenan embedding method

Carrageenan is highly stable as fine powder and does not hydrolyze even when heated. 4 g carrageenan was poured at 8% concentration into 50 mL distilled water and shaken. This was disinfected and dissolved in a sterilizer and allowed to cool. After cooling to about 45 ℃, the mixed bacterial suspension was embedded.

### 4.3.5. Determination of EPS using the phenol sulfuric acid method

Pure glucose (4 mg) was dissolved in double-distilled water, quantitatively transferred to a 100 mL volumetric flask, diluted with double-distilled water to the container mark line, and shaken well to obtain a final concentration of 40 μg/mL glucose.

Standard samples of different concentrations were prepared in 8 20 ml fixed tubes. The absorbance (OD) of the sample was measured at 490 nm using a spectrophotometer. The standard glucose quasi-curve was prepared by taking the liquid in tube number 0 (blank) as a reference, the glucose concentration as the

abscissa, and the corresponding OD value as the ordinate. The linear regression equation y = 0.0095x-0.0067 and the correlation coefficient $R^2$ = 0.9955 were obtained.

### 4.3.6. MD34 dialysis bag treatment and use method

Pretreatment of dialysis bags were to cut 150 mm dialysis bags and boil them in deionized water for 10 minutes.

Then the dialysis bag was treated by washing the used dialysis bag with deionized water, and the washed dialysis bag was boiled in solution A for 10 min, taken out, and washed with deionized water. The dialysis bag was then boiled in solution B for 10 min, washed with deionized water, and finally stored in 20% ethanol at 4 ℃ or used directly.

The beaker was filled with 800 mL of double-distilled water, and one end of the treated dialysis bag was clamped with a dialysis clip, and then double-distilled water was charged to check for water leakage (including whether the clip was caught or the dialysis bag was damaged). After the leak detection was completed, the bag was marked with a black marker on the clip, and 20 times diluted EPS solution was added to the dialysis bag and checked for leaks again. After confirming the error, dialysis was carried out for 12 h.

### 4.3.7. Dialysate post-treatment

The dialyzed dialysate was poured into a sterile test tube and a number was assigned to it. To a corresponding number of sterile test tubes, 1 mL of the treated dialysate was added separately, and 2 mL of double-distilled water was added to one

of the tubes numbered 0, as a blank control experiment. Then, to each tube, 1 mL of deionized water followed by 1 mL of 5% phenol solution, and 5 mL of 95% concentrated sulfuric acid were added to each tube and allowed to stand for 10 min. Finally, EPS was determined by the phenol sulfuric acid method.

### 4.3.8. Optimization of protein removal conditions

The polysaccharide purification process involves the removal of impurities from the extracted crude polysaccharide so that a single polysaccharide component can be obtained. The main impurities in the LAB EPS are proteins, pigments, and some small molecules, which make it difficult for the subsequent extraction of EPS. Therefore, the key point of purifying the LAB fermentation broth was the removal of proteins. For this, the trichloroacetic acid solution was added at a one-fifth volume of the fermentation broth, shaken well, and placed at 4 ℃ for 12 h to settle.

### 4.3.9. Experimental procedure

The process flow chart of EPS separation and purification is shown in Figure 4-1.

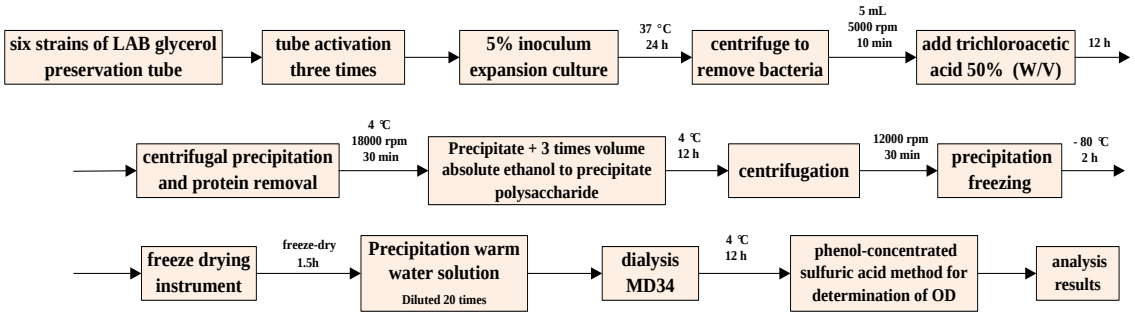



**Figure 4-1.** Process flow chart of EPS separation and purification

### 4.3.10. Statistical analysis

Every measurement was repeated at least 2 times. The results were subjected to ANOVA test and using the Tukey test, for the significant difference $p<0.05$ was considered (Origin 2021).

## 4.4. RESULTS AND DISCUSSION

### 4.4.1. Screening of the antibacterial properties of LAB

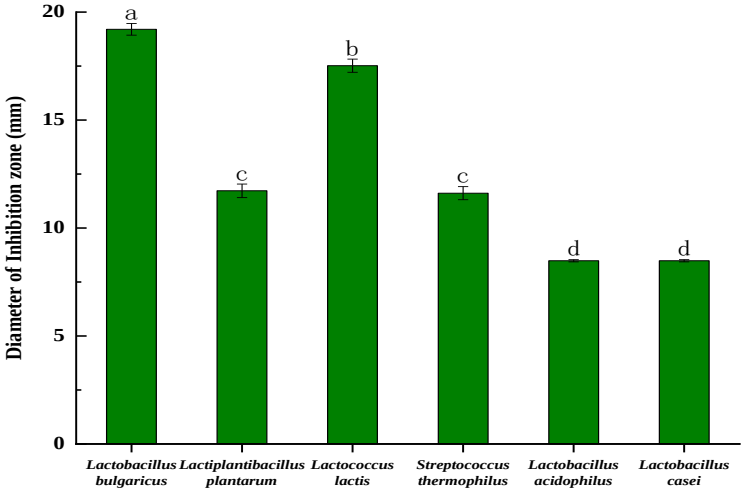



**Figure 4-2.** Inhibitory effect of 6 LAB on *Bacillus subtilis*, as shown by zone of inhibition diameters. [a, b, c, d] *statistically significant different by Tukey test.*

A total of 6 LAB strains (*Lactobacillus bulgaricus* CGMCC1.6970, *Lactobacillus acidophilus* CGMCC1.1878, *Lactiplantibacillus plantarum* GIMCC1.191, *Streptococcus thermophilus* CGMCC1.2718, *Lactobacillus casei* CGMCC1.3206, and *Lactococcus lactis subsp. latis* CICC6242 LL9) were activated and fermented on MRS medium. The antibacterial activities of the 6 LAB were tested against *Bacillus subtilis* using the agar diffusion method. Most LAB could inhibit the growth of *Bacillus subtilis*, although the antibacterial effects varied (Figure 4-2). Among the

strains, *Lactobacillus bulgaricus* strain exhibited the strongest inhibitory effect against *Bacillus subtilis*, with a measured inhibition zone diameter of 19.20 mm. *L. plantarum* to produce antimicrobial peptides was not the most prominent with a measured inhibition zone diameter of 11.72 mm.

### 4.4.2. Screening of extracellular polysaccharide produced by LAB

If the EPS produced by LAB cells is widely used, the first task in actual production is to obtain EPS high-yielding strains to reduce EPS production costs. In this experiment, the EPS producing ability of six strains of LAB was studied, and high EPS-yielding strains were screened. For each LAB, 5% inoculum was fermented in MRS medium for 24 h, and the EPS yield was measured after extraction and purification. The results are shown in Figure 4-3.

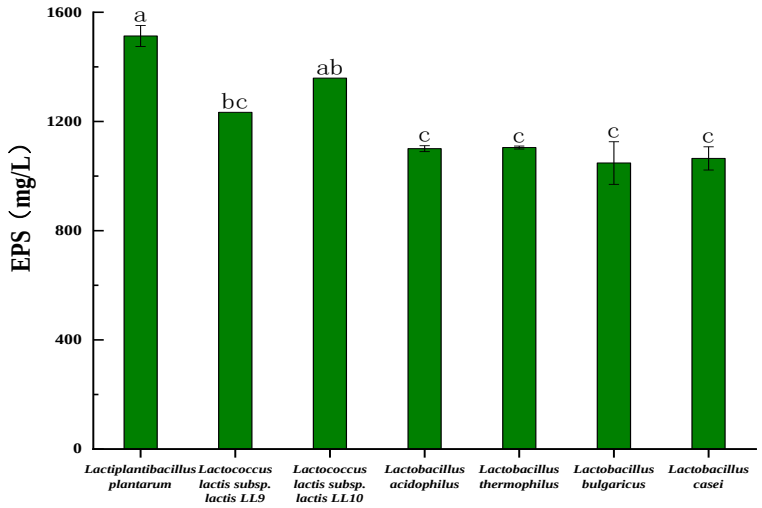



**Figure 4-3.** EPS produced of 6 LAB at 5% inoculum each. *[a, b, c] statistically significant different by Tukey test.*

According to Figure 4-3 that the six strains of LAB can synthesize EPS, but at different capacities. The highest production EPS was by *L. plantarum* at 1513.1 mg/L, and the lowest yield was by cheese LAB, *L. casei* at 1064.6 mg/L. The EPS

production by the other five strains was somewhere in between. *L. plantarum* was identified as a high EPS producing strain (M. Y. M. Imran, et al., 2016). *L. plantarum* strain was thus selected for follow-up tests.

### 4.4.3. Single-factor impact experiment

#### 4.4.3.1. Effect of immobilized materials on the production of extracellular polysaccharides by *L. plantarum*

*L. plantarum* was immobilized in four different carrier types, with 5% inoculum, and fermented in a shake flask at 37 ℃. Then EPS extraction and purification were conducted, and finally, the EPS yield was measured by a spectrophotometer.

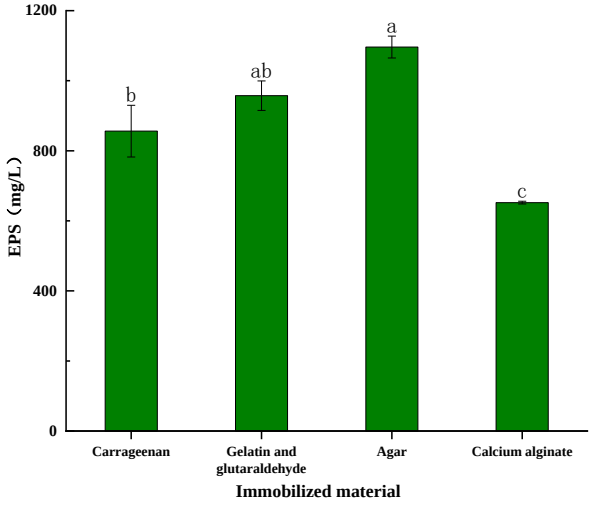



**Figure 4-4.** Effect of immobilized materials on EPS yield. *[a, b, c] statistically significant different by Tukey test.*

The bar graph (Figure 4-4) shows that the EPS yield of *L. plantarum* decreased after treatment with immobilized materials, and the agar embedding method had the highest EPS productivity by *L. plantarum*, which was 1096.2 mg/L; therefore, agar was selected as the immobilization material to further explore the optimum process conditions.

#### 4.4.3.2. Effect of temperature on the content of extracellular polysaccharides by *L. plantarum*

Immobilization of microorganisms sometimes changes their optimum temperature of the fermentation. Generally, the optimum temperature of the immobilized enzyme is higher than that of the free enzyme, so the optimum fermentation temperature also increases. We investigated the optimal temperature of EPS synthesis after immobilizing microbial cells. The EPS yield measured after fermentation of immobilized *L. plantarum* at different temperatures is shown in Figure 4-5.

As can be seen from the Figure 4-5, the optimum temperature for fermentation of immobilized *L. plantarum* was 37 ℃. When the fermentation temperature was lower than 37 ℃, the EPS yield reduced significantly and was the lowest at 30 ℃. The results showed that the optimum temperature of fermentation for maximum yield of EPS by *L. plantarum* did not change, and was 37 ℃.

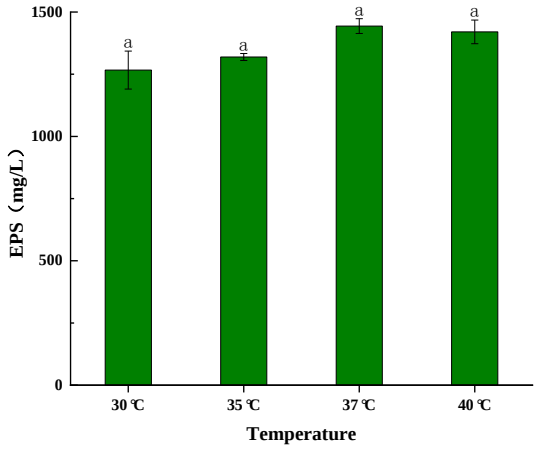



**Figure 4-5.** Effect of temperature on EPS yield. *[a] statistically significant different by Tukey test.*

#### 4.4.3.3. Effect of amount of inoculum on the production of extracellular polysaccharides by *L. plantarum*

Microorganisms require optimal inoculum for fermentation and productivity. A very large or very small size of inoculum affects product synthesis. Here, we studied

the effect of different amounts of inoculum (15%, 13%, 10%, 8%, and 5%). The effect of inoculum size on the production of EPS by *L. plantarum* is shown in Figure 4-6.

As can be seen from Figure 4-6, when the inoculum amount was 5%, the EPS yield of LAB was the highest at 1443.6 mg/L, indicating that the inoculation amount was optimal and the most beneficial for the production of EPS by LAB fermentation.

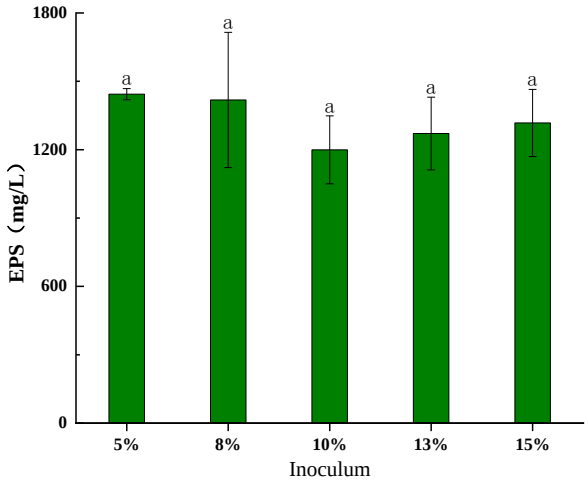



**Figure 4-6.** Effect of inoculum size on EPS yield. [a] *statistically significant different by Tukey test.*

#### 4.4.3.4. Effect of immobilization using agar at different concentrations on the production of extracellular polysaccharides by *L. plantarum*

The concentration of agarose for immobilization can have a significant impact on EPS yield. A low concentration of agar results in low strength of the colloid after embedding, causing brittleness and affecting dispersibility, and a possible cell leakage leading to embedding failure. At high agarose concentration, the agar colloid strength is not easy to break, but it affects the contact efficiency between the nutrient and the bacteria, causing slow growth of the cells, and a decrease in the efficiency of EPS productivity. Moreover, the EPS cannot diffuse into the fermentation broth through the agar, and little or no detection was registered in the fermentation broth, thus, greatly affecting the yield of LAB EPS. We studied the effect of different

concentrations of agar (15, 20, 25, 30, and 35 g/L). The effect of agar concentration on EPS production by *L. plantarum* is shown in Figure 4-7.

When the agar concentration was 35 g/L, the LAB EPS yield was 1458.3 mg/L. This indicates that the agar strength does not significantly affect the LAB EPS productivity of *L. plantarum* and the culture solution. It does not affect the absorption of nutrients by the cells, as well as the diffusion of EPS into the medium solution. Therefore, the agar concentration of 35 g/L was selected as the optimum colloid strength.

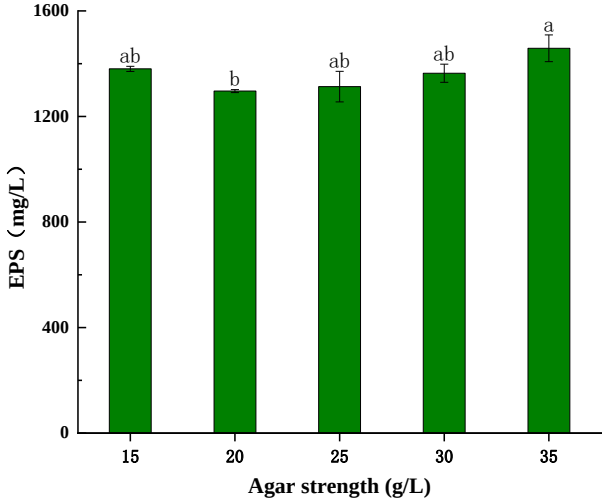



**Figure 4-7.** Effect of agar strength on EPS yield. *[a, b] statistically significant different by Tukey test.*

#### 4.4.3.5. Effect of the diameter of agar particle on the production of extracellular polysaccharides by *L. plantarum*

The size of the encapsulated agar block has a large effect on the EPS yield of *L. plantarum*. Excessively large size is not conducive to the exchange of substances between the LAB and the medium and is too small to be conducive to experimental operation and actual fermentation production. Here, we tried the beads of the immobilized carrier material at different sizes (5, 10, and 15 mm) and the effect on

EPS production is shown in Figure 4-8.

When the agar particle diameter was 1.0 cm, the EPS yield of *L. plantarum* was 1477.3 mg/L and the highest among all the tested agar particles of different diameters. This indicates that the microorganisms embedded in the agar can exchange materials with the medium and that the effect of agar immobilization on LAB EPS productivity was minimal. Therefore, 1.0 cm was selected as the optimum particle size for immobilization.

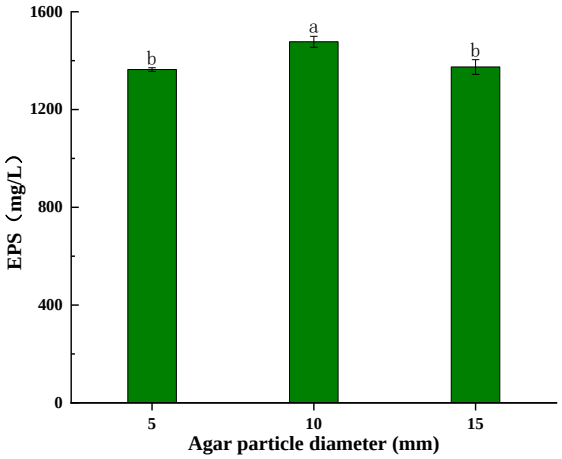



**Figure 4-8.** Effect of agar particle diameter on EPS yield. *[a, b] statistically significant different by Tukey test.*

### 4.4.4. Orthogonal experimental results

From the afore-mentioned single factor experiments, three conditions of fermentation temperature, agar strength, and inoculum size could be selected for the orthogonal experiment (Table 4-1). The results of the orthogonal experiment are shown in Table 4-2.

The analysis of the R-value from the orthogonal experiments shows that the influence of a single factor on the EPS yield of LAB was in the order of inoculum size > agar intensity > temperature. The optimum fermentation conditions were agar

strength of 30 g/L, fermentation temperature of 37 °C, and inoculum size of 3%.

**Table 4-1.** Orthogonal experimental factors.

| Factor and level | A Agar strength (mg/L) | B Temperature (℃) | C Inoculation amount (%) |
|---|---|---|---|
| 1 | 30 | 35 | 3 |
| 2 | 35 | 37 | 5 |
| 3 | 40 | 40 | 8 |

**Table 4-2.** Orthogonal experiment results

| Experiment Number Factor | A Agar strength (mg/L) | B Temperature (℃) | C Inoculum (%) | Productivity of EPS (mg/L) |
|---|---|---|---|---|
| 1 | 30 | 35 | 3 | 1487.8 |
| 2 | 30 | 37 | 5 | 1418.5 |
| 3 | 30 | 40 | 8 | 1401.5 |
| 4 | 35 | 35 | 5 | 1399.4 |
| 5 | 35 | 37 | 8 | 1399.4 |
| 6 | 35 | 40 | 3 | 1489.9 |
| 7 | 40 | 35 | 8 | 1302.5 |
| 8 | 40 | 37 | 3 | 1485.7 |
| 9 | 40 | 40 | 5 | 1361.5 |
| K1 | 4307.6 | 4189.7 | 4463.4 | |
| K2 | 4288.7 | 4303.6 | 4179.4 | |
| K3 | 4149.7 | 4252.9 | 4103.4 | |
| K1 | 1435.9 | 1396.6 | 1487.8 | |
| K2 | 1429.6 | 1434.5 | 1393.1 | |
| K3 | 1383.2 | 1417.6 | 1367.8 | |
| R | 52.7 | 37.9 | 120.0 | |

## 4.4.5. Verification through a parallel experiment

The optimal combination mentioned above was not observed in the orthogonal experiment, therefore, parallel experiments were carried out in triplicate for verification and the results are shown in Figure 4-9. LAB fermentation was carried

out using an inoculum size of 3% at optimum agar strength of 30 g/L, at 37 °C. The average yield of EPS in the parallel experiment was 1489.9 mg/L at 3% inoculum amount, and 30 g/L agar for immobilization at 37 ℃.

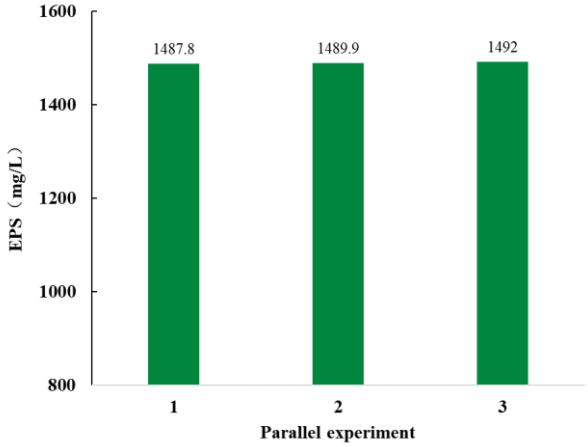



**Figure 4-9.** Verification of optimum fermentation condition of LAB through parallel experiments.

## 4.5. CONCLUSIONS

In this study, we screened six strains of LAB (*L. bulgaricus*, *L. plantarum*, *L. casei*, *L. acidophilus*, *L. lactis* subsp. *lactis LL9*, and *L. thermophilus*). Among them, *L. plantarum* was not the most prominent in antimicrobial peptide production with a measured inhibitory zone diameter of 11.72 mm. However, the highest EPS production was found in *L. plantarum*. The highest yield of EPS was 1513.1 mg/L using a 5% inoculum of *L. plantarum* fermented at 37 ℃ in a shake flask. *L. plantarum* can produce antibacterial substances and prolong the storage time of food.

The selection of the optimal immobilized carrier for *L. plantarum* was carried out using calcium alginate colloid, carrageenan, gelatin, and agar as carriers. The agar-embedded method gave the best immobilization effect, and the yield was 1096.2 mg/L. Compared with other immobilization methods, this method has the least effect on EPS yield; therefore, it was selected as the best embedding vector for EPS

production by *L. plantarum*. The process conditions of immobilized fermentation of *L. plantarum* were investigated. The optimal conditions were agar strength of 30 g/L, particle size of 1.0 cm, and fermentation of 3% of the inoculum at 37 ℃ for 24 h; the optimal condition resulted in the highest yield of extracellular polysaccharide at 1489.9 mg/L.

The immobilized cells of *L. plantarum* yielded almost the same yield of EPS as the fermentation with free cells, probably because the diffusion of the product in the immobilized carrier was limited, which affected the yield increase. However, from a process perspective, the immobilization method greatly simplifies the process. Immobilization reduces the need to activate the strain multiple times, avoiding the step of cell separation in the fermentation liquid, which simplifies the subsequent product extraction. In addition, the immobilized LAB cells can be well maintained and used repeatedly, which effectively increases the utilization rate of the strain and reduces the production cost.

EPS production of LAB is generally low, and there are many influencing factors. In recent years, based on the understanding of the genetic basis, biochemical type, biosynthetic pathway, metabolic model and physiological activity of EPS-producing lactic acid bacteria, metabolic engineering method has become a hotspot in this field. The strain and technology of this study also laid a good foundation for EPS production by LAB.

## 4.6. References


Idris, A., & Suzana, W. (2006). Effect of sodium alginate concentration, bead diameter, initial pH and temperature on lactic acid production from pineapple waste using immobilized Lactobacillus delbrueckii. *Process Biochemistry*, *41*(5), 1117-1123.

Imran, M. M., Reehana, N., Jayaraj, K. A., Ahamed, A. P., Dhanasekaran, D., Thajuddin, N., Alharbi, N. S., & Muralitharan, G. (2016). Statistical optimization of exopolysaccharide production by Lactobacillus plantarum NTMI05 and NTMI20. *International Journal of Biological Macromolecules*, 731-745.

Ju, H., Chen, H., Xiang, A., Wang, Y., Yue, T., & Yuan, Y. (2021). Identification and characterization of Lactobacillus paracasei strain MRS-4 antibacterial activity against Alicyclobacillus acidoterrestris. *LWT*, *150*, 111991.

Lee, I., Caggianiello, G., van Swam, I. I., Taverne, N., Meijerink, M., Bron, P. A., Spano, G., Kleerebezem, M., & Schaffner, D. W. (2016). Strain-Specific Features of Extracellular Polysaccharides and Their Impact on Lactobacillus plantarum-Host Interactions. *Applied and Environmental Microbiology*, *82*(13), 3959.

Menchavez, R. N., & Ha, S. H. (2019). Fed-batch acetone-butanol-ethanol fermentation using immobilized Clostridium acetobutylicum in calcium alginate beads. *Korean Journal of Chemical Engineering*, *36*(6), 1019.

Miao, M., Ma, Y., Huang, C., Jiang, B., Cui, S. W., & Zhang, T. (2015). Physicochemical properties of a water soluble extracellular homopolysaccharide from Lactobacillus reuteri SK24.003. *Carbohydrate Polymers*, *131*, 377-383.

Mozzi, F., Giori, G., & Valdez, G. (2010). UDP-galactose 4-epimerase: a key enzyme in exopolysaccharide formation by Lactobacillus casei CRL 87 in controlled pH batch cultures. *Journal of Applied Microbiology*, *94*(2), 175-183.

Patel, S., Majumder, A., & Goyal, A. (2012). Potentials of Exopolysaccharides from Lactic Acid Bacteria. *Indian Journal of Microbiology*, *52*(1), 3-12.

Thakur, A., Panesar, P. S., & Saini, M. S. (2019). L(+)-Lactic Acid Production by Immobilized Lactobacillus casei Using Low Cost Agro-Industrial Waste as Carbon and Nitrogen Sources. *Waste and Biomass Valorization*, *10*(5), 1119-1129.

# CHAPTER V

# DETERMINATION OF ATP-RELATED COMPOUNDS BY HPLC TO STUDY THE EFFECT OF CELL-FREE SUPERNATANTS OF *LACTOBACILLUS PLANTARUM* ON THE SHELF LIFE OF SLICED DRY-CURED HAM

## 5.1. ABSTRACT

This study aimed to provide insight regarding the effects of ATP-related compounds and novel bacteriostatic packaging film on sliced dry-cured ham preservation. Determination of ATP-related compounds by HPLC was employed to quantify ATP-related compounds in sliced dry-cured ham wrapped in a novel antibacterial packaging film containing the cell-free supernatant (CFS) of a probiotic *L. plantarum* strain. The contents of ATP-related compounds within 7 days of storage of ham wrapped in the novel antibacterial packaging film containing CFS of *L. plantarum* were significantly lower than those of ham samples wrapped in commercial PE packaging, which proves that the novel antibacterial packaging film has remarkable antibacterial activity. Thus, the evaluated CFS could be considered as a natural preservative with good activity when used as a packaging material, which was shown to prolong the shelf life of sliced ham. This study presents an effective method to assess the quality of sliced ham, which might also contribute to the adoption of safer and more effective anti-corrosion measures by the industry.

## 5.2. INTRODUCTION

Due to the SARS-CoV-2 (COVID 19) pandemic, the demand for primary and secondary packaging increased significantly last year, and there was more attention paid to antibacterial packaging. Food packaging has made great strides in recent decades. Antibacterial packaging interacts with the product in a positive way, extending the life of the food (Moncao, Grisi, de Moura Fernandes, Souza, & de Souza, 2022). Antibacterial packaging is a novel development that incorporates antimicrobial agent into polymer film to suppress the activities of targeted microorganisms. However, antibacterial packaging is still an extremely challenging technology and there are only a few commercialized products found in the market. Edible films and coatings are a new trend for active food packaging (Trajkovska Petkoska, Daniloski, D'Cunha, Naumovski, & Broach, 2021).

Lactic Acid Bacteria have been isolated from various food and human sources. Many LAB strains have probiotic properties with well-characterized genomes and secretory systems (Huang, Nzekoue, Silvi, Sagratini, & Verdenelli, 2020). In addition, certain LAB strains can secrete active antibacterial compounds which have high efficacy and broad-spectrum activity (Arrioja-Breton, Mani-Lopez, Palou, & Lopez-Malo, 2020), being thus considered ideal antibacterial agents to be applied in food packaging materials (Huang, et al., 2020). Several studies have shown that the direct application of LAB or cell-free supernatants (CFS) obtained from LAB strains could inhibit undesirable bacteria in many food matrices, such as chicken breast, beef, bread, and cheese (Arrioja-Breton, et al., 2020). Most studies focused on the application of probiotic strains and CFS as food additives to enhance food safety or extend food shelf-life (Yong, Hamidon, Rajangan, Soh, & Min, 2016). However, few

studies have attempted to employ CFS as preservatives as part of active food packaging material.

In the present study, CFS as a novel antimicrobial obtained from a LAB strain was used to prepare an antimicrobial film, of which the efficacy as an antibacterial food packaging material in sliced ham conservation was examined (Figure 5-1). The results discussed herein provide effective technical guidance that can be applied to the quality control of sliced ham, with prospects for popularization and widespread application.

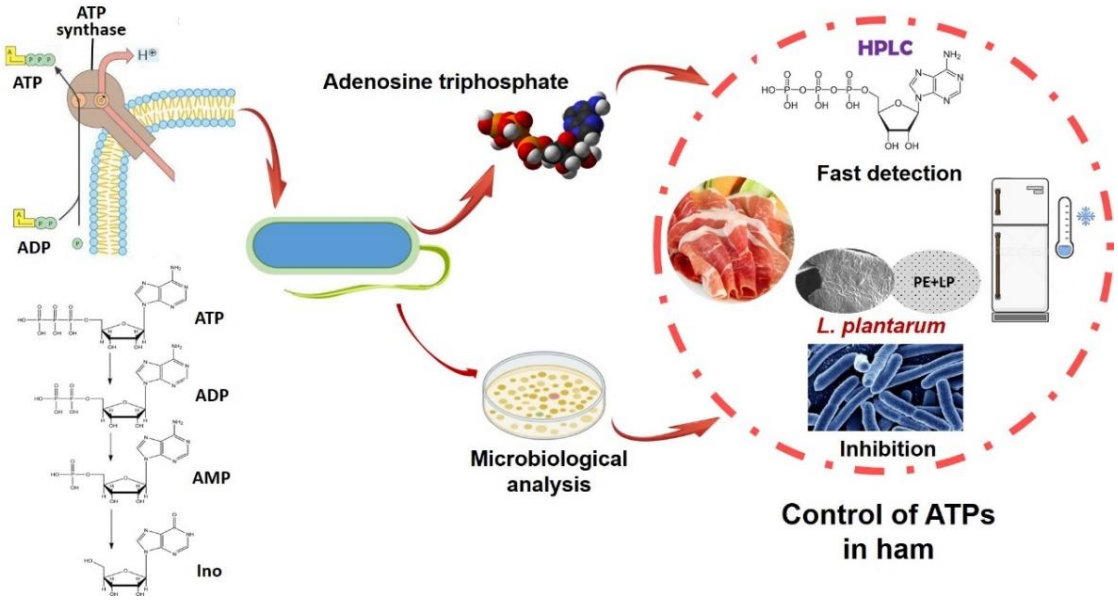



**Figure 5-1.** Schematic diagram of the effect of *L. plantarum* CFS on shelf life of sliced dry-cured ham.

## 5.3. MATERIALS AND METHODS

### 5.3.1. Materials and reagents

The samples of ham were sliced Italian Parma dry-cured ham, which were purchased at Maile food store (Nanan, China). *Lactiplantibacillus plantarum* CICC 20022 was obtained at the China Center of Industrial Culture Collection.

### 5.3.2. Sample and antibacterial packaging film preparation

Packages of vacuum-sealed sliced ham were opened, further divided, and wrapped in a previously prepared commercial polyethylene (PE) fresh-keeping bag (20 × 30 cm) kindly provided by a local supermarket (Zhengzhou, China). Prior to packaging, the surface of PE bags was sterilized under an ultraviolet lamp for up to 3 h.

To prepare the antibacterial packaging film, the CFS derived from the probiotic strain *L. plantarum* CICC 20022 was used. This strain was chosen based on its well-studied safety, antimicrobial properties, and adaptability. After flask fermentation in MRS broth at 37 ℃ for 24 h with shaking at 200 rpm using a 5% inoculum size (*v/v*), 30 mL of *L. plantarum* CICC 20022 suspension was submitted to centrifugation at 3000 rpm for 15 min. The obtained CFS of *L. plantarum* CICC 20022 was incorporated by spraying onto the internal surface of the UV-decontaminated commercial PE films. After spraying, drying was conducted in an oven (Heraeus, Hanau, Germany) at 45 ℃ until the film weight reached a constant value.

Ham samples were stored at 4 ℃ either in sterile PE packaging bags (control) and in the PE bags sprayed with CFS of *L. plantarum* (PE+LP). Samples were then studied for microbiological, chemical, and sensorial analyses on days 0, 2, 5, and 7.

### 5.3.3. Determination of ATP-related compounds, microbiological analysis

The methods used in the present study were the same as described in Chapter 3.

### 5.3.4. Recovery

The recovery of all five ATP-related compounds was determined by the spiking of 5 μg/mL of respective standards to both packaged ham samples. After fortification

and extraction, analyses were performed, and recoveries were calculated by comparison on days 2 and 7 of storage.

### 5.3.5. Statistical analysis

The results of the test were expressed as mean ± standard deviation. SPSS Statistics version 25.0 was used for statistical analysis. Significant differences were determined by Student's *t* test (*p<=0.05, **p<=0.01, ***p<=0.001) with Origin 2021. Each experiment was performed in twice.

## 5.4. RESULTS AND DISCUSSION

### 5.4.1. Validation of the method

Ham slice samples were sub packed, one was normally packed in PE fresh-keeping bag and the other was sealed in a new antibacterial packaging bag (PE+LP) sprayed with *L. plantarum* CFS. To study the recovery of the procedure the sample was added to known quantities of ATP, ADP, Ino, AMP and Hx. The sample was analyzed in duplicate before and after the additions (Table 5-1). The recoveries of ATP, ADP, AMP and Hx varying between 51% and to 212% indicated that the repeatability of the procedure was good. Sensitivity determined was also found satisfactory. In fact, for Ino this value is lower than that found before, however, for ATP and AMP the results obtained with our method are clearly higher. The low Ino recovery rates may be due to the interference of sample extraction process on Ino and its instability in the extraction conditions.

**Table 5-1.** Recovery of both packaged ham samples (at stored T2 and T7).

| Analyte | Time | Package | 0μg/mL[a] | 5μg/mL[a] | Theoretical value | Recovered[a] | Recovery (%) |
|---|---|---|---|---|---|---|---|
| ATP | T2 | PE | 171.99±1.75 | 181.68±9.87 | 5 | 10.62 | 212% |
| | | PE+LP | 143.02±11.07 | 152.43±5.54 | 5 | 9.41 | 188% |
| | T7 | PE | 162.36±4.52 | 167.30±8.52 | 5 | 4.94 | 99% |
| | | PE+LP | 158.83±12.79 | 164.83±6.68 | 5 | 6.00 | 120% |
| ADP | T2 | PE | 6.69±1.00 | 12.12±1.50 | 5 | 5.43 | 109% |
| | | PE+LP | 5.33±1.13 | 7.89±1.56 | 5 | 2.56 | 51% |
| | T7 | PE | 7.91±2.59 | 10.72±1.50 | 5 | 2.81 | 56% |
| | | PE+LP | 6.86±0.57 | 9.34±1.17 | 5 | 2.66 | 53% |
| Ino | T2 | PE | 0.06±0.08 | 0.32±1.04 | 5 | 0.26 | 5% |
| | | PE+LP | 0.04±0.05 | 0.14±0.03 | 5 | 0.10 | 2% |
| | T7 | PE | 0±0 | 0±0 | 5 | 0.00 | 0% |
| | | PE+LP | 0±0 | 0±0 | 5 | 0.00 | 0% |
| AMP | T2 | PE | 107.32±2.78 | 117.47±7.39 | 5 | 9.68 | 194% |
| | | PE+LP | 88.4±10.43 | 95.31±5.22 | 5 | 6.91 | 138% |
| | T7 | PE | 118.51±3.00 | 123.82±3.17 | 5 | 5.31 | 106% |
| | | PE+LP | 97.92±7.90 | 107.42±6.18 | 5 | 9.50 | 190% |
| Hx | T2 | PE | 10.59±0.17 | 20.28±4.09 | 5 | 9.68 | 194% |
| | | PE+LP | 10.14±0.82 | 12.72±2.41 | 5 | 2.58 | 52% |
| | T7 | PE | 10.88±0.20 | 12.52±1.20 | 5 | 5.03 | 101% |
| | | PE+LP | 9.66±0.91 | 13.28±1.12 | 5 | 3.62 | 72% |

[a] Each value corresponds to twice replicates at each concentration level.

### 5.4.2. Comparison of the ham samples stored in an antibacterial packaging film and PE packaging.

Considering ATP-related compounds as chemical quality markers in sliced hams, their content was monitored in ham samples stored in two packaging systems, a PE packaging film (control) and an antibacterial packaging made with a CFS of *L. Plantarum* CICC 20022, which was incorporated by spraying onto the internal surface of PE packaging. This study was performed to assess the effectiveness of CFS in food

packaging application for ham preservation.

Levels of ATP-related compounds in differentially packed sliced ham samples on days 0, 2, 5, and 7 of storage were determined by HPLC (Figure 5-2). The contents of ATP-related compounds within 7 days of storage in ham wrapped in the novel antibacterial packaging film containing CFS of *L. Plantarum* were significantly lower than those of ham samples wrapped in commercial PE packaging, which proves that the novel antibacterial packaging film has remarkable antibacterial activity. The inhibitory effect of pure CFS from *L. Plantarum* could be due to the existence of acetic acid, valeric acid, alcohol, maltol, and furanone derivatives with antimicrobial properties (Kuley, Kuscu, Durmus, & Ucar, 2021). CFS of *L. Plantarum* culture in liquid nutrient medium exhibits growth inhibition activity against Gram-positive and negative pathogenic bacteria (Danilova, et al., 2019). Similarly, the bacteriostatic activity of CFS from various *L. Plantarum* strains against a broad range of food pathogens were reported in several studies (Kim, et al., 2020; Oldak, Zielinska, Rzepkowska, & Kołożyn-Krajewska, 2017). In this study, for both packaged hams, relatively high ATP and AMP levels and low ADP and Hx levels were observed. The two packagings showed the greatest difference on day 2, with levels of ATP-related compounds in the antibacterial packaging 32% lower than those in the control packaging, indicating that CFS of *L. Plantarum* showed the highest bacteriostatic effect in the first two days. The difference in the levels of the ATP-related compounds caused by the two packagings began to decrease on day 5. The difference in ATP levels between the two packagings decreased to 12.0% on day 5 and 7.7% on day 7 (Table 5-2). It is considered that the bacteriostatic effect of CFS of *L. plantarum* began to weaken with the increase of time. The bacteriostatic effect of the improved packaging is concentrated in the first two days, which meets the needs of daily ham

storage.

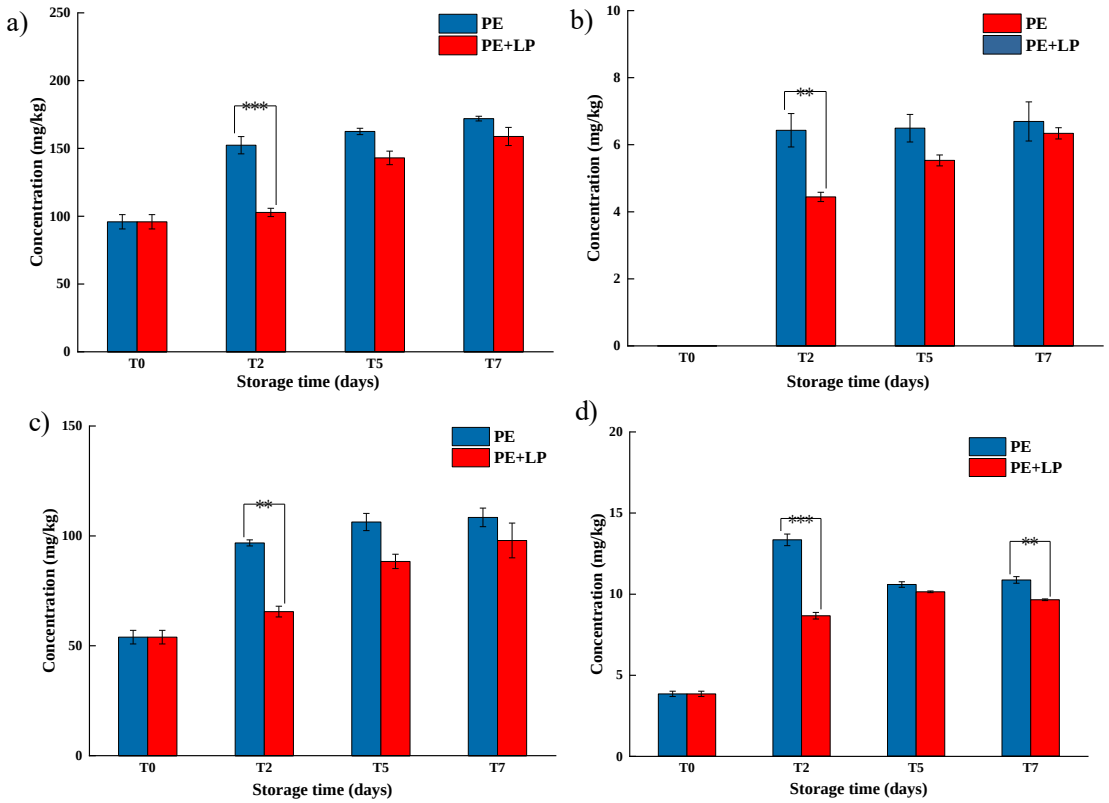



**Figure 5-2.** Changes in the concentration of ATP-related compounds in sliced Italian Parma ham samples wrapped in polyethylene (PE) packaging and novel antibacterial packaging film containing CFS of *L. plantarum* (PE+LP). a) adenosine triphosphate (ATP); b) adenosine diphosphate (ADP); c) adenosine monophosphate (AMP); d) hypoxanthine (Hx). Significant differences (*p<=0.05, **p<=0.01, ***p<=0.001) between the two packaging in each day are indicated by the asterisk.

**Table 5-2.** Comparison of the concentrations of ATP-related compounds (mg/kg) in ham samples stored in different packagings from T0 to T7

| Concentration | T0 | | T2 | | T5 | | T7 | |
|---|---|---|---|---|---|---|---|---|
| (mg/kg) | PE | PE+LP | PE | PE+LP | PE | PE+LP | PE | PE+LP |
| ATP | 95.9 ± 5.3 | 95.9 ± 5.3 | 152.4 ± 4.5 | 102.3 ± 3.1 | 162.5 ± 2.3 | 143.0 ± 5.0 | 172.0 ± 1.8 | 158.8 ± 6.7 |
| ADP | n.d | n.d | 6.5 ± 0.4 | 4.4 ± 0.1 | 6.7 ± 0.6 | 5.3 ± 0.2 | 6.4 ± 0.5 | 6.3 ± 0.2 |
| AMP | 53.9 ± 3.1 | 53.9 ± 3.1 | 96.8 ± 1.4 | 65.6 ± 2.4 | 106.3 ±2.8 | 88.4 ± 3.3 | 108.4 ± 3.0 | 97.9 ± 7.9 |
| Hx | 3.9 ± 0.2 | 3.9 ± 0.2 | 13.4 ± 0.4 | 8.7 ± 0.2 | 10.9 ± 0.2 | 10.1 ± 0.1 | 10.9 ± 0.2 | 9.7 ± 0.1 |

n.d: non detected (<LOQ)

### 5.4.3. Relationship between the content of adenosine triphosphate related compounds and the number of microorganisms in ham during storage

Changes in TBC of ham samples on day 2 of storage were measured to confirm the chemical results and to determine the efficacy of the proposed HPLC method to detect ATP-related compounds as a quality test for ham samples. Figure 5-3 depicts a comparison between TBC and the total contents of ATP-related compounds in ham wrapped in two packaging materials on day 2 of storage. The lowest values of TBC and ATP-related compounds were found in ham stored in antimicrobial packaging film with the CFS of *L. Plantarum*. These results confirm the antimicrobial activity associated with the CFS of *L. Plantarum* in packaging materials, as reported in the literature (Divyashree, Anjali, Somashekaraiah, & Sreenivasa, 2021; Kuley, et al., 2021). Interestingly, the antimicrobial packaging film with the CFS of *L. Plantarum* remarkably inhibited spoilage microorganisms. The TBC of ham stored in the antibacterial packaging material on day 2 of storage was significantly lower (21.4%) compared to control stored ham, while the result of HPLC measurements of the contents of ATP-related compounds showed a difference of 35.0 %, hence closely related to TBC. The relationship between the ATP assay and traditional microbial culture methods appears to agree well with lower numerical differences. Although ATP assay is not an alternative to culture methods, as it assesses compounds rather than cells, it is a potentially useful and rapid method to assess the effectiveness of packaging materials in preserving microbial contamination of foods. Thus, the contents of ATP-related compounds can be used as a marker to quickly determine the quality of sliced ham as well as to screen antimicrobial agents. In conclusion, the

novel antibacterial packaging film containing the active components of probiotic *L. Plantarum* strain had a remarkable antibacterial effect on spoilage microorganisms, thus contributing to the preservation of ham and effectively prolonging the shelf life of sliced ham.

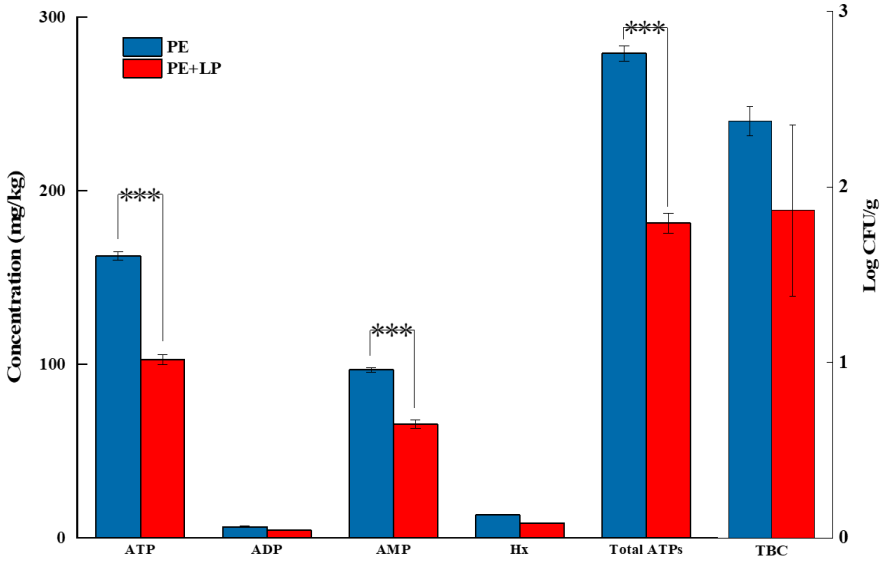



**Figure 5-3.** Total of the concentrations of four ATP-related compounds (ATP, ADP, AMP, and Hx) and total plate count in sliced Italian Parma ham samples wrapped in polyethylene (PE) packaging and novel antibacterial packaging film containing CFS of *L. plantarum* (PE+LP). Legend: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), hypoxanthine (Hx), total of the concentrations of four ATP-related compounds (total ATPs) and total plate count (TBC). Significant differences (*p<=0.05, **p<=0.01, ***p<=0.001) between the two packaging in each day are indicated by the asterisk.

## 5.5. CONCLUSIONS

The effect of a new antibacterial packaging film enhanced with *Lactiplantibacillus plantarum* CFS on ATP content was studied. The results showed that four ATP-related compounds (ATP, ADP, AMP and Hx) were successfully detected in dry ham samples using the validated method, while Ino was not measurable in ham. Moreover, the recovery of ATP-related compounds in ham samples was in accordance with the

application detection range. *Lactiplantibacillus plantarum* CFS as a natural microbial preservative was able to rescue ATP levels when it was used as a natural microbial preservative to improve the packaging material of dry-cured ham and delay food corruption. The CFS of *L. Plantarum* CICC 200022 showed activity as a natural preservative in dry cured ham and could potentially be used to improve food packaging materials and thus prevent food spoilage.

## 5.6. References


Arrioja-Breton, D., Mani-Lopez, E., Palou, E., & Lopez-Malo, A. (2020). Antimicrobial activity and storage stability of cell-free supernatants from lactic acid bacteria and their applications with fresh beef. *Food Control*, *115*, 107286.

Danilova, T. A., Adzhieva, A. A., Danilina, G. A., Polyakov, N. B., Soloviev, A. I., & Zhukhovitsky, V. G. (2019). Antimicrobial activity of supernatant of Lactobacillus plantarum against pathogenic microorganisms. *Bulletin of Experimental Biology and Medicine*, *167*(6), 751-754.

Divyashree, S., Anjali, P. G., Somashekaraiah, R., & Sreenivasa, M. Y. (2021). Probiotic properties of Lactobacillus casei-MYSRD 108 and Lactobacillus plantarum-MYSRD 71 with potential antimicrobial activity against Salmonella paratyphi. *Biotechnology Reports*, *32*, e672.

Huang, X., Nzekoue, A., Silvi, S., Sagratini, G., & Verdenelli, C. (2020). Lactobacillus Strains Treatment on Commercial Packaging Paper as Preliminary Study for Extending the Shelf-Life of Chicken Meat.

Kim, S. W., Kang, S. I., Shin, D. H., Oh, S. Y., Lee, C. W., Yang, Y., Son, Y. K., Yang, H., Lee, B., & An, H. (2020). Potential of Cell-Free supernatant from lactobacillus plantarum NIBR97, including novel bacteriocins, as a natural alternative to chemical disinfectants. *Pharmaceuticals*, *13*(10), 266.

Kuley, E., Kuscu, M. M., Durmus, M., & Ucar, Y. (2021). Inhibitory activity of Co-microencapsulation of cell free supernatant from Lactobacillus plantarum with propolis extracts towards fish spoilage bacteria. *LWT*, *146*, 111433.

Moncao, E. D. C., Grisi, C. V. B., de Moura Fernandes, J., Souza, P. S., & de Souza, A. L. (2022). Active packaging for lipid foods and development challenges for marketing. *Food Bioscience*, *45*, 101370.

Oldak, A., Zielinska, D., Rzepkowska, A., & Kolozyn-Krajewska, D. (2017). Comparison of antibacterial activity of Lactobacillus plantarum strains isolated from two different kinds of regional cheeses from Poland: Oscypek and Korycinski cheese. *Biomed Research International*, *2017*.

Trajkovska Petkoska, A., Daniloski, D., D'Cunha, N. M., Naumovski, N., & Broach, A. T. (2021). Edible packaging: Sustainable solutions and novel trends in food packaging. *Food Research International*, *140*, 109981.

Yong, H. P., Hamidon, F., Rajangan, C., Soh, K. P., & Min, T. L. (2016). Application of Probiotics for the Production of Safe and High-quality Poultry Meat. *Korean Journal for Food Science of Animal Resources*, *36*(5), 567-576.

# CHAPTER VI

# ASSESSMENT OF SHELF-LIFE OF CHICKEN BREAST MEAT STORED IN THE NOVEL COMPOSITE GRAPHENE AND PROBIOTIC PAPER-PLA PACKAGING

## 6.1. ABSTRACT

In food applications, graphene-based composites have been used as toxin/pesticide detectors and as antimicrobial packaging for the storage of food. In such circumstances, our aims are to investigate the potentials of graphene-based composites together with probiotic strains in food preservation applications. Paper-based material is widely used for storing beverages, bakery products as it is being biodegradable, recyclable and ecofriendly. Chicken breast meat is rich in polyunsaturated fatty acids that are sensitive to the presence of oxygen. Furthermore, high moisture, nutrient-rich, neutral pH properties of chicken meat favor the growth of microbial contaminate that further accelerating the spoilage process. In order to extend raw chicken meat shelf-life, to maintain the biodegradability of the packaging, an active packaging composed by paper-PLA based material, containing composites and probiotic bacteria - *L. plantarum* was used to store raw meat. The efficiency of active paper-PLA active package was evaluated on meat quality parameters. The characterization of the physio-mechanical properties of the packaging put in evidence that the incorporation of composites improved the general structural and mechanical properties compared to

plain paper-PLA package, but the barrier properties of modified paper/PLA packages were slightly weaker than those of polyethylene. The modification of *L. plantarum* IMC 509 enhanced the antimicrobial properties to active paper/PLA package against the growth of tested pathogenic strains - *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*. Regarding the packaging efficiency of paper-PLA packages, the microbial analysis revealed that the samples generally showed higher amount of total aerobic mesophiles bacteria and lactic acid bacteria when stored in *L. plantarum* IMC 509 - treated package as part of *L. plantarum* transferred from package surface to meat surface. The amount of meat hygiene and spoilage indicators - Enterobacteriaceae and *Pseudomonas* spp. - were lower in samples stored in probiotic modified packages during the first two and 5 days of storage. The pH value of the meat was not affected by the type of package during storage. Probiotic packages exerted positive roles in preserving meat sensorial attributes by inhibiting the biogenic amines production through various mechanisms.

In addition, the chemical analysis showed that sample in *L. plantarum*-modified package had higher B.A.I. values than those in paper-PLA 0.5% package, but the B.A.I. values remained acceptable up to 7 days in both packages.

The comparison of commercial meat packages with the studied active packages demonstrated that the two systems had different effect on meat microbial groups. The increased amount of B.A.I. suggested that active packages were more efficient during the first days of study, but this effect was not kept for longer storage period. In general, the antimicrobial effect of probiotic packages was compromised by the presence of food components and the initial microbial content on food.

The studies subject of the current thesis is included in the framework of the project "Active GRAphene based Food packaging for a modern society". This project

involved five partners from different European countries, including Technical University of Cluj Napoca (Romania), Ceprohart SA (Romania), National Institute of Chemistry (Slovenia), Andaltec (Spain), University of Camerino (Italy), Synbiotec Srl (Italy). The main objective of the project was to design and develop a pilot-scale active food packaging prototype based on using paper and polylactic acid (PLA), containing composites of graphene and nano-Ag-$TiO_2$ as well as probiotic bacteria.

## 6.2. INTRODUCTION

For food applications, graphene derivatives are considered as interesting active fillers to enhance the general performance of polymers-based packages. Furthermore, graphene composites can be chemically modified with other alloys to produce various derivatives for specific applications. Among the functional properties, the inhibitory ability of graphene derivatives has been widely investigated against a variety of microorganisms. As we mentioned above, the antimicrobial actions of graphene-based composites require direct contact of composites and bacteria, which interacting with functional groups (proteins) and structural components (phospholipids) and further damaging cell structure, inducing oxidative stress. In addition, researchers find that graphene sheets can also wrap around microorganisms and inhibit their metabolic activity and proliferation (Mohammed, et al., 2020).

Packages made by renewable sources are considered as sustainable packaging trends in replacing fossil-based materials and reducing environmental impacts (Vytejckova, et al., 2017). Paper and its based materials are one of the most used packaging materials in food industry globally. It possesses many advantages such as ecofriendly, recyclability, biodegradability as well as providing information of the

product. The use of paper-based materials is commonly for storing beverages,popcorns, bakery products, eggs. Commercially, paper is often laminated and reinforced with additives such as polyethylene, wax, aluminium foil as the poor barrier and mechanical properties of plain paper packaging (Vytejckova, et al., 2017). To maintain the biodegradability of paper packaging, polylactic acid can be used as alternative to replace polyethylene as well as to enhance the physio-mechanical properties of paper-based package.

Chicken breast meat was rich in polyunsaturated fatty acids and thus it needs to be preserved in packaging material with strong barrier properties that prevent from oxidation process. Furthermore, the neutral pH and high-water activity of raw chicken meat favor the growth of microbes that contaminate meat surface through various routes (Rawdkuen, Punbusayakul, & Lee, 2016). The development of active packaging with antimicrobial and antioxidant properties helps to efficiently delay the spoilageprocess and reduce the risks associated with contaminated meat consumption. Besides the functional roles of active packaging, its property in exhibiting and preserving organoleptic characteristics of meat is of paramount importance to consumers (Min & Ahn, 2012).

The aims of the present study were to characterize the preservation efficiency (meat chemical parameters, microbial profile, pH value,) of novel composite graphene and probiotic *L. plantarum* IMC 509 paper-PLA sandwich packaging film on raw chicken meat.

# 6.3. MATERIALS AND METHODS

## 6.3.1. Graphene composites and probiotic strains

Graphene-based composites were prepared by collaborators from Technical University of Cluj Napoca (Romania) through processes, including inclusion, immersion, reduction, drying and thermal treated (or not). After processing, all composites are supplied in powder form (Figure 6-1). The characteristics of the graphene composites were evaluated by National Institute of Chemistry Ljubljana Slovenia (NIC) through analyses: FTIR, reflectance UV-Vis, Eg, XRD, XPS, EDS, Raman, STEM-HAADF, porosity. The graphene composites used in each test with be listed in the following tables.

**Figure 6-1.** Samples of graphene-based composites

*L. plantarum* IMC 509 - was chosen based on their human-origin, well-characterized functionalities and safety (Min & Ahn, 2012). The strains were supplied by SYNBIOTEC S.r.l. (Camerino, Italy) in freeze-dried powder form, and subsequently sub-cultured in de Man Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) for 24-48h. Bacterial inoculum was prepared by suspending a loopful of bacterial colonies inside 5 ml sterile saline solution and the final concentration of bacterial suspension was adjusted to $10^8$ CFU/ml.

### 6.3.2. Paper-PLA sandwich packaging film preparation

Packages of paper-PLA sandwich structure were prepared, including paper (H)-PLA (H- PLA), H-PLA with incorporation of 0.5% Ag-GN-$TiO_2$ on PLA surface, paper with 0.5% composite and PLA with 0.5% (Ag-GN-$TiO_2$) (Figure 6-2).


**Figure 6-2**. H-PLA: paper and polylactic acid sandwich; H-PLA 0.5%( H-PG0.5%): paper and polylactic acidwith 0.5% Ag-GN-$TiO_2$; H 0.5%-PLA 0.5% ( HG0.5%-PG0.5%): paper with 0.5% Ag-GN-$TiO_2$ and polylactic acidwith 0.5% Ag-GN-$TiO_2$

All the packages were fabricated andcharacterized by our partners from Technical University of Cluj Napoca, Romania (UTCN), Ceprohart (Romania), and Andaltec (Spain). For modifying H-PLA/composite packages with the probiotic strain *L. plantarum* IMC 509 (LP), all inner surface of H-PLA packaging film was sterilized by UV lamp up to 3 h under Biohazard hood (FASTER, Milan, Italy). 0.5 g of *L. plantarum* IMC 509 lyophilized powders ($10^{11}$ CFU/g) (SYNBIOTEC Srl, Camerino, Italy) were dissolved in 10 mL of sterile PBS buffer. After spraying, the drying of the probiotic film was done in lab oven (Heraeus, Hanau, Germany) at 45 °C until the weight of the film reached a constant value. In the present study, six kind of sandwich packages are included: H-PLA (whole paper and PLA), H-PG0.5% (whole paper and PLA with 0.5% graphene), HG0.5%-PG0.5% (paper and PLA with 0.5% graphene),

H-PLA-LP (whole paper and PLA, added with probiotic *L. plantarum* IMC 509), H-PG0.5%-LP (whole paper and PLA with composite, further added with probiotic), HG0.5%-PG0.5%-LP (paper and PLA with composite, with addition of probiotic).

### 6.3.3. Meat and packaging preparation

Slices of fresh breast meat derived from chicken, purchased from a local supermarket, were immediately transferred to the laboratory, where they were further divided, wrapped and stored in previously prepared packages in a refrigerator at 4 °C. At 0, 2, 5, 7 days of storage, the corresponding sample was taken out from fridge and analyzed for the following parameters: chemical analysis, microbial counts, pH measurement and sensorial.

### 6.3.4. Microbiological analysis

The parameters analyzed comply with the standards of CeIRSA (2017) and the applied procedures comply with their respective ISO guidelines. Chicken breast meat microbiota was monitored by determining total aerobic mesophiles, Enterobacteriaceae, LAB, coagulase-positive staphylococci, presumptive *Pseudomonas* spp., β-glucuronidase-positive *Escherichia coli*, sulfite-reducing anaerobes, *Clostridium perfringens*. Five grams of meat were taken from each sample and subsequently homogenized in 45 ml of saline solution in a sterile Stomacher bag (Stomacher® 80, Seward, UK). Perform a continuous 10-fold dilution of each replicated sample and spread out and inoculate into selective agar medium with 0.1 mL of the corresponding dilution. Under aerobic conditions at 30 °C, perform 72 h aerobic mesothermal bacterial count (ISO 4833) on Plate Counting Agar (PCA Oxoid,

Basinstoke, UK). For the counts of LAB, de Man, and Rogosa, used Sharpe agar (MRS Agar at pH 5.7, VWR, Leuven, Belgium) (ISO 15214-1998). Detection and counting of Enterobacteriaceae on violet red bile glucose agar (VRBGA, VWR), which was inoculated with samples at 37 ℃ for 24 h (ISO 21528-2). Count of *Pseudomonas* spp. by aerobic inoculating samples into Pseudomonas 210 Selective Agar (CFC, Liofilchem s.r.l., Roseto degli Abruzzi, Italy) at 25℃ for 44±4 h (ISO 13720:2010). Count of sulfide-reducing bacteria (ISO 15213-2003) by incubating for 48 h under anaerobic conditions at 37±1 ºC by using iron sulfite agar plates (Liofilchem). *Cl. perfringens* count (ISO 7937-2004) was performed on trypsin-sulfite-cycloserine (TSC) agar (VWR) for 20±2 h under 37 ºC anaerobic conditions.

Standard pathogenic strains - *Staphylococcus aureus* ATCC 25923 was purchased from ATCC. Stock strains stored at -20℃ in 15% (w/w) glycerol was re-activated in Tryptic soy broth (TSB) (Oxoid) at 37℃ for 24-48h. After incubation, single colonies of each pathogenic strain were isolated on Tryptic soy agar (TSA, Oxoid). All selected bacteria were cultured in corresponding broth for 24-48 h at 37 ℃ under aerobic conditions (ISO 6888- 1:1999). Tryptone Bile X205 glucuronide Agar (TBX, VWR) for the detection of β-glucosidase-positive *E. coli* was aerobically cultured at 44 ℃ for 18-24 h (ISO 16649-2).

### 6.3.5. Chemical analysis, pH measurement

The methods used in the present study were the same as described in Chapter 2.

### 6.3.6. Sensory characteristic evaluation of the chicken meat

Ten members were selected and trained on meat description following the methods reported by (Baston & Barna, 2010) and to familiarize the specific vocabulary and corresponding characteristic of meat. The panelists were asked to judge the aspect (presence of slime), odor, color and elasticity according to its intensity, they were also asked for the over- all acceptability.

The sensory evaluation was conducted in the open laboratory, where each sample was served on a clean white plate, with its corresponding code. The sensory analysis was based on a three-point hedonic scale ranging from 1 (poor) to 3 (excellent) (Table 6-1).

**Table 6-1.** Sensorial attribute values for raw chicken meat (modified from) (Baston & Barna, 2010)

| Attributes | Description | Values* |
|---|---|---|
| Aspect | Without slime | 3 |
| | Present in some parts slime | 2 |
| | All surface with slime | 1 |
| Odour | Characteristic | 3 |
| | Off-odours | 2 |
| | Foreign | 1 |
| Elasticity | Fast return | 3 |
| | Slow return | 2 |
| | No return | 1 |
| Overall acceptability | Excellent | 3 |
| | Acceptable | 2 |
| | Unacceptable | 1 |

** Intensity:1-3*

### 6.3.7. Statistical analysis

The results were subjected to ANOVA test and using the Tukey test, for the

significant difference $p<0.05$ was considered (IBM SPSS Statistics - Version 23 and Origin 2021). All experiments were performed in triplicate.

## 6.4. RESULTS AND DISCUSSION

### 6.4.1. Packaging characterization

Compared to the polyethylene-based meat packaging bag, the developed paper/graphene composite packaging showed higher permeability to grease and water vapor. To enforce the barrier properties of packaging, a sandwich structure composed by paper and polylactic acid (PLA) layers that blended with graphene-based composite was developed and characterized. The structural and mechanical properties of the sandwich package were analyzed by our partners UTCN and CEPROHART (Romania).

The thickness, grammage and smoothness were ranked in the following order: HG0.5%- PG0.5% > H-PG0.5% > H-PLA. The addition of graphene composites in both layers increased packaging thickness, grammage and smoothness.

Moreover, regarding the physical-mechanical properties of the complex, although incorporation of 0.5% graphene composites in paper and PLA layer had reduced the elongationcharacteristic of the film, other properties - tear resistance, tensile strength, folding endurance,ash content, and Gurley porosity - were generally improved.

Active H-PLA (with inclusion of 0.5% graphene composites) showed higher value of pH, redox potential and conductivity than pure H-PLA package. In general, H-PLA packages were more permeable to water vapor and grease than polyethylene-based package, and the permeability was highly affected by tested temperature. The antimicrobial activity of H-PLA packages was also evaluated through shaking flask

method, the results showed that the probiotic - *L. plantarum* IMC 509 treated packages was able to inhibit the growth of *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*.

### 6.4.5. Chemical analysis of sample in active paper-PLA packages

To evaluate the biogenic amines production during storage, four indexes were taken into consideration such as the Total of the monitored Biogenic Amines (Total BAs), Biogenic Amine Index (BAI), Chemical Quality Index (CQI) and Spermidine/Spermine ratio (SPD/SPM). Each index was calculated and compared between samples. Six samples were studied in the experiment: H-PLA, H-PG0.5%, HG0.5%-PG0.5%, H-PLA-LP, H-PG0.5%-LP, HG0.5%-PG0.5%-LP (Figure 6-3, 6-4, 6-5, 6-6).

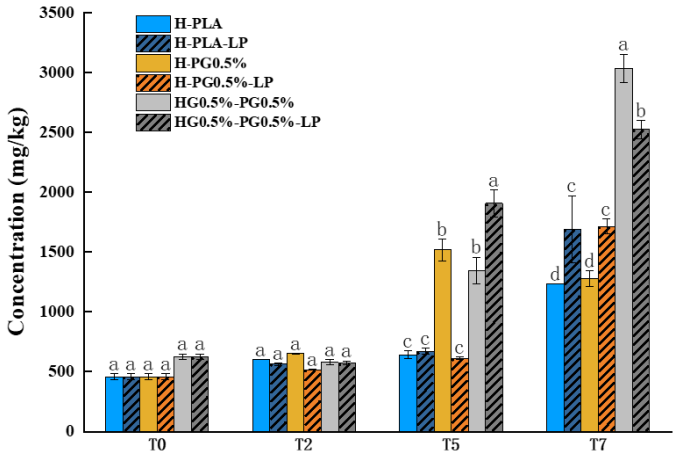



**Figure 6-3**. The total of the monitored biogenic amines (Total BAs) of chicken samples during 7 days of storage at 4°C. $^{a, b, c}$ *statistically significant different by Tukey test.*

As shown in Figure 6-3, an increase in total BAs levels during storage was observed in all samples. Figure 6-4 showed at T2, the B.A.I. in chicken sample stored into H-PLA-LP had the lowest amount compared to all other samples, followed by

H-PG0.5%-LP. Their levels were statistically significant lower ($p < 0.05$) than either H-PLA or H-PG0.5%. At T5, chicken samples into H-PG0.5%-LP showed the lowest BAI compared to the other samples. At T7, H-PG0.5% presented significantly lower BAI compared to H-PG0.5%-LP, but there was no statistical difference with the other samples. There was also no statistical difference between the BAI of chicken sample stored into the different packaging at T2 and T5. At T7, chicken samples stored in HG0.5%-PG0.5%-LP showed the lowest BAI ($p < 0.05$) compared to all othersamples (HG0.5%-PG0.5%-LP > HG0.5%-PLA 0.5% = H-PLA).

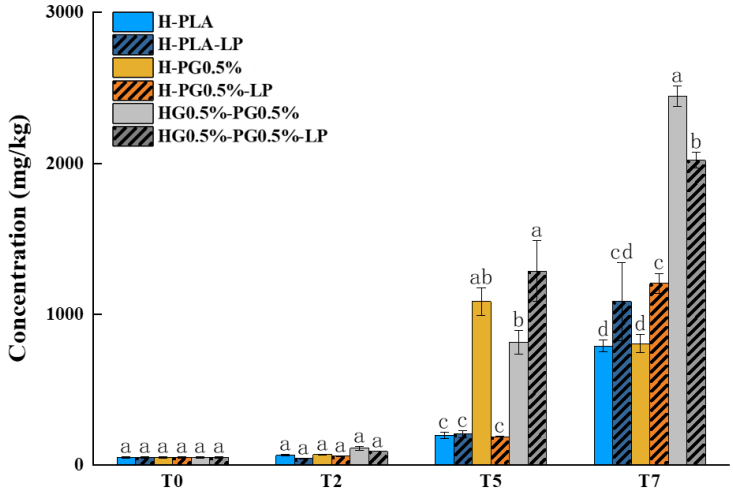



**Figure 6-4**. Biogenic Amine Index (BAI) of chicken samples during 7 days of storage at 4°C. *a, b, c, d statistically significant different by Tukey test.*

Regarding the Spermidine/Spermine ratio, this is considered one of the most important indicators for assessing the quality of chicken meat, as it is independent of flora type (Sirocchi, et al., 2013). During the shelf life study, the values of 6 samples of packaged meat were very constant for the first 2 days (Figure 6-5). Differences began on day 5, as the prevalence of SPM during the 14-day storage period was higher than that of SPD levels due to microbes using this polyamine as a nitrogen source. CQI is acceptable between 1-10 mg/kg, so the quality of the chicken is

guaranteed in all packages for the first 7 days (Figure 6-6). Finally, a comparison of total BAs, CQI, BAI concentrations, and SPD/SPM ratios of six packaged chickens showed H-PLA-LP and H-PG0.5%-LP samples were lower at day 5.

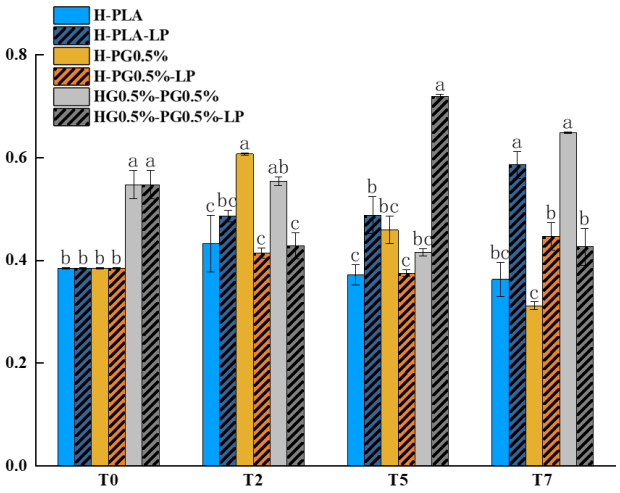



**Figure 6-5**. Spermidine/Spermine ratio (SPD/SPM) of chicken samples during 7 days of storage at 4°C. [a, b, c] *statistically significant different by Tukey test.*

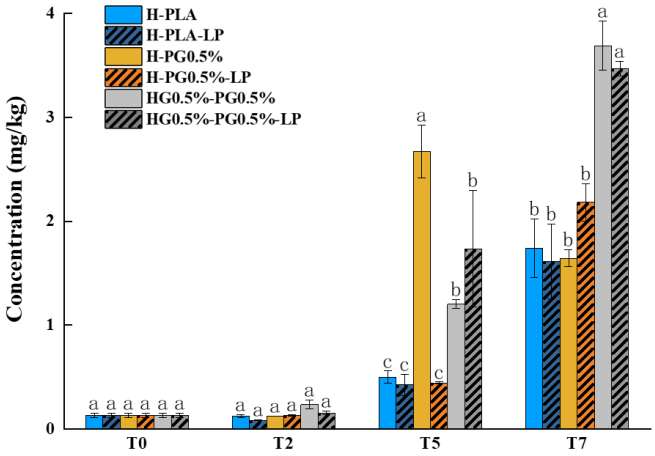



**Figure 6-6**. Chemical Quality Index (CQI) of chicken samples during 7 days of storage at 4°C. [a, b, c] *statistically significant different by Tukey test.*

## 6.4.2. Microbiological analysis of active paper-PLA packages

Figure 6-7 shows an increasing trend of the total aerobic mesophiles counts in all samples during storage period. In general, samples in LP modified packages showed relatively higher amount of total mesophile counts than samples stored without LP

packaging due to the incorporation of *L. plantarum*. The number of total mesophiles in H-PLA and H-PG0.5% samples reached the threshold at 5 days of storage. Due to the deliberately addition of *L. plantarum* on packaging, the number of LAB on meat stored in LP-modified packs exceed thethreshold only after 2 days and the level was maintained during the following days, whereas the counts of LAB in H-PLA/H-PLA-GN meat remained relatively stable (Figure 6-8). In fact the assay of probiotic viability on this packaging surface showed around 8 log CFU/cm$^2$, whichwas similar to the count of LAB in the present study, confirming their contribution to the total counts. LP-modified samples revealed lower amount of Enterobacteriaceae (Figure 6-9) than the respective package without LP modification, but this effect was not maintained during the following time. Only meat stored in H-PLA revealed less Enterobacteriaceae counts (under threshold) at 5 days of storage. The high initial level of Enterobacteriaceae revealed that meat was contaminated through second handling by operators. H-PG0.5%-LP was able to maintain the level of presumptive *Pseudomonas* spp. counts (Figure 6-10) for 2 days and increased to around 5.5 log at day 5 and day 7. Moreover, the sample in the H-PLA, H-PLA 0.5%, H- PG0.5%-LP did not exceed the threshold during the whole storage time.

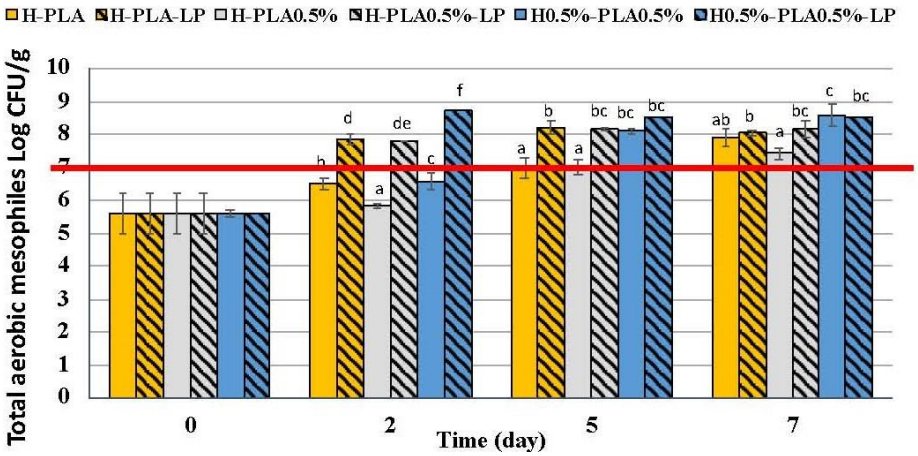



**Figure 6-7**. Bacterial counts of total mesophiles in chicken breast meat samples during 7 days of storage at 4°C. $^{a, b, c, d, e, f}$ *statistically significant different by ANOVA test.*

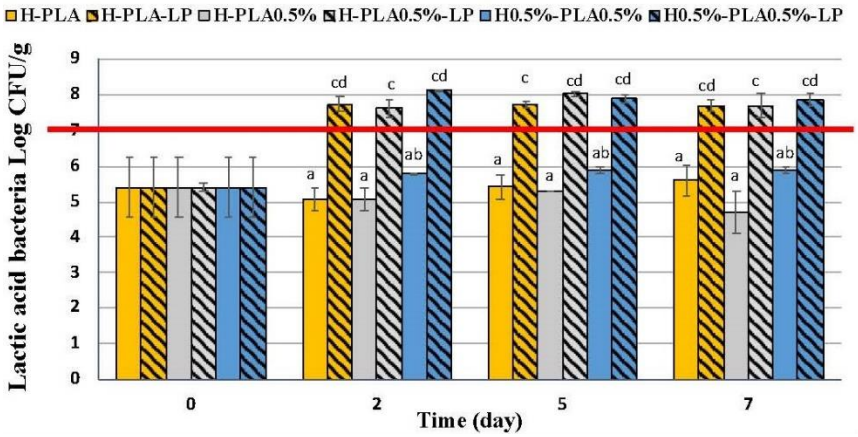



**Figure 6-8**. Bacterial counts of Lactic acid bacteria in chicken breast meat samples during 7 days of storage at 4°C. $^{a, b, c, d}$ *statistically significant different by ANOVA test.*

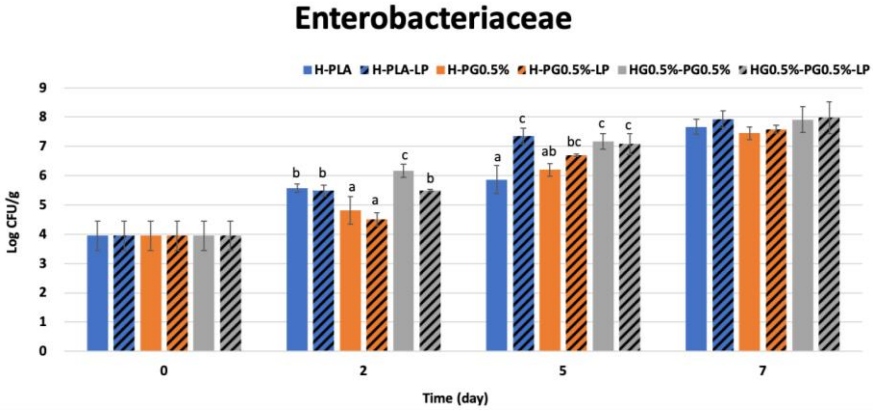



**Figure 6-9**. Bacterial counts of Enterobacteriaceae in chicken breast meat samples stored in active packages with recycled composites during 7 days of storage at 4°C. $^{a, b, c}$ *statistically significant different by ANOVA test.*

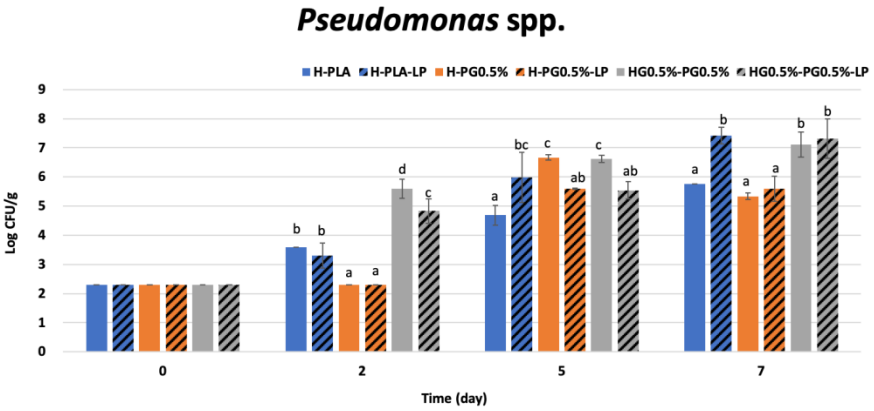



**Figure 6-10**. Bacterial counts of *Pseudomonas* spp. in chicken breast meat samples stored in active packages with recycled composites during 7 days of storage at 4°C. $^{a, b, c}$ *statistically significant different by ANOVA test.*

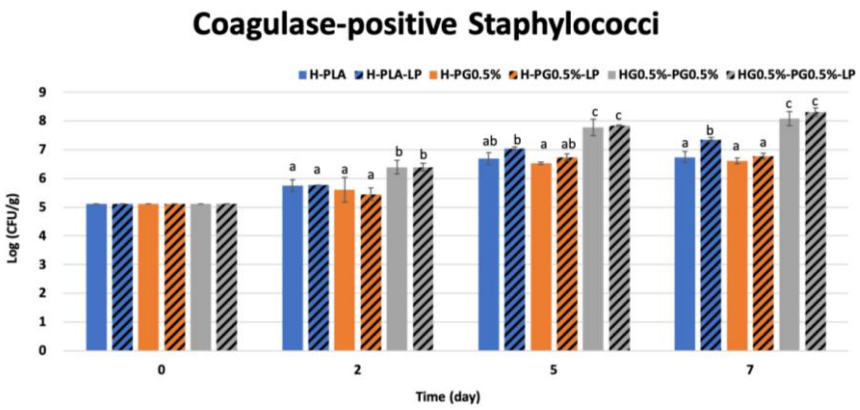



**Figure 6-11**. Bacterial counts of Coagulase- positive staphylococci in chicken breast meat samples stored in active packages with recycled composites during 7 days of storage at 4°C. $^{a, b, c}$ *statistically significant different by ANOVA test.*

The bacterial groups of β-glucuronidase-positive *Escherichia coli*, sulfite-reducinganaerobes, *Clostridium perfringens* were under detection limit.

### 6.4.3. pH values

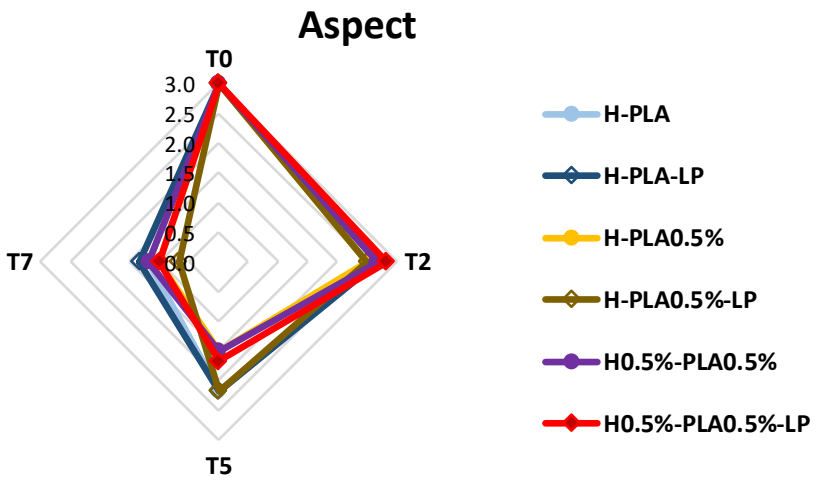
The pH values presented similar variation in all meat samples no significant difference was found between samples (data not shown).

### 6.4.4. Sensorial analysis

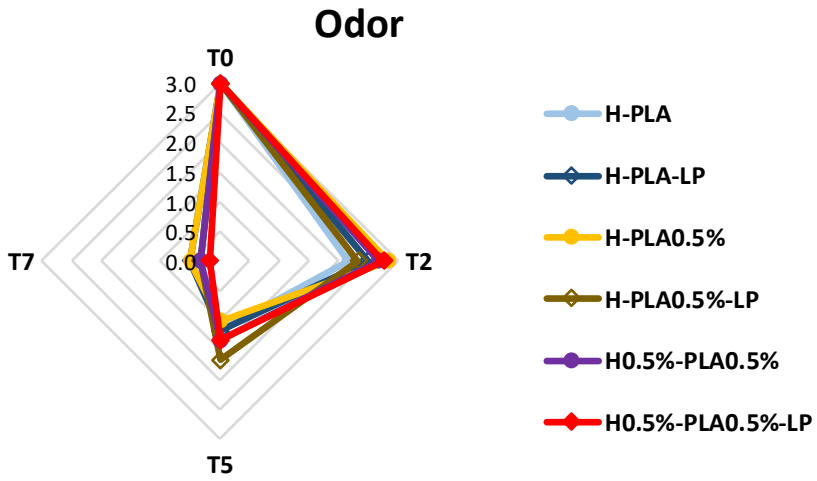
A general evaluation of raw chicken breast meat was performed based on the following characteristics: Aspect, Odor, Color, Elasticity at 0, 2, 5, 7 days (Figure 6-12). The values of each meat attribute either sprayed with *L. plantarum* IMC 509 (darker lines) or without decreased during storage. Compared to other samples, H-PLA-LP (dark blue) showed to be able to maintain better-higher value in meat aspect (Figure 4A) during study period. While forodor and elasticity, during the first 5 days, H-PG0.5%-LP (brown line) sample was higher in values than other samples. After another two days storage, meat stored in H-PG0.5% (yellow line) demonstrated

better in odor than other samples. H-PLA (light blue line) had a protective effect on meat color (Figure 4C) during time. For the overall acceptability, the score of H-PLA-LP was the highest at 7 days storage (Figure not shown).

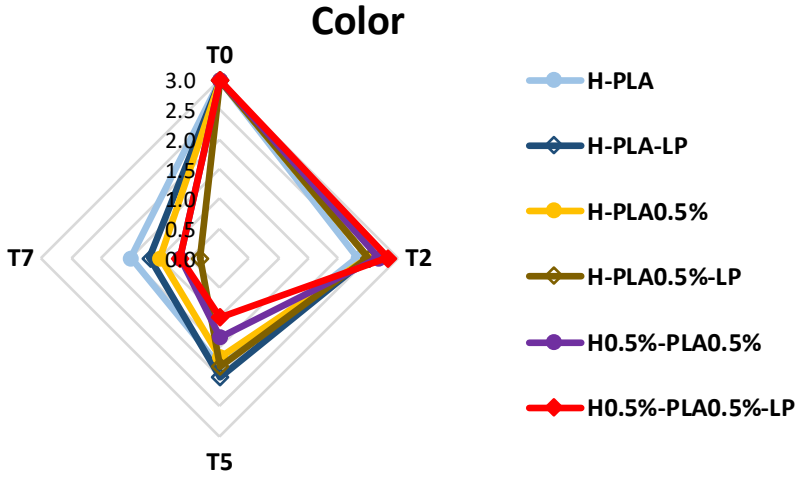
a)




b)




c)



d)

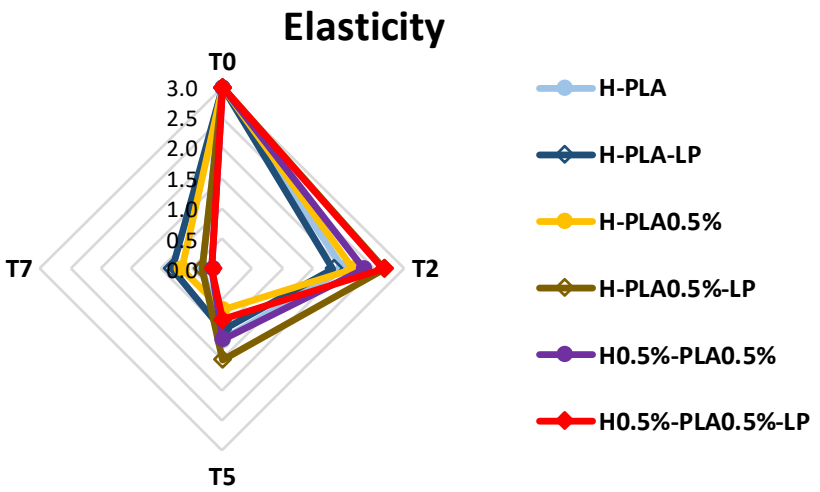



**Figure 6-12**. Sensorial analysis of meat stored in the different sandwich paper-PLA based packaging for 7 days. a-Aspect, b-Odor, c-Color, d-Elasticity. Hedonic scale: 0-3.

## 6.5. CONCLUSIONS

The incorporation of composite - Ag-GN-$TiO_2$ in paper-PLA based packaging material showed improvements in the structural and mechanical properties such as thickness, smoothness, tear resistance, strength, porosity. But the barrier property of H-PLA packaging material is sensitive to temperature, and it is more permeable to water vapor compared to polyethylene film. Furthermore, the probiotic modification of H-PLA complex showed inhibitory activity against the growth of tested pathogenic bacterial strains.

From the meat microbial quality point of view, the counts of total aerobic mesophiles and lactic acid bacteria were generally high in meat stored in LP-containing packages (*L. plantarum* IMC 509-sprayed surface in touch with meat surface) than other samples. However, chicken breast meat stored in H-PG0.5% and H-PG0.5%-LP had lower amounts of Enterobacteriaceae and coagulase-positive staphylococci (data not shown) during study period compared to other samples, with statistical

significance at T2, T5 and T7. For *Pseudomonas* spp. that are common food spoilage indicators, H-PLA 0.5% and H-PG0.5%-LP packaging showed positive effects on limiting the increment of this group of bacteria for 2 days, although there were some variations, both were able to maintain the counts lower than acceptable limit for 7 days under cold storage. Meat pH was remained during study period. For sensorial characteristics, generally samples packed in *L. plantarum* IMC 509-treated packaging showed relatively better results than non-LP-treated. In the chemical study, in correspondence with sensorial graphs, *L. plantarum* IMC 509 seemed to be able to inhibit the production of biogenic amines compared to other types of H-PLA discs.

From the microbial and chemical comparison of commercial meat packages with active packages, a different impact of packages on meat microbial proliferation rate was noticed. Factors, such as barrier properties and incorporation of composites and probiotic bacteria, can positively or negatively affect microbial proliferation. The increased amount of B.A.I. revealed that active packages exhibited better efficiency for the first days of storage, while as the enforced barrier properties of commercial packages (polyethylene) limited the bacterial growth and metabolic acidity, reducing the amount of biogenic amines produced during extended storage.

The use of paper-PLA based packaging with incorporation of graphene-based composite and probiotics offered an ecological approach for preserving meat. Moreover, although the addition of *L. plantarum* on packaging did cause a significant increase in meat microbial load, the probiotic strain had positive effects on meat quality such as inhibiting undesirable microbial groups, preserving meat sensorial quality. The current study also compared the microbial and chemical variations of meat stored in different packages, summarizing the main variations among samples. According to the different analysis, we may conclude that the active packaging was more efficient than

commercial ones in preserving meat quality during the first days of storage, but the barrier properties need to be enhanced, as well as the probiotic incorporation method needs to be improved to increase the overall efficiency in preserving food.

## 6.6. References


Baston, O., & Barna, O. (2010). Raw chicken leg and breast sensory evaluation. *Annals food science & technology*.

CeIRSA (2017). Linee Guida per l'analisi del Rischio nel campo della microbiologia degli alimenti (allegato B). In: Protocollo tecnico della Regione Piemonte per l'effettuazione dei controlli microbiologici ufficiali sugli alimenti e l'interpretazione e gestione degli esiti analitici (updated 21.08.2017).

Deshwal, G. K., Panjagari, N. R., & Alam, T. (2019). An overview of paper and paper based food packaging materials: health safety and environmental concerns. *Journal of Food Science and Technology*, *56*(10), 4391-4403.

Min, B., & Ahn, D. U. (2012). 4 - Sensory properties of packaged fresh and processed poultry meat. In J. P. Kerry (Ed.), *Advances in Meat, Poultry and Seafood Packaging* (pp. 112-153): Woodhead Publishing.

Mohammed, H., Kumar, A., Bekyarova, E., Al-Hadeethi, Y., Zhang, X., Chen, M., Ansari, M. S., Cochis, A., & Rimondini, L. (2020). Antimicrobial mechanisms and effectiveness of graphene and graphene-functionalized biomaterials. A scope review. *Frontiers in Bioengineering and Biotechnology*, *8*, 465.

Rawdkuen, S., Punbusayakul, N., & Lee, D. S. (2016). Chapter 17 - Antimicrobial Packaging for Meat Products. In J. Barros-Velazquez (Ed.), *Antimicrobial Food Packaging* (pp. 229-241). San Diego: Academic Press.

Sirocchi, V., Caprioli, G., Cecchini, C., Coman, M. M., Cresci, A., Maggi, F., Papa, F., Ricciutelli, M., Vittori, S., & Sagratini, G. (2013). Biogenic amines as freshness index of meat wrapped in a new active packaging system formulated with essential oils ofRosmarinus officinalis. *International Journal of Food Sciences and Nutrition*, *64*(8), 921-928.

Verdenelli, M. C., Coman, M. M., Cecchini, C., Silvi, S., Orpianesi, C., & Cresci, A. (2014). Evaluation of antipathogenic activity and adherence properties of human Lactobacillus strains for vaginal formulations. *Journal of Applied Microbiology*, *116*(5), 1297-1307.

Vytejckova, S., Vapenka, L., Hradecky, J., Dobias, J., Hajslova, J., Loriot, C., Vannini, L., & Poustka, J. (2017). Testing of polybutylene succinate based films for poultry meat packaging. *Polymer Testing*, *60*, 357-364.

# CHAPTER VII

# GENERAL DISCUSSION AND SUMMARY

Lactic acid bacteria are ubiquitous in foods, and their functions include fermentation, flavor development, and preservation. Recently, the antibacterial effect of LAB and its active metabolites has been extensively studied and reviewed. As biopreservatives, their applications in food have advantages such as less food texture degradation, safety and health benefits for consumers.

This study was conducted to explore the concept of evaluating the quality of meat and meat products during storage, with the aim of designing and developing a prototype antibacterial food packaging based on lactic acid bacteria cells/metabolites and composites of graphene and nano-Ag-$TiO_2$ as bacteriostatic agents. We firstly characterized the efficacy of commercial (currently used) packaging on selected food foams. Secondly, research was conducted to develop a suitable method for detecting ATP in hams. Thirdly, the efficacy of packaging films spiked with LAB CFS was successfully evaluated using this method. Suitable graphene matrix composites and *L. plantarum* were then used to develop novel antibacterial packaging. The efficacy of the novel antibacterial packings in chickens was demonstrated and compared with commercial counterparts.

The main empirical results are chapter specific, and the empirical evidence obtained to meet the requirements of each specific objective is summarized in this section.

**1. Characterization of the preservation efficiency - biogenic amines and microbial load - of commercial packaging materials (multilayer packaging with**

**metal layer) on 8 different species for raw and cooked ham.**

a. The analytical method based on extraction, derivatization, purification and determination in HPLC-DAD allows us to quantify the content of biogenic amines in ham contained in different types of packaging. The results obtained show that all the samples analyzed have a chemical quality index lower than 3. The total content of BAs shows slight differences during shelf life. Chemical, microbiological and pH analyzes confirmed the equivalence of the different packaging. It can be concluded that the packages provide a high level of food safety to protect the consumer.

b. The three packages (Pack 1 - multilayer sheet + multilayer bag, Pack 6 - polycoupled sheet + metallized bag and Pack 7 - polycoupled sheet + copper bag) have exceptionally high efficiency compared to the other packages studied. The samples stored in these packages did not show any significant increase in BAs during the study. The packaging system can help distinguish the development and correlation of biogenic amines and spoilage-related microorganisms in the product. The indicators of microorganisms and BAs resulting from packaging conditions could be distinguished from PC1 by PCA analysis. The results of PCA applied to the mean values of the parameters in packages 1, 6, and 7 showed that tyramine, putrescine, and cadaverine could be used as spoilage indicators for ham. PC1 was the most important variable related to the differences between packaging conditions, as it accounted for 46%, 52.4%, and 53.6% of the total variability in Pack 1, Pack 6, and Pack 7, respectively. PC1 was positively related to microbial analysis and protein change (parts of biogenic amine content).

c. The changes in biogenic amines, microbial content, and pH during ham preservation compared to raw meat were no significant difference, probably due to the reduction in moisture and increased NaCl concentration in the ham. Studies have

shown that NaCl concentration has a significant effect on the endogenous microbiota (Lactic acid bacteria, mesophilic aerobic bacteria, psychrotrophic bacteria, *Staphylococcus* spp. and Enterobacteriaceae) and biogenic amine content (histamine, tyramine, putrescine, cadaveramine and spermatine) in dry-cured meat. The higher NaCl cluster had a lower content of BAs. A negative correlation between microbial count and BAs content in the lower NaCl cluster suggests that the higher BA's content in the lower NaCl cluster may be the result of a stress response mechanism. On the other hand, the salt concentration in the higher NaCl cluster had an inhibitory effect on the formation of BA except histamine. The collective results suggest a NaCl threshold that minimizes the formation of BAs in dry cured meats.

**2. Investigate the correlation between biogenic amines and microorganisms, ATP and microorganisms in different foods to select the appropriate shelf life markers for ham.**

a. An HPLC-based method for the separation and quantification of ATP-related compounds was proposed. SunFire$^{TM}$ C18 (4.6 mm×mm, 5μm) was used and the mobile phase was V (0.05mol/L $KH_2PO_4$-$K_2HPO_4$) : V (methanol) = 9:1 buffer at a flow rate of 0.6 mL/min, and was detected by high performance liquid chromatography at 254 nm and quantified by external standard method.

b. The developed HPLC method had high sensitivity as well as good linearity and repeatability. The validated method successfully detected four ATP-related compounds (ATP, ADP, AMP and Hx) in dry ham samples, while Ino was not measurable in ham. Moreover, the recovery of ATP-related compounds in ham samples was in accordance with the application detection range.

c. A positive correlation was found between ATP content and microbial load during storage in ham samples. Thus, the method developed here can be generally

applied for the determination of four ATP-related compounds in dry ham.

**3. Selection of probiotic bacteria with potential use in food and optimization of fermentation conditions.**

a. We studied six strains of LAB (*L. bulgaricus*, *L. plantarum*, *L. casei*, *L. acidophilus*, *L. lactis* subsp. *lactis LL9*, and *L. thermophilus*). Among them, *L. plantarum* was not the most prominent in antimicrobial peptide production with a measured inhibitory zone diameter of 11.72 mm. However, the highest EPS production was found in *L. plantarum*. The highest yield of EPS was 1513.1 mg/L using a 5% inoculum of *L. plantarum* fermented at 37 ℃ in a shake flask. *L. plantarum* can produce antibacterial substances and prolong the storage time of food.

b. The selection of the optimal immobilized carrier for *L. plantarum* was carried out using calcium alginate colloid, carrageenan, gelatin, and agar as carriers. The agar-embedded method gave the best immobilization effect, and the yield was 1096.2 mg/L. Compared with other immobilization methods, this method has the least effect on EPS yield; therefore, it was selected as the best embedding vector for EPS production by *L. plantarum*. The process conditions of immobilized fermentation of *L. plantarum* were investigated. The optimal conditions were agar strength of 30 g/L, particle size of 1.0 cm, and fermentation of 3% of the inoculum at 37℃ for 24 h; the optimal condition resulted in the highest yield of extracellular polysaccharide at 1489.9 mg/L.

c. The immobilized cells of *L. plantarum* yielded almost the same yield of EPS as the fermentation with free cells, probably because the diffusion of the product in the immobilized carrier was limited, which affected the yield increase. However, from a process perspective, the immobilization method greatly simplifies the process. Immobilization reduces the need to activate the strain multiple times, avoiding the

step of cell separation in the fermentation liquid, which simplifies the subsequent product extraction. In addition, the immobilized LAB cells can be well maintained and used repeatedly, which effectively increases the utilization rate of the strain and reduces the production cost.

d. EPS production of LAB is generally low, and there are many influencing factors. In recent years, based on the understanding of the genetic basis, biochemical type, biosynthetic pathway, metabolic model and physiological activity of EPS-producing lactic acid bacteria, metabolic engineering method has become a hotspot in this field. The strain and technology of this study also laid a good foundation for EPS production by LAB.

**4. To obtain the efficacy of ATP as a shelf life marker in modified packages and to study the cell-free supernatant of *L. plantarum*, which can be used as part of an antibacterial package to extend the shelf life of ham.**

a. The effect of a new antibacterial packaging film enhanced with *L. plantarum* CFS on ATP content was studied. The results showed that four ATP-related compounds (ATP, ADP, AMP and Hx) were successfully detected in dry ham samples using the validated method, while Ino was not measurable in ham. Moreover, the recovery of ATP-related compounds in ham samples was in accordance with the application detection range.

b. *L. plantarum* CFS as a natural microbial preservative was able to rescue ATP levels when it was used as a natural microbial preservative to improve the packaging material of dry-cured ham and delay food corruption. The CFS of *L. Plantarum* CICC 200022 showed activity as a natural preservative in dry cured ham and could potentially be used to improve food packaging materials and thus prevent food spoilage.

**5. Prepare and evaluate different prototypes of PLA/paper packaging with addition of graphene-based composite and probiotic bacteria - *L. plantarum* IMC 509 - on raw chicken meat.**

a. Incorporation of the composite - Ag- GN -$TiO_2$ - into the paper-PLA-based packaging material showed improvements in structural and mechanical properties such as thickness, smoothness, tear resistance, strength and porosity. However, the barrier properties of the H-PLA packaging material are temperature dependent and it is more permeable to water vapor compared to polyethylene film. In addition, the probiotic modification of the H-PLA complex showed an inhibitory effect on the growth of the tested pathogenic bacterial strains.

b. Regarding the microbial quality of the meat, the number of total aerobic mesophiles and lactic acid bacteria was generally higher in meat stored in LP -containing packages (*L. plantarum* IMC 509-sprayed surface in contact with the meat surface) than in other samples. However, chicken breast meat stored in H-PG0.5% and H-PG0.5%-LP had lower levels of Enterobacteriaceae and coagulase-positive Staphylococci during the study period (data not shown) than other samples, with statistical significance at T2, T5, and T7. For *Pseudomonas* spp. which are common indicators of food spoilage, the H-PG0.5% and H-PG0.5%- LP packages showed a positive effect on limiting the proliferation of this bacterial group for 2 days, although there was some variation, both were able to maintain counts below the acceptable limit for 7 days in cold storage. The pH of the meat remained constant during the study period. Regarding sensory characteristics, samples packed in packages treated with *L. plantarum* IMC 509 generally showed better results than those not treated with LP. In the chemical study, *L. plantarum* IMC 509 appeared to be able to inhibit the production of biogenic amines compared to other types of H-PLA discs, in

agreement with the sensory diagrams.

c. Microbial and chemical comparisons of commercial meat packaging with active packaging revealed a differential impact of packaging on meat microbial multiplication rates. Factors such as barrier properties and the inclusion of composites and probiotic bacteria can positively or negatively influence microbial proliferation. The increased amount of BAI showed that active packaging had better efficiency during the first days of storage, while the enhanced barrier properties of commercial packaging (polyethylene) limited bacterial growth and metabolic acidity, reducing the amount of biogenic amines produced during prolonged storage.

d. The use of paper PLA-based packaging with a graphene-based composite and probiotics provided an ecological approach to meat preservation. Although the addition of *L. plantarum* on the packaging resulted in a significant increase in the microbial load of the meat, the probiotic strain had positive effects on meat quality by inhibiting undesirable microbial groups and preserving the sensory quality of the meat. The current study also compared the microbial and chemical changes of meat stored in different packages and summarized the main differences between the samples. The various analyses suggest that the antibacterial packaging was more efficient than the commercial packaging in maintaining meat quality during the first days of storage. However, the barrier properties still need to be improved, as well as the method of probiotic insertion, to increase the overall efficiency in food preservation.

# CONCLUSIONS

For the storage of certain foods, ham and raw chicken, we characterized and tailored the antibacterial packaging to meet the specific composition and requirements

of the food while maintaining the bioactivity and antibacterial effect of the packaging. We thought about the active metabolites of probiotic bacteria as alternatives. The tests performed showed that the cell metabolites have a broad spectrum of inhibition, suggesting a potential role in food processing and preservation. Exploring the preservation potential of LAB and its metabolites allowed us to understand their interactions with food components and packaging materials, evaluate their impact on food quality, and expand their applications in the food sector.

The food packaging currently under study was only a prototype for laboratory-scale studies. To realize large-scale production, packaging performance and methods for incorporating the probiotic bacteria/active metabolites need to be improved. In addition, evaluations of food chemistry parameters - gas composition in the air space, humidity variations, macromolecule variations (lipid oxidation) - and the effects on the food under different storage conditions (temperature variations, light exposure) can also provide valuable insights and expand knowledge on packaging characterization. In the absence of food shelf life guidelines covering the most important aspects, we can only draw conclusions based on specific criteria. Future work is needed to standardize methods and make connections between the different aspects of food and packaging studied. Recently, the well-documented bioactivities and health benefits of probiotic metabolites, their protective function, and their technical advantages have also attracted the attention of researchers. In the future, the use of these natural preservatives can be validated and tailored to food matrix properties. Another important role of graphene composites is their antiviral activity through physical damage. One study has shown that SARS-CoV-2 can remain active on meat and fish at cold (4°C) and freezing temperatures (-10°C to -80°C) for up to 21 days, leading to possible spread of the disease through contaminated food. It may be

interesting to study the antiviral activity of a decontamination treatment with graphene-based packaging on food.

# List of publications

1. **Jing Tao**, Xiaohui Huang, Feiyue Ling, Bilian Yu, Xiao Zhou, Qing Shen, Gianni Sagratini (2022). Immobilization of Lactic acid bacteria for production of extracellular polysaccharides. *Food Science and Technology*. https://doi.org/10.1590/fst.99021
2. Franks kamgang Nzekoue, Gulzhan Khamitova, Simone Angeloni, Alba Nacher Semperec, **Jing Tao**, Filippo Maggi, Jianbo Xiao, Gianni Sagratini, Sauro Vittori, Giovanni Caprioli (2020). Spent coffee grounds: A potential commercial source of phytosterols. *Food Chemistry 325(4):126836.* https://doi.org/10.1016/j.foodchem.2020.126836
3. Dianbo Zhao, Shaodan Wang, Yushan Hua, Xiao Liu, **Jing Tao**, Gianni Sagratini, Qisen Xiang (2022). Inactivation of Escherichia coli O157:H7 by lauric arginate: Role of membrane disruption and oxidative stress. *LWT.* https://doi.org/10.1016/j.lwt.2022.113449
4. Juan Du, Jialei Liu, Kai Liu, Dianbo Zhao, Gianni Sagratini, **Jing Tao**, Yanhong Bai (2022). Development of a fluorescent test strip sensor based on surfacepositively charged magnetic beads separation for the detection of Listeria monocytogenes. *Analytical Methods.* https://doi.org/10.1039/D2AY00384H
5. Laura Alessandroni, Giovanni Caprioli, Federico Faiella, Dennis Fiorini, Renzo Galli, Xiaohui Huang, Giacomo Marinelli, Franks Nzekoue, Massimo Ricciutelli, Serena Scortichini, Stefania Silvi, **Jing Tao**, Alessandro Tramontano, Daniele Turati, Gianni Sagratini (2021). A shelf-life study for the evaluation of a new

biopackaging to preserve the quality of organic chicken meat. *Food Chemistry 371(5):131134.* https://doi.org/10.1016/j.foodchem.2021.131134

6. **Jing Tao**, Bilian Yu, Qing Shen, Xiao Zhou, Shaopeng Chen, Gianni Sagratini, Sauro Vittori, Giovanni Caprioli, Hua Zhang, Yanhong Bai, Franks Kamgang Nzekoue (2022). Determination of ATP-related compounds by HPLC to study the effect of cell-free supernatants of Lactobacillus Plantarum on the shelf life of sliced dry-cured ham. *Food control– under submission*
7. **Jing Tao**, Xiaohui Huang, Manuella Lesly Kouamo Nguefang, Gianni Sagratini, Giovanni Caprioli, Sauro Vittori, Stefania Silvi, Hua Zhang, Yanhong Bai, Franks Kamgang Nzekoue (2022). Formation of biogenic amines and growth of spoilage-related microorganisms in sliced raw ham and cooked ham stored under different packaging conditions applying PCA – *In preparation*

## Patents

1. **Tao J,** Li H, Ling F, Zhang Z, Han L, Zhang B, Song J, Zhou A. "Immobilization of *L. plantarum* for production of extracellular polysaccharides" has been authorized as a national patent by the State Intellectual Property Office of China. ZL 201810953538.2
2. **Tao J,** Yu B, Shen Q, Feng X, Yang J, Zhou X, Niu Y, Lei Y. "A preparation method of new antibacterial packaging film and application in ham preservation" has been patent application acceptance by the State Intellectual Property Office of China. 202110953843.3

## Participation in international/national conferences

1. 15-17.04.2019 18th European Young Cereal Scientists and Technologists Workshop, Sede di Servizio, Italy
2. 23-24.05.2019 Food shelf life: challenges, pitfalls and packaging innovation, Università degli Studi di Udine, Udine, Italy
3. 09.07.2019 Cibo e nutraceutici: parola chiave "caratterizzazione", Camerino, Italy
4. 25-27.09.2019 6th MS food day, University of Camerino, Camerino, Italy
5. 30.03-01.04.2021 Workshop ZZULI-UNICAM Building Research Bridges, Virtual conference
6. 02.04.2022 Deutsch-chinesische Gesellschaft fur Molekulare Medizin und Molekulare Pharmakologie (DCMP), Virtual conference

## POSTERS

1. **Jing Tao**, Xiaohui Huang, Feiyue Ling, Bilian Yu, Xiao Zhou, Qing Shen, Gianni Sagratini. Application of bio-microcapsule immobilization technology for Lactic acid bacteria fermentation to produce extracellular polysaccharide (Oral presentation). Poster at Workshop ZZULI-UNICAM-: Building Research Bridges, Virtual conference. 30.03.2021 – 01.04.2021.
2. Laura Alessandroni, Giovanni Caprioli, Federico Faiella, Dennis Fiorini, Renzo Galli, Xiaohui Huang, Giacomo Marinelli, Franks Nzekoue, Massimo Ricciutelli, Serena Scortichini, Stefania Silvi, **Jing Tao**, Alessandro Tramontano, Daniele Turati, Gianni Sagratini. Organic chicken meat in a compostable biopackaging solution: a comparative shelf-life study. Poster at XXI EuroFoodChem Conference, On-line Conference. 22.11.2021-24.11.2021.

## Attendance of lectures and courses

1. 11.06.2019 Il bello della scuola di scienze del farmacoe dei prodotti della salute, Campus universitario, Sala Convegni Rettorato, La Scuola di Scienze del Farmacoe dei Prodotti della Salute si presenta, Camerino, Italy.
2. 12.06.2019 Il bello del dottorato di ricerca, Campus universitario, Sala Convegni Rettorato, Camerino, Italy.
3. 28.06.2019 Seminar, Room C, chemistry department, University of Camerino, Synthetic approaches to generic active principles, by Dr. Daniele Ciceri Inden.
4. 30.09-04.10.2019 SAS activities, University of Camerino, Camerino, Italy.
5. 25-26.02.2020 SAS activities, University of Camerino, Camerino, Italy.
6. 04.04.2022, Seminar, ChIP-Research Center, University of Camerino, Phytochemical Extracts of Red Grapes from Organic and Conventional Vineyards as Bioactive Compounds Sources in Cosmetic Formulations, by Prof. Cristiana Radulescu, Valahia University of Targoviste, Camerino, Italy.
7. 05.04.2022, Seminar, ChIP-Research Center, University of Camerino, Dry Skin Emollient Cream with Skin / Seed Extract (Vitis Vinifera L. Organic Culture), A New Concept for the Treatment of Atopic Dermatitis, by Prof. Cristiana Radulescu, Valahia University of Targoviste, Camerino, Italy.

## Acknowledgments

I would like to express my sincere gratitude to the following people for their immense contribution during the course of my Ph.D.

Firstly, I am deeply grateful to my supervisor, Prof. Gianni Sagratini, Prof. Hua Zhang and Prof. Yanhong Bai for their inestimable supervision, endless support,

mentorship, and confidence in me throughout my Ph.D. studies. I would like to extend my sincere thanks to Prof. Sauro Vittori and Dr. Giovanni Caprioli for their precious advice, continuous assistance, and encouragement during my academic research and daily life. My gratitude extends to Prof. Stefania Silvi and Prof. Elena Vittadini, for the opportunity they gave me to collaborate and learn in their respective research fields. Additionally, I would like to offer my special thanks to Dr. Franks Nzekoue and Dr. Xiaohui Huang for their technical support and counseling in all the steps of my Ph.D. thesis.

This work would not be materialized without the financial and technical support of the University of Camerino and Zhengzhou University of Light Industry. I would like to recognize the important role of Dr. Cristina Soave, Dr. Simone Angeloni, Dr. Laura Alessandroni, Dr. Ke Li, Dr. Yanting Zuo and Dr. Gulzhan Khamitova.

My appreciation also extends to all my laboratory colleagues, the Doctorals and Masters students with who I worked for their kind help and friendship. Special thanks to Dr. Manuella Lesly Kouamo Nguefang, Dr. Lixia Yuan, Dr. Chuanpan Guo, Dr. Fenghe Duan, Dr. Yue Sun.

To my parents, husband and son, thank you for your years of love, patience and the embodiment of love. Your devotion over the years has allowed me to reach this point.

The research described in this thesis was obtained the joint support by the University of Camerino and Zhengzhou University of Light Industry.