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Active GRAphene based FOOD packaging systems for a modern society

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To my mother 张敏

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CHAPTER I

GENERAL INTRODUCTION

1.1 | POLYLACTIC ACID BASED FOOD PACKAGING

1.1.1 | PLA as food packaging material

During recent years, the increased public awareness together with policies boost the exploration and development of bio-based and biodegradable materials globally. Moreover, government also takes measures to limit the usage of traditional plastics and also promote the widespread of biodegradable materials, achieving transition from oil-based plastics to biodegradable materials. Among the potential candidates, Polylactic Acid (PLA) is considered as one of the most promising commercial bioplastics, because of its safety, tailorable mechanical properties, biocompatibility, relatively good barrier properties and availability of sources [1].

PLA is an eco-friendly, biodegradable polymer which can either be produced both by bacterial fermentation or chemical synthesis using lactic acid monomers (2-hydroxy propionic acid) [2]. Lactic acid isomer has different forms L-lactic acid and D-lactic acid, in which the former is considered as safe and can be commonly found in body as metabolism products. On the contrary, the D-isomer cannot be digested by human due to lack of specific enzyme and its intake is limited in adults and strictly banned in infant formula.

There is rapid growing of PLA production and applications in global market. Like most thermoplastics, PLA can be mold into different forms- fibers, films, sheets. Its flexibility together with the GRAS (Generally Regarded as Safe) status, PLA has been successfully launched in food sector as packaging films or containers for direct contact with various products, such as vegetables, chips, yogurt, water bottles [3, 4]. To date, food packaging is considered as the major application of PLA and its marketing is still growing. As the bio-based nature, PLA can be composted together with the foods and thus facilitating the waste management [5]. However, to fulfil the different requirements of food products, the physical and chemical

stability of PLA package needs to be assessed and monitored on real food [4]. Despite the advantages possessed by PLA, their widespread usage is still limited by its poor thermal stability, high gas transmission rate, brittleness, hydrophobicity, toughness, restrict the widespread use of PLA-based materials. Although PLA is generally considered as safe, its oligomers or the presence of residue of plasticizer still need to be cautious. For food packaging, high-quality PLA is regarded safe as it contains much lower free lactic acids compare to food ingredients [6]. Based on the abovementioned limitations, active fillers seem to be good solutions in improving the material properties as well as confer additional benefits to PLA-based packaging.

1.1.2 | PLA polymers synthesis and degradation

The synthesis of PLA involves the direct polymerization of lactic acid monomers into PLA as well as through the formation of intermediate substance-lactide then the intermediate is converted to PLA (microbial fermentation). The former process is direct and easier, but the process is difficult to manipulate and the final PLA product generally has low molecular weight. The second method is considered a better choice for mass production as the product obtained with high-molecular-weight, which exhibits better mechanical and thermal properties with wider applications [7]. The commonly used synthesis techniques include: extrusion, blow molding, injection molding, thermoforming as well as 3D printing technique to produce different shapes, like film, water bottles. Among them, injection molding can be used for quick mass production with relatively low labor cost, whereas 3D printing is more useful for processing PLA objects with tailored demands or requirements. Compared to the common plastics, the biodegradability is one the major attractive property of PLA. Under natural conditions, the degradation is initiated by hydrolysis and then followed by the enzymatic or microbial degradation reactions, causing fragmentation and further digested to harmless materials [8]. Microbial degradation activity is considered as a safe and economic method of PLA waste management. Microorganisms, especially Actinobacteria are being considered as major potential PLA degrader in nature, using the serine protease to hydrolysis PLA molecules [9]. The filamentous fungi are found to be able to grow and penetrate the surface of poly lactic acid plate. However, the diversity and abundance of the degrader microorganisms vary according to different environmental conditions, which could also affect the degradation rate [10]. Several factors can accelerate or decelerate the degradation process, including the intrinsic properties of PLA-molecular weight, stereocomplex formation, crystallinity as well as environmental factors-hydrophilicity, acidity, moisture, light, oxygen, temperature, solvent presence [11]. Generally, PLA with high-molecular-weight and complex crystallinity shows more resistant to the attach of hydrolysis, whereas the extended exposure to heat and UV can severely damage the PLA materials [12].

1.1.3 | Active PLA food packaging

Currently, consumers are not satisfied with the basic functions of food package and the use of synthetic preservatives that could be unnatural and harmful to health. Active package containing natural preservatives is gaining popularity in food market. Natural fillers (fibers, proteins, starch, enzymes) and nano-additives (nanocomposites) are widely investigated and considered as their interactions with PLA molecules could potentially enhance its packaging performance and further bring additional functions, such as antimicrobial and antioxidant activities [13]. The antimicrobial capacity of package is important to control and limit the growth of microorganisms and thus to maintain food quality and safety during transportation and storage. Antimicrobial packaging involves the addition of inhibitory substances that may come from microbial (organic acids, bacteriocins, enzymes), plant (extracts and essential oils), chemical sources (metals, nanocomposites, EDTA) into matrix. However, the high temperature encountered during thermal processing may negatively impact the efficiency of heat sensitive and high volatile substances such as enzymes, bacteriocins, and plant extracts. Therefore, the selection of proper active compounds and tailored processing techniques are essential to the final efficiency of desired packaging. Efforts have been made to overcome the heat-sensible nature of active fillers, including the addition of plasticizer (lower processing temperature) [14], microencapsulation technique (protect fillers) [15,16], and using proper casting method. For example, solvent casting is considered as a suitable method as it requires only a suitable solvent to dissolve antimicrobials, whereas extrusion method is more proper for producing of PLA films containing heat resistance substances such as metals and nanoparticles [17]. Interestingly, novel technology-nanotechnology could not only improve the intrinsic properties of the package but also allow a controlled release of active agents and therefore realizing extended food protection [18]. However, the final efficiency of active package may subject to various factors: incorporation methods-coating methods showed limited interactions and potential effects, the properties of active compounds (size, compatibility with PLA, thermal sensitivity, high volatility), processing techniques (high temperature, shear force, acidity), storage environments (temperature fluctuation, light exposure), foods characteristics may affect the efficiency of the active PLA film [19, 20]. Therefore, a good selection of effective antimicrobials and a suitable processing technique are essential to produce a successful active PLA film [1].

1.2 | GRAPHENE AND FOOD APPLICATIONS

1.2.1 | Graphene

Among the nanoparticles applied in PLA film, graphene composites and its derivatives in developing active PLA film have attracted a lot of attention. Graphene is the carbon atoms that connected through covalent bounds with the formation of one-atom-thick hexagonal lattice [21, 22]. Monolayer graphite can assemble to ball, nanotube, and stacked graphite through van der Waals forces [21, 23]. Graphene can be synthesized from graphitic sources via micromechanical cleavage, chemical exfoliation, reduction of graphite oxide; and from non-graphitic sources via epitaxial growth and chemical vapor deposition as illustrated in Figure 1 [24, 25, 26]. Other sources can come from plant, insects, foods, and even waste [27-29]. Graphene particles can be chemically modified to form graphene derivatives, such as graphene oxide via graphene chemical oxidation and micromechanical exfoliation [30] or reduced graphene oxide. With its unique high surface area, graphene possesses excellent thermal conductivity, electron mobility, flexibility, optical properties. All these properties make graphene ideal material to be designed and manufactured into a variety of devices such as electronic parts, biosensors, transistors, biomedical instruments [24, 31]. One of the most attractive characteristic of graphene composites is their antimicrobial property as the major inhibitory mechanisms are through physical interactions that unlikely cause resistance. The growing interests and number of publications have unfolded the antimicrobial mechanisms of graphene-based antimicrobials, researchers used various instruments in order to visualize the graphene-microbial interactions, such as transmission electron microscopy (TEM) and scanning electron microscope (SEM) to visualize the morphology change after interaction [30]. Assays for analyzing cell viability and oxidative status were used to quantify the bactericidal efficiency and oxidation stress [33]. In general, graphene composites showed a relatively broad antimicrobial-spectrum, including the common investigated microorganisms: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Streptococcus mutans, Porphyromonas gingivalis, Klebsiella pneumoniae, Salmonella typhymurium* as well as yeast-*Candida albicans* [34-37]. Apart from the excellent antimicrobial capacity of graphene derivatives, graphene oxide was active in preventing the formation of biofilm as well as reducing the preformed biofilm [38]. Moreover, graphene composites also were active against fungi via ultrastructural changes, inhibition mycelia synthesis and protozoans via oxidative stress [39, 40]. Recently, as the surge of the coronavirus worldwide, the anti-viral property of graphene-based materials have been extensively studied and reviewed as solutions to combat this pandemic. Like the mechanisms exert on bacterial, graphene composites can interact with the negative groups on virus surface and subsequently causing physical damages and local rupture of the virus. Therefore, the number of negatively charged groups on virus and the sharpness of graphene

composites are decisive factors for its final efficiency. Recently, one study reviewed and highlighted the potential medical applications-biosensors, antiviral coatings, face mask, surface plasmon resonance (SPR) substrates-using graphene composites [41].

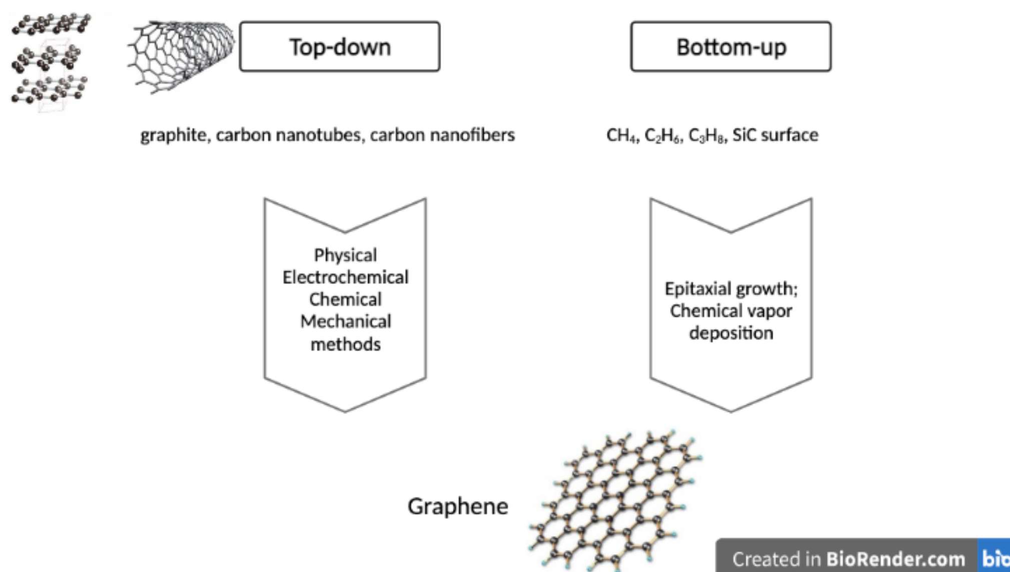


Figure 1. Graphene synthesis methods.

1.2.2 | Graphene antibacterial properties

As mentioned above, the main bactericidal mechanisms reported by different authors are through physically damaging cell membrane and other compartments that require the interactions between graphene and microbes [42, 43]. Similar to other nanoparticles, graphene nanoparticles initiate the weak linkage to bacterial cell membrane via van der Waals forces and electrostatic attractions. Then, the graphene-bacteria connection is reinforced through hydrophobic, ligand-bindings. Once these connections have stabilized, nanoparticles use the sharp edges to damage cell membrane and thus allowing itself to enter inside cell where it can bind to cell components - DNA, proteins, ribosomes- disrupting normal function and causing oxidative stress to bacteria. All these activities trigger/facilitate the formation of increased membrane permeability, metabolism disruption and subsequently cell death [44]. Briefly, the major bactericidal actions can be summarized into: 1) damaging cell membranes by the sharp edges of graphene composites; 2) inducing the production of ROS and oxidative stress; 3) depriving bacterial membrane of electrons and leading to loss integrity; 4) wrapping around cells and separating cells from nutrients and air; 5) interfering DNA replications [42, 43, 45]. Among the proposed mechanisms, physical damage and ROS induction are the mostly accepted. Regarding the oxygen deprivation-wrapping capacity of graphene composites, an interesting study revealed that apart from the good biocompatibility, graphene oxide could act as anaerobic

scaffold that promote the proliferation of gut beneficial bacteria - *Bidifidobacterium adolescentis*, and also enhance its antagonistic activity against pathogens [46]. Several factors can affect the final antimicrobial efficiency of graphene-based composites, including: **1) the intrinsic properties of the composites** - the carbon radical density, particle size, layer, arrangements, functional groups (oxygen-containing) the concentrations, interactions between molecules, the processing methods, compatibility, dispersity, and distribution in different matrixes [44, 47]; 2**) the target microorganisms-species, membrane properties and growth stages**. One study examined the inhibitory activity of graphene oxide on the growth of Gram positive bacteria - *Staphylococcus aureus*, Gram negative bacteria - *Pseudomonas aeruginosa* as well as yeast - *Candida albicans.* The results demonstrated that graphene oxide showed good antimicrobial activity on *S. aureus* (time-dependent), while weak and transient activity on *P. aeruginosa*, and the efficiency against *Candida albicans* was observed at late stage of growth (24h). As reviewed by AI-Jumaili *et al*. (2017), in most studies, the graphene-polymer films showed greater bactericidal activity against *S. aureus* than against *E. coli* as the complex cell structure of Gram negative bacteria inhibit the attachment and accessibility of composites [48]. To enhance the performance as well as to reinforce the antimicrobial efficiency of graphene composites, metal nanoparticles-silver, titanium dioxide, zinc oxide, copper, gold is used to form graphene-metal composites or graphene-metal oxide nanoparticles [49, 50]. Silver is a pronounced antimicrobial metal used for centuries. The graphene-based Ag nanocomposites showed stronger bactericidal activity compared to graphene composite alone [51, 52]. Silver nanoparticle anchored graphene oxide (Ag-GO) demonstrated strain-specific interactions, revealing that composite exhibited membrane-damaging action on *E. coli*, whereas the cell division-inhibition was found on *S. aureus* [53]. TiO_2 is another suitable candidate for the functionalization of graphene as its high photocatalytic activity that can enhance the ROS generation performance of graphene composites leading to higher antimicrobial performance. Moreover, TiO_2 is an economic material with high stability and low toxicity, but it is less reactive under visible light, the modification with graphene composites improved its photocatalytic efficiency under visible light, and thus enhanced antimicrobial activity was observed [54, 55]. However, the ratio between graphene and metals is decisive for the final efficiency of the composite, unbalanced ratio will lead to composite agglomeration or weak functionality as less functional groups of the composite. Moreover, organic molecules can also be combined with graphene composites with enhanced antimicrobial ability. Enzyme (lysozymes) [56], protein-lactoferrin, bacteriocin (nisin), and chitosan, have been used to functionalize graphene-based materials with improved bacteriostatic or bactericidal effects against *E. coli, S. aureus, Pseudomonas* spp. [57-61].

1.2.3 | Graphene applications in food sector

The applications of graphene are widely studied in different sectors. For medical purposes, graphene nanocomposites can act as drug carrier to control the release and enhance the efficiency of drugs [62]. In food sector, graphene-based sensors were developed to detect the presence of undesirable substances: synthetic chemicals, colorants, and toxins in foods [63]. Graphene sensors can also be used to check food quality by monitoring the level of food components such as cholesterol, glucose, volatile organic compounds [63-68]. As abovementioned, the excellent thermal, optical, mechanical as well as antimicrobial properties make graphene composites ideal candidates as fillers into polymer matrix for fabricating active food packaging films. The functional groups present on polymers and graphene composites can interact and thus affect the overall properties of the graphene-polymer mixture. The polymers prevent graphene composites from agglomerating/self-assembly, meanwhile, graphene composites could enhance the physiochemical and functional properties of the polymer-based material. Currently, the common polymers used to blend with graphene composites with great antimicrobial functionality include polyvinylcarbazole (PVK), polylactic acid (PLA), polyamide (PA), polyurethane (PU), polyvinyl alcohol (PVA), polysulfone (PSU), polyallylamine hydrochloride (PAH), polyvinylidene fluoride (PVDF), polydopamine (PDA) [69]. Moreover, the blended graphene-polymers demonstrated showed better antimicrobial ability and less cytotoxicity than the graphene composites alone [70-73]. Compared to pure PLA film, the addition of graphene composites improved gas impermeability, mechanical properties, stiffness and strength of the graphene-PLA film [74]. Some authors suggested that the enhancement of the performance was ascribed to the covalent and non-covalent interactions between functional groups of graphene nanocomposites and polymer matrix [75].

1.2.4 | Graphene – active compound in active packaging systems

The addition of graphene nanocomposites not only improve the thermal, physio-mechanical properties of the packaging film, but also bring additional antimicrobial property to pure PLA film. Biopolymers of PLA and polyurethane show good biocompatibility and potentials in tissue engineering, the incorporation of GO (graphene oxide) nanosheets into PLA/polyurethane (PU) film greatly enhanced the antimicrobial efficacy compared to the PLA/PU film on the attachment and growth of tested strains (*E. coli* and *S. aureus*). The activity is increased with increasing concentration of graphene oxide in film. The SEM image of bacterial cells (after contact with film) revealed that, after contact the active film, bacterial strains irreversibly lost their morphology. Therefore, authors presumed that the underlying mechanisms are through disrupting cell physical structure and inducing oxidative stress. Moreover, the active film also demonstrated good biocompatibility and no toxicity which

makes it suitable for tissue implants [73]. The incorporation of graphene nanoplatelets improved strength, toughness, thermal properties of plasticized PLA film. The active graphene-PLA film also successfully inhibited the proliferation of *E. coli*, *Salmonella typhimurium*, *S. aureus*, and *Listeria monocytogenes* as the high aspect ratio of nanocomposites that inactive microorganisms [76]. In another study, although PLA-based film modified with graphene nanoplatelets showed no activity on the growth of Gram positive bacteria - *Micrococcus luteus* through agar diffusion test, graphene nanoplatelets realized a sustained release of incorporated antibiotics as well as improved the rigidity and elastic modulus of PLA film [77]. Active film composed by PLA/GO and ZnO nanoparticles effectively reduced the counts of inoculated strains - *S. aureus* and *E. coli* - under light and dark conditions. The antimicrobial activity was ascribed to the synergistic effects of GO and ZnO that shows high photocatalytic efficiency, good biocompatibility, environment-friendly [78]. Furthermore, the interactions between GO-ZnO nanocomposites with PLA matrix contributed to enhanced rigidity (storage modulus), increased glass transition temperature as well as UV shielding property [79]. PLA film containing 2wt% of silver-graphene oxide (Ag@GO) showed improvements in film flexibility, bactericidal activity against gram-positive strain (*S. aureus*) and gram-negative strain (*E. coli*). Furthermore, the results showed that the processing method, *in situ* polymerization method was more suitable for processing this Ag@GO-PLA film than the direct mechanical blending method [80]. The addition of reduced graphene oxide (rGO) (0.5%) and cellulose nanocrystals (CNC) (1%) had positive effects on the overall biocompatibility, tensile strength, thermal stability, antimicrobial ability of PLA-based film. The active film showed no toxic effects to tested fibroblast cells with hydrophilic property that even support the growth of cells. Compared to gram negative bacteria, gram positive strain was more sensitive to the activity of the rGO/CNC/PLA film [81].

1.2.5 | Graphene safety issues

As the strong ROS generation ability against microbes of graphene composites are ascribed to its high surface mass ratio. It also raises the worry of whether graphene composites could cause some damages on mammal cell lines, such as damaging cell structures, inducing oxidative stress and causing cell apoptosis. The cytotoxicity has been tested on fish cell lines, the results showed that GO demonstrated mild toxicity in terms of delaying hatching process and inhibiting fish embryo growth, disturbing cellular metabolisms; higher concentration of graphene composites can even stop the development of zebrafish embryo [82, 83]. Damaged cell organelles, disrupted cell metabolisms, increased amount of reactive oxidative species were possible mechanisms leading to the overall toxicity to cells [84]. In adult fish model, graphene exposure leading to disrupted immune system-increased inflammatory cytokines, oxidative stress, lipid peroxidation, modified gut structure and dysbiosis in fish gut microbiota [85]. Under high dose of GO, processes like elevated level of lipid oxidation (inactivation of

antioxidant enzymes, promotion of oxidation processes), reduced lysosomal stability, increased DNA damages occurred in earthworms (*Eisenia fetida*) for longer exposure (14-21 days) [86]. One study conducted on PC12 cells, under the exposure to graphene, the amounts of ROS generated by cells increases as the concentration and exposure time increasing [87, 88]. Therefore, the cytotoxicity of graphene composites is depending on the composite's characteristics, the concentrations tested, the exposure time. In case of designing graphene composites for human applications, the hazard assessments need to be carefully evaluated [89].

1.3 | LACTIC ACID BACTERIA AND METABOLITES AS BIO-PRESERVATIVES

1.3.1 | Lactic acid bacteria as bio-preservatives

Food preservation methods, such as fermentation, salting and drying, have been mastered by human since ancient time. Although the roles of bacteria in fermentation remained unknown, it was found that by transferring a part of previously fermented foods to raw material-back slopping, a more flavored and stable food can be obtained, which leading to increased variety of foods, such as yogurt, cheese, sausages and fermented vegetables [90]. The discovery of food fermentation techniques and agents uncovered the roles of fermentative bacteria lead to the characterization of lactic acid bacteria (LAB). In general, LAB are composed by cocci or rod shaped gram positive bacteria that are catalase-negative, lactic acid production, resistant to acidic environment [91]. The most relevant genera in foods include *Streptococcus, Leuconostoc, Lactobacillus, Pediococcus, Aerococcus* [90]. In particular, LAB are generally used as starter cultures for fermenting foods, adjunct cultures for producing additional flavors or improving textures, bio-preservatives for maintaining food safety [92]. During fermentation process, LAB uses the enzymes through metabolic activities-glycolysis, proteolysis, lipolysis-to transform food molecules into flavoring compounds. As LAB can produce organic acids, therefore, contributing to lowered pH in the food environment, leading to unfavorable environment for the growth of other microbes, such as pathogens [93]. Recently, there is a growing interest in isolating and characterizing the beneficial bacteria from food and using them and/or their active metabolites for *in situ* protection. Numerous researches have documented the microbial control ability of LAB and their metabolites, also the underlying mechanisms have been extensively studied. However, in certain cases, for example, on raw meat, the acid-production ability of LAB may cause undesirable sensorial alteration, such as sour taste [94]. Another technical advantage of LAB as food bio-preservative is their tolerance to low temperature, oxygen level that normally occurred at storage conditions. Certain species of *Lactobacillus* and *Bifidobacterium* are considered as beneficial bacteria for promoting heath. The beneficial activities of probiotic are through their antagonistic activities and metabolites that positively

influence host metabolism as well as negatively influence the pathogens. The major health benefits of probiotics are ascribed to their antimicrobial, antioxidant, and immune protection capacities. *In vivo* studies demonstrated that probiotic bacteria - *Lactobacillus* spp. help to ameliorate the oxidative stress induced by ROS (reactive oxygen species) through their innate antioxidant system and also boost the host antioxidant system to regulate the oxidative state [95]. The health-associated benefits together with food preservation potentials also promote the selection of proper LAB in certain foods in order to extend food shelf-life, in the meanwhile, providing health benefits to consumers. Recently, numerous studies focused on the isolation of food origin *Lactobacillus* spp., the characterization of antimicrobial properties, and the application of live bacteria or their metabolites as bio-preservatives to extend food shelf-life [96].

1.3.2 | Postbiotics as bio-preservatives

However, the use of LAB as natural preservative is not always an optimal option for all kinds of food as the proliferation and metabolism may be difficult to control, and thus leading to unpleasant results. For instance, the roles of LAB on raw meat quality remain controversial as some authors suggested that the proliferation of LAB may cause unpleasant aspect and odor changes to meat [97, 98], while others argued that certain LAB can be considered as natural bio-preservatives as they had less impact on meat organoleptic properties while protecting meat from spoilage through their antagonistic activities [99, 100]. Moreover, environmental variations may also impact the preservation efficacy of LAB on food matrices. Although probiotics are generally considered as safe and beneficial bacteria, people with a weakened immune system needs to be cautious to the ingestion of foods containing live bacteria. Therefore, to avoid such potential impacts, the use of postbiotics derived from food-grade LAB, especially that have been included in EFSA QSP lists, can be considered as alternative food preservatives. The definition of postbiotics, as proposed by the International Scientific Association for Probiotics and Prebiotics (ISAPP), is "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [101]. As the definition suggests, postbiotic implies the use of lifeless food-grade microorganisms that still possess functional effects. The lifeless microorganisms can be obtained through heat or non-heat treatments (ionization, UV, pressure, sonication and so on) that are normally used for pasteurization. Although the microbial cells have been deliberately inactivated, but their components, such as cell wall fragments, enzymes, may still possess immune-regulatory, antimicrobial and antioxidant capacities [102, 103]. Regarding their safety for food applications, a variety of research have investigated their health-associated benefits through *in vivo* and *in vitro* - on animal models and human subjects [101]. Compared to live bacteria, postbiotics also have technical advantages, such as stability and resistance to common food processing techniques-thermal treatment. However, before the application on foods, it is necessary to

evaluate whether the treatment could affect postbiotics efficacy and their safety to human consumption through *in vivo* test [104].

1.3.3 | LAB metabolites as bio-preservatives

In addition to the application of both live and lifeless food-grade LAB, their active metabolites have also been widely studied as a natural strategy for food preservation. As we know, LAB exert antagonistic activity mainly through cell-cell competition as well as through active metabolites production. Depending on the bacteria species and available substrates, LAB can produce various valuable metabolites, including organic acids, exopolysaccharides (EPSs), biosurfactants, short-chain fatty acids, hydrogen peroxide, ethanol and also proteinous compounds-bacteriocins and bacteriocin-like substances [105, 106]. For food applications, cell metabolites were used either as a heterogenous mixture-cell-free supernatants that contain multiple products; or as purified form of bacteriocins, exopolysaccharides and so on [107]. Cell-free supernatant (CFS) is the exometabolites-containing liquid broth that are separated from its producer after incubation. Recently, researchers have studied extensively the antibacterial efficacy of *Lactobacillus*-CFS against common food pathogens and spoilage species using agar and food models [108, 109]. However, due to the complex nature of foods, to achieve the desired microbial control, higher amount of CFS is needed to achieve the same efficacy on foods than on agar surface [102]. Moreover, CFS also possess other properties that could be useful for food preservation and functional foods, like biofilm eradication, antioxidant, and immune-regulatory [110]. Despite the above-mentioned advantages, characteristics, such as the strong color of CFS (normally prepare in broth medium), sensitivity to alkaline conditions (organic acids production) may limit its usage in many foods. In addition, CFS generally contain various metabolites, which needs to be identified and characterized to understand better its mechanisms and efficacy. Moreover, metabolites in purified form can also be used as bio-preservative, such as bacteriocins, exopolysaccharides, antioxidant enzymes [111]. Bacteriocins are proteinous substances secreted by certain bacteria to kill closely-related species as a competition act. To date, the most well-studied bacteriocins include: nisin, pediocin and plantaricin. Nisin is mainly produced by probiotic bacteria - *Lactococcus* spp. and *Streptococcus* spp.- and shows effectivity mainly against gram-positive pathogens and spore outgrowth [112, 113]. Forming pores on cell membrane and disrupting cell wall synthesis are the main antimicrobial mechanisms exerted by nisin [114]. With the approval of FDA and GRAS (generally recognized as safe) status, nisin is currently used as food preservatives for different food products in many countries [115]. Pediocin is thermostable, pH-resistant bacteriocin that mainly produced by Pediococci, its usage is also obtained GRAS status in food applications. Like nisin, pediocin is also effective to most studied food pathogens and spoilage species, especially *Listeria monocytogenes* [116]. In this case, pediocin, such as PA-1, is also called Listeria-active bacteriocins [117]. The main bactericidal mechanism of pediocin is via

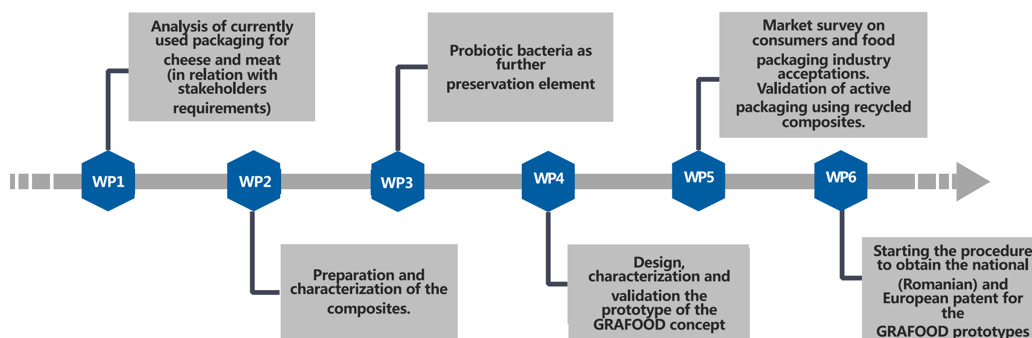
destabilizing cell membrane and subsequently cell death [114]. Although the application of pediocin (produced by Pediococci) the main inhibition mechanisms exerted by bacteriocins are through binding to cell membrane, forming pores and leading to cell leakage and death [113]. Other well-studied bacteriocins include plantaricins (*Lactobacillus plantarum*) [117], reuterin (*Lactobacillus reuteri*) [114], and so on. The food applications of bacteriocins on dairy, meat, seafood and beverages have been extensively reviewed by Verma [113]. However, due to the proteinous nature of bacteriocins, their activity is highly susceptible to the presence of proteolytic enzymes that can be found in foods. Therefore, the application on certain protease-rich foods may limit bacteriocin preservation efficiency. Also, bacteriocins can affect closely-related species - *Lactobacillus*, dairy products that require starter cultures to start fermentation may affected by the addition of bacteriocins [118]. Thus, based on these limitations, active packaging system with incorporation of bacteriocins have been investigated. The active coating or film may protect active compounds from interacting with food ingredients and thus losing its efficiency [119, 120]. Exopolysaccharides (EPS) are produced by bacteria with the aim of forming biofilm structure, which allows gene exchange and quorum sensing between species and protects bacteria community protection from hostile conditions and antibiotics [121]. Moreover, due to the polysaccharides nature, EPS is able to retain water that hydrates the microbial community in biofilm. This property is also utilized by food industry as additives to improve food and beverage rheological properties. Considering the safety issues related to producer strains, LAB derived EPSs are generally used by industrial food applications [121, 122]. However, limitations - the sensitivity of LAB in storage conditions, the number of metabolites they produced, sensitivity to different food compounds, inhibitory spectrum - may weaken the preservation efficacy. Therefore, combining with other hurdle technologies and incorporating into active packaging are considered as good strategies to both enhance its validity and preservation efficacy [118]. In summary, probiotic and food-grade LAB and their metabolites possess great potential to be used as food preservative with aims of maintaining food microbial quality, preventing oxidation and deterioration of food quality. Moreover, apart from being natural bio-preservatives, LAB and their metabolites can also be added into products as functional foods because of their immune-regulation capacity. But a thorough understanding of the active compound, their efficiency both *in vitro* and *in vivo*, a proper food matrix and storage conditions as well as the safety issues related to added amount need to be carefully evaluated by food professionals.

1.4 | GRAFOOD PROJECT OVERVIEW

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. The specific objectives of our part in the project are described below in outline of the thesis sections.

GRAFOOD-WORKPLAN



1.5 | OUTLINE OF THE THESIS

The objectives of the thesis were: 1) to characterize the preservation efficiency - microbial and chemical parameters - of commercial available packaging materials (polyethylene-based) on fresh ricotta cheese and paper-based packaging film for the preservation of chicken breast meat; 2) to select the proper graphene composites and probiotic bacteria with potential food applications, and to examine their combination to be used as part of active packaging for extending food shelf-life (ricotta cheese and raw chicken meat); 3) to prepare and evaluate different prototypes PLA/paper packages with addition of graphene-based composite, probiotic bacteria - *Lactobacillus plantarum* IMC 509 - on food models; 4) to obtain the circular economy, the preservation efficiency of active package with recovered graphene composites were also evaluated on fresh chicken meat. 5) to evaluate the viability of probiotic bacteria on package during storage conditions and 6) market survey of package prototypes.

The content of each chapter are summarized in below: **Chapter 2** – a review on natural preservatives from plants in cheese preservation; **Chapter 3** – shelf-life evaluation of ricotta cheese stored in four types of commercial in use polyethylene packages; **Chapter 4** – shelf life assessment of chicken breast meat stored in commercial in use paper packaging systems; **Chapter 5** – selection of graphene composites and probiotic strains used for food packaging;

Chapter 6 – *Lactobacillus* strains treatment on commercial packaging paper as preliminary study for extending the shelf-life of chicken meat; **Chapter 7 –** assessment of ricotta cheese shelf-life stored in PLA-based composite packaging bag and comparison with commercial package; **Chapter 8 –** assessment of chicken breast meat shelf-life stored in paper-PLA composite packaging; **Chapter 9 –** assessment of probiotics viability on packages surface; **Chapter 10 –** market survey on novel composite-probiotic PLA/paper-PLA packaging; **Chapter 11 –** potential of *Lactobacillus*-derived cell-free supernatants as food bio-preservatives; **Chapter 12 –** final conclusion.

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CHAPTER II

NATURAL PRESERVATIVES FROM PLANTS IN CHEESE PRESERVATION

2.1 | ABSTRACT

The current chapter has emphasized and focused on the cheese preservation because our first food model of the project (Grafood) was ricotta cheese, therefore, to understand better the interplays between packaging and cheese matrix.

As the appealing flavor, nutritious profile, wide diversity characteristics, dairy products – in particular cheese - are consumed worldwide. The microbial profile that originates from milk or from environment play crucial roles in flavor development as well as safety issues. Cheese products with raw milk sources, mild pH value, high moisture, are highly susceptible to the spoilage or pathogenic microorganisms, which not only causing economic loss but posing health risks to consumers. Currently, the raised awareness of consumers on healthy diet and health concerns on foods additives are transforming the use of synthetic ones to natural derived preservatives. Under such conditions, the objectives of the present chapter are to highlight the natural antimicrobials from plants in cheese preservation during recent years and to provide some views for future studies on cheese preservation. In this context, plant antimicrobials (essential oils and plant extracts) and the major actions on cheese are being discussed. Moreover, to enhance their efficiency on food during storage, the packaging systems and the combined hurdle techniques involved are also summarized here. In addition, their major efficiency and potential impacts on cheese are also considered. As the importance of microorganisms on cheese quality and human health, the chapter mainly focuses on the preservatives with potent antimicrobial properties. Furthermore, the limitations and future perspectives are also being discussed.

Under such circumstances, the chapter aims to address and highlight the following points: 1) the main preservation potentials of plant-derived preservatives; 2) the packaging and hurdle techniques; 3) the interactions between antimicrobials and cheese matrices; 4) the limitations and future perspectives. The studies and research papers published in the last decade were selected and referred.

2.2 | INTRODUCTION

2.2.1 | Cheese

Cheese are popular milk-based products that with diverse organoleptic characteristics produced by different regions of the world [1, 2]. The typical flavor, aroma, texture and nutrition profile are affected by various factors - the places of the farm, animal feeds and milk sources, starter cultures, processing techniques and ripening conditions [1, 3]. The consumption of cheese varies greatly in different countries, high amounts of cheeses have been consumed in many European nations, whereas relatively low amounts in some Eastern countries, such as China and Japan. The economic globalization contributes to the increased cheese production and consumption worldwide [4]. In the early times, the cheese making was discovered by chance and the primary goal was to preserve milk under warm temperature. Similar to other fermented foods, as the special aroma and flavor are accepted and appreciated by human, cheese become a common cuisine in our daily life. From the nutritional point of view, cheese is a nutritious food and as a good dietary source of valuable protein, digestible fat [5], conjugated linoleic acid (CLA) [6], vitamins (vitamin A, riboflavin, vitamin B_{12}, folate) [7], and minerals [8].

2.2.2 | Cheese classification

Cheese classification varies among different studies and authors. According to Fox *et al.* [2], the most widely accepted criterion is to sort cheese products according to its texture/moisture into: very hard, hard, semi-hard, semisoft, soft. Other classifications include the sources of milk (cow, sheep, buffalo), microorganisms contained or deliberately added, coagulation process (enzymes or microbes). However, there is not a satisfactory universal cheese classification documented up to now. Cheese can also be made by using raw milk or pasteurized milk, raw milk cheese is appreciated by some consumers as the raw milk cheese have richer and intense flavors than pasteurized cheese as a result of more diverse cheese microbiota and less processing techniques. But many foodborne outbreaks have linked to the raw milk cheese that contaminated by common animal associated pathogens, such as *Listeria*

monocytogenes, verocytotoxin-producing *Escherichia coli, Mycobacterium avium* subsp. *paratuberculosis,* enterotoxin-producing *Staphylococcus aureus, Coxiella brunetii* [1, 9, 10].

2.2.3 | Cheese manufacture

The most common milk sources for cheese production include cow, sheep, goat and buffalo. Among which, cow is currently the most commonly used for cheese-making worldwide, but the other types contribute diversity and appealing characteristics to cheese products. Similar to other type of fermented products, the final product quality and flavor is a result of series of chemical and microbial activities and the interactions among different molecules produced during processes. Although cheese types vary greatly in different area and tradition, for most cheeses, their manufacture processes involved in five major steps, which include: milk pretreatment, solid curd formation, whey removal, curd processing and cheese ripening and aging [11]. Pasteurization is an important procedure to eliminate both pathogens and spoilage microorganisms (coliforms and psychrophiles) that may cause some defects to final quality. Although pasteurization is necessary to ensure cheese quality and safety, some argued that the process (heat treatment) may severely damage cheese flavor by eliminating the functional microorganisms, altering enzymatic profile, resulting unflavored product, and also contributing to extended ripening time [12, 13]. One study compared the microbial composition of raw milk cheese and pasteurized milk cheese, the results showed different microbial profile of two types of cheese, with higher level of mesophilic non-starter lactobacilli and enterococci in the raw, whereas higher amount of starter culture - *Lactococcus lactis* found in pasteurized cheese. Moreover, the authors also found that the pasteurization process had no effect on cheese physicochemical profile [14].

2.2.4 | Cheese microbiota

Microorganism composition and metabolism play important roles in determining cheese final quality and safety. Factors, including the geographic area and conditions of farms, animal species and health status, milk types, operators handling and processing techniques all can shape the composition and metabolic activities of cheese microbiota [15]. The latter contains intrinsic species that originate from milk or from animal; or functional species that are deliberately added as starter and nonstarter cultures; or contaminants that are from environmental conditions [15]. In industrial or large-scale cheese production, for safety concerns and quality management, milk needs to be firstly pasteurized to eliminate the indigenous microbes, then functional cultures will be added to start cheese transformation. The functional cultures can be categorized based on their function, such as primary starters that mainly are lactic acid bacteria (LAB), which are involved in acid production and milk

coagulation. The main species are *Lactococcus lactis*, *Leuconostoc* species, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*, and *L. helveticus* [16]. Non-starter culture, or secondary culture, is also used to enrich the flavor and aroma as well as to form typical characteristics of certain cheese. In general, non-starter LAB, *Propionibacteria*, *Coryneforms*, *Staphylococcus*, yeasts and molds are included in this group. *Propionibacterium freudenreichii* was found to be essential in the formation of round holes (eyes) for Swiss type cheeses through the production of CO_2 by lactate fermentation [17]. *Penicillium roqueforti* forms the veins structure in blue cheeses [18].

In general, cheese spoilage species include *Pseudomonas* spp., *Acinetobacter* spp., Enterobacteriaceae that are notorious for their proteolytic and lipolytic activities [19]. Moreover, due to the high tolerance to acidity and harsh storage conditions (low water, temperature, oxygen level) of spoilage molds, their proliferation can lead to quality defects, off-flavor, visual defects, bitter tastes that making consumers rejection, then affecting negatively dairy industry economics. Commonly identified spoilage molds are *Penicillium* (*P. comune*, *P. nalgiovense*, and *P. roqueforti*), with exception of blue-veined cheese, in which *P. roqueforti* acts as a functional specie [20].

Soft cheese made by raw milk acts as reservoir of pathogens that contaminate cheese through various routes, ranging from animal to storage conditions. *Salmonella* spp*., S. aureus, Listeria monocytogenes,* Shiga toxin-producing *E. coli (STEC)* are the main microbial hazards associated with contaminated cheese, together with spore-forming strains, such as *Clostridium* spp. [21].

2.2.5 | Fate of cheese microbiota

There are numerous factors that determine the fate of cheese microbiota. D'Amico classified these factors into two groups including intrinsic factors that related to cheese properties, such as moisture content, acidity, nutrient profile, redox potential, antimicrobials (added or naturally) and the presence of competitive microbes; whereas extrinsic factors that represent environmental conditions include packaging materials; atmosphere; temperature and time of handling and storage process [22]. Moreover, the intrinsic properties of cheese microbiota, indicating the responses to stressed conditions, can also affect its survival on cheese [21].

2.3 | Natural antimicrobials

Living organisms are equipped with various mechanisms to protect and defend themselves against the invasion of external predators and pathogens. Plants can accumulate secondary

metabolites with antimicrobial properties such as phytoanticipins and phytoalexins to protect themselves from microbial attack [23]. The immune system of animals and the secondary metabolites of microorganisms are mechanisms against the invasion of foreign substance. Recently, the active components isolated from biological systems have drawn researchers and food industries' attention with the purpose of developing natural foods preservation as they possess several advantages over traditional synthesized preservatives, such as non-toxic (many have been approved as GRAS status), biological-derived (natural), less processed and with additional health benefits [24]. Substances that have been isolated from plant, animal, bacteria, algeae, mushrooms possess antimicrobial properties are defined as natural antimicrobials [25, 26].

2.4 | Plant-origin antimicrobials and their antimicrobial mechanisms

Medicinal plants, culinary herbs and spices together are the major sources of bioactive compounds which provide antimicrobial, antioxidant and protective role to food and human health. Plants and their extracts have a long history in cheese production, with the function of flavoring compounds, coagulation substances, packaging materials. As reviewed by Dupas [27], the plant species commonly used - peppers, thyme, cloves, curcumin, garlic/onion, oregano, olive, basil, nuts-in cheese making not only providing additional aroma, also preserving raw milk cheese quality. In addition, the secondary metabolites of plant, such as essential oils (EOs), alkaloids and phenolics, are of preservation potentials as the potent antimicrobial and antioxidant effects they possess. EOs are mainly composed by terpenes, as well as terpenoids and aromatic compounds [28]. Recently, there is a growing trend of incorporating EOs into film/coating solutions to form active food packaging in order to both protect EOs from degradation and extend food shelf-life [29]. EOs exert bactericidal activity mainly through affecting multiple cellular targets such as cell membrane, peptidoglycan layer, enzymes, bacterial metabolism, ROS generation, growth and division [30]. Moreover, EOs can also cause damages or inhibit the formation of cell wall structure, cytoplasm, conidia, hyphae, and conidiophores of fungi [31-33].

2.4.1 | Plant extracts, essential oils active compounds used for cheese

Table 1 shows the main plants or essential oils, or plant by-products investigated for cheese applications during the last years. There are mainly three major categories of plant-derived compounds, including 1) EOs, such as oregano, thyme, perilla, pink pepper tree fruit, lemongrass oil, ginger (*Citrus lemon* var., *Pompia gamarda* leaf, *Pimpinella saxifrage*, moringa, *Laurus nobilis*, *Rosmarinus officinalis*); 2) plant extracts, derivatives and proteins (thyme, pomegranate peel, olive oil by-product, Roselle calyx, white tea, yerba mate, *Santolina*

chamaecyparissus L. solid by-product, curcumin solution, bitter vetch protein); 3) extracted chemical compounds are catechins, cinnamaldehyde, gallic acid, citric acid. One of the attractive compounds in *Pimpinella saxifrage* essential oil is anethole that is a terpenoid compound that possess antimicrobial and antioxidant properties. In addition, anethole is widely used in food as a sweet flavoring agent [34]. Cinnamaldehyde is a generally recognized as safe α, β-unsaturated aldehyde mainly isolated from cinnamon species, contributing to the characteristic cinammon odor [35] Moreover, cinnamon has a long history in medicine and food additives.

2.4.2 | Antimicrobial activity and mechanisms of plant extracts

Due to the reported foodborne diseases caused by contaminated cheese, the majority studies focused on the antagonistic activity of plant-derived antimicrobials on pathogens (Table 1), such as *L. monocytogenes, L. innocua, E. coli, S. aureus, S. typhimurium*. Moreover, spoilage microorganisms can cause significant economic loss, the major species include *Pseudomonas fluorescens*, Enterobacteriaceae, coliforms, fungi (*P. expansum, Aspergillus niger, P. aurantiogriseum, A. parasiticus*). Interestingly, we also found several studied that examined the potential impacts of plant extracts on cheese native or functional microflora, such as total mesophilic bacteria, lactic acid bacteria, yeasts and molds.

Among the listed studied, due to the well-documented properties, essential oils have been widely studied as preservative agents. In general, there are more than two chemicals – terpenes, terpenoids, phenylpropenes - present in EOs [30]. The hydrophobic property of these chemicals facilitates their interaction with bacterial lipids, which permeating into cell compartments and subsequently leading to cell lysis and death [36]. In addition, EOs can modify cell membrane fatty acid profile, alter ATP production, interfere metabolism, interrupt bacterial communications [30]. Pink pepper essential oil (PPEO) exerted antimicrobial ability by increasing cell membrane permeability, leading to cell lysis [37]. It is interesting to notice that catechin act as a double-edged sword on the growth of bacteria. At low concentrations, it favored the growth of bacteria, whereas higher amounts demonstrated bactericidal effects through the generation of reactive oxygen species leading to membrane damage [38]. Gallic acid is a phenolic acid that cause irreversible changes membrane properties – hydrophobicity and charges – and form pores on bacterial membrane [39]. After treating *L. monocytogenes* with *Citrus limon* var *Pompia* leaf essential oil (PLEO) gas, the SEM and TEM image revealed that PLEO gas mainly target on cell wall and membrane by coagulating membrane and cytoplasm proteins, damaging of fibrillar structure, and thinning cell wall [40]. Similar to PLEO, moringa oil can disrupt cell structures of *L. monocytogenes* and *S. aureus.*

2.4.3 | Factors affect plant-derived antimicrobial efficiency

There are several factors that can affect the antimicrobial efficacy of plant-derived antimicrobials, including chemical composition, concentration, exposure time; target microbes, such as cell wall structure and charges, single strain or mixture of strains, microorganisms species; cheese matrix properties, and packaging method.

2.4.3.1 | Chemical composition of plant antimicrobials

Regarding the structure, the amount and position of hydroxyl groups on their structure determine the antimicrobial strength as the increasing in hydroxylation results in increased toxicity [41, 42]. Regarding the species, the antimicrobial efficiency was ranked by the following order: clove oil > cinnamon oil > oregano oil [42]. On the contrary, López [43] showed vapor-phase of oregano EO demonstrated the higher antimicrobial efficacy than thyme and cinnamon EO vapors. *Laurus nobilis* EO showed stronger antimicrobial activity than *Rosmarinus officinalis* EOs; their efficiency increased through storage time [44]. The observed differences could be caused by different state of EOs tested and the extraction method. Moreover, one study evaluated and compared the antimicrobial efficiency (*E. coli*) using plant extracts, essential oils and individual active components, the results showed that essential oils had the highest antimicrobial efficacy, followed by the single active component that was more potent than plant extract. The inhibitory efficacy also depends on the concentration and exposure time [42]. It is interesting to note that catechin, when applied at low concentrations, it stimulated the proliferation of bacteria, whereas at high concentrations it exerts bactericidal effects through generating reactive oxygen species [38]. Higher concentration of thyme and oregano EOs significantly reduced the survival period of *L. monocytogenes* and *E. coli* O157:H7, with higher reduction on *E. coli* [45]. In addition, the state of plant antimicrobials can also impact its efficiency. The gaseous *Citrus limon* var *pompia* leaf essential oil and cinnaldehyde showed enhanced antimicrobial efficiency against foodborne pathogens compared to liquid state, and further thus the inactivation process does not need direct contact [43]. The enhanced activity was presumed by higher amount and volatility of the chemical compounds in vapor state EOs that are more accessible to microorganisms, and thus facilitating the attachment and actions [46-50].

2.4.3.2 | Target microorganisms

Moringa oil reduced 3 log reduction of *S. aureus*, while only 1.5 log on *L. monocytogenes* at 4°C. It exerted bactericidal activity on *S. aureus* (1 log reduction) and bacteriostatic effect on *L. monocytogenes* at 25°C [51]. On the contrary, Göksen [44] observed that 1,8 cineole rich

EOs was more active against *L. monocytogenes* than *S. aureus* at the same concentration. As reported by many researchers, due to the thick peptidoglycan layer and simple cell wall structure of Gram-positive bacteria, hydrophobic molecules such as EOs can easily across the cell wall and target on cell membrane [29]. Active film containing pink pepper essential oil (PPEO) under all tested concentrations showed inhibitory on Gram-positive pathogens, while only film containing the highest concentration of PPEO was active against Gram-negative pathogens [37]. In addition, the number and composition of target microorganisms can also determine the inhibitory efficacy. The gaseous *Citrus limon* var *pompia* leaf essential oil (PLEO) exhibited bactericidal activity on single *L. monocytogenes*, whereas only bacteriostatic activity was found on mixture strains of *L. monocytogenes* [40].

Natural microbiota and starter culture are essential in development of cheese texture and flavor. The starter culture is normally composed by lactic acid bacteria, which are also important for cheese quality and safety through their competition mechanisms [52]. Compared to pathogenic bacteria, few studied addressed the potential impacts of EOs on cheese microbiota, especially starter cultures. de Carvalho [53] compared the impacts of *Thymus vulgaris L.* essential oil (TVEO) on both pathogenic and starter culture bacteria, the results showed starter culture bacteria – *Lact. lactis* subsp. *lactis*, *Lact. lactis* subsp. *cremoris* were more susceptible than tested pathogens (*L. monocytogenes* and *S. aureus*) with the same concentration. On the contrary, Mushtaq [54] revealed that the Pomegranate peel extract (PPE)-zein film boosted the growth of cheese lactic acid bacteria. However, Fancello [40] showed that although gaseous PLEO strongly inhibited the pathogenic bacteria (*L. monocytogenes*), it did not affect the number of total mesophiles and LAB for 30 days storage. During 7 days of chilled storage, *Pimpinella saxifrage* essential oil (PSEO) coating reduced around 1.4 log of total mesophile and around 0.7 log of LAB [55]. After storage in yerba mate (YM) and white tea extracts (WPI), fresh soft rennet-curd cheese showed decreased LAB counts [56].

Edible film containing oregano essential oil (2.5%) inhibited the growth of psychrophilic bacteria and molds and yeasts for 6 days and 24 days, respectively [57]. Interestingly, one study revealed that a polyphenolic extract from olive oil by-product had protective role on Fior di latte cheese through extending lag phase and reducing maximum growth rate of *Ps. fluorescens* and Enterobacteriaceae [58]. *Laurus nobilis* essential oils had higher impacts on the growth of aerobic mesophiles than *Rosmarinus officinalis* essential oils. In addition, compared to *L. monocytogenes* or *S. aureus,* total mesophiles were more susceptible to EOs-loaded film [44].

Similar to the mechanisms on bacterial cells, EOs mainly terpenes or terpenoids act on fungal cell membrane, interfering sporulation and germination, disrupting metabolism, and causing death [60]. On cheese applications, cinnamaldehyde-gliadin film delayed the growth of fungal (*P. expansum A. niger*) for 30 days compared to only 15 days of control sample [61].

Active film containing perilla oil successfully extended cheese shelf-life by delaying the growth of cheese microorganisms – total bacteria and fungi – and preventing moisture loss [62]. Nano-encapsulated oregano oil demonstrated antifungal activity in limiting the growth of *Cladosporium* sp. *Fusarium* sp. *Penicillium* sp on Minas Padrão cheese [63] On soft cheese, *Thymus* oil showed efficiency on *P. aurantiogriseum, A. parasiticus* [64-65]. The appearance of molds and yeasts on roselle calyx (RE) extract-coated cheese after 3 months of storage, whereas only one month shelf-life of control cheese. Moreover, there was no significant differences in *Streptococcus*, *Lactobacillus* and total bacterial counts [66].

2.4.3.3 | Cheese matrix properties

Moreover, cheese properties, such as moisture level, may facilitate or prevent the diffusion of compounds and thus affecting the preservation efficiency of antimicrobials. Studies have compared the plant antimicrobials effect both on liquid cheese suspension and solid cheese model, and they found that the stronger inhibition effects were observed on liquid cheese broth model. This effect was presumed by the low moisture in solid cheese that inhibit the dispersion of active compounds [53, 67].

2.4.4 | Delivery methods for plant preservatives

The methods involved for incorporating plant-derived antimicrobials can also affect its efficiency. As we mentioned above, the main inhibitory mechanisms are through permeating cell membrane, therefore, methods that facilitate the accessibility of antimicrobials to bacteria are more efficient in controlling the microbial quality of cheese. Depend on the type of cheese and state of plant derivates, gaseous essential oils can consider as active atmosphere for cheese preservation. To prevent moisture loss, gaseous EO and cheese model were further sealed using parafilm [45, 63]. Moreover, liquid form of plant-derived ingredients can be added directly in governing solution and coated on cheese as active coatings/films. For high moisture cheese ("Fior di latte") that normally stored in governing solution, the active compounds can be added into the governing liquid that facilitate the dispersion and interactions between antimicrobials and cheese microbes. However, this method is often limited due to its possible impacts on cheese flavor and its high volatility that result in reduced efficiency [52].

Active coatings and films imply the incorporation of active compounds in polymer materials to form film or to coat on cheese surface, aiming to allow direct contact between compounds-cheese and also maintain active compounds efficiency during storage. In the current review, gliadin, zein, mung bean starch, water chestnut starch, cellulose acetate, mandarin fiber, soy protein, sodium alginate, gelatin, furcellaran and whey protein, and chitosan are used as carrier matrix to develop active coatings/films. They possess good

biocompatible and biodegradable (gliadin, zein, cellulose acetate), antimicrobial properties (chitosan), nutritional properties (mandarin fiber, mung bean starch and water chestnut starch). Zein is a group of alcohol-soluble prolamine storage proteins in maize with GRAS status [68-69] Zein molecules can auto-assembly to form a meshwork structure during film forming process and thus providing hydrophobicity property and good water and gas permeabilities to zein film. However, the mechanical and barrier properties of zein film can be tailored by casting method, solvent, drying temperature and relative humidity [70]. Furthermore, zein film and gliadin-based films can be used as a delivery matrix for various active compounds to obtain a controlled release and thus a prolonged effect of active substances. The addition of wax can modify the release rate of compound releasing [71-73]. Polymers with antimicrobial property has been considered as proper matrix to enhance active packaging efficiency, chitosan-based gallic acid film exhibited sustained protection by enhancing the mechanical, antioxidant and anti-*E. coli* activities [74]. Chitosan-*Sonneratia caseolaris (L.) Engl.* leaf extract active film showed remarkably efficiency in limiting the growth of spoilage bacteria *Ps. aeruginosa* [75]. Moreover, edible films made by mandarin fiber, mung bean starch and water chestnut starch, can also function as prebiotics by boosting probiotic bacteria and bringing additional health effects to human [57, 76-77].

Recently, applications of polymer nanotechnologies enhanced the stability and efficiency of active food packaging. Due to the large surface-volume ratio, these nanosized particles showed enhanced physical, chemical and biological properties as well as reduced toxicity [78]. For cheese applications, nanoemulsion-edible coating composed by oregano essential oils, mandarin fiber and sodium alginate have successfully extended low-fat cut cheese [57]. Nanofibers possess a large interaction surface structure that create a greater interaction surface and control the release of active substance. Electrospinning is considered as a propriate method to load antimicrobial substances on nanofibers [79]. Electrospun nanofibers containing gelatin and chitosan nanoparticles loaded with moringa oil showed efficiency in eliminating pathogens from cheese surface [51]. The chitosan/polyethylene oxide/cinnamaldehyde (5%) nanofiber mats quickly released cinnamaldehyde in both vapor and liquid state, which inactivated *P. aeruginosa* and *E. coli* in a short period [48]. As previously said, the high volatility and instability of EO limit its efficiency for longer storage, encapsulation techniques are considered as methods to protect and maintain EO activities [80]. Liposomes are good carrier for both polar and nonpolar compounds to facilitate compounds distribution into target system and maintaining their bioactivity [81-82]. As reported by Ortan [83], liposomes loaded with EOs can be stable for more than half-year at 4 ± 1°C. In addition, the release of active compounds can be triggered by defined signal, the release of lemongrass oil from liposome was controlled by the presence of listerolysin O [67].

2.4.5 | Hurdle technologies

Hurdle technologies are often used in combination with natural antimicrobials because generally natural antimicrobials cannot guarantee complete absence of pathogens. Therefore, non-thermal hurdle techniques, such as high-pressure processing (HPP), X-ray irradiation, Pulsed light (PL), light-emitting diode 460-470nm (LED 460-470nm) treatment, were normally applied to weaken the barrier properties of microorganisms and facilitated the actions of active compounds in order to obtain synergist efficiency. High pressure processing is considered ideal treatment on certain foods as inactive cheese enzymes and microflora without affecting nutritional and organoleptic parameters [84]. In addition, combined treatments of high-pressure processing (HPP) and thyme extracts showed accelerated and enhanced reduction of *L. monocytogenes* on cheese than thyme extract treatment alone [98]. However, researchers found that higher pressure and extended exposure time were associated with compromised cheese texture and reduced antimicrobial efficiency [85-87]. X-ray irradiation is another high efficiency non-thermal strategy used to inactive microbes through ROS generation and membrane disruption in various foods such as vegetables, ham, seafood and meat [88]. Park and his team (2020) verified the antimicrobial efficiency of the X-ray irradiation in combination with curcumin on *L. monocytogenes* and *S. aureus* through PBS and cheese models. They found the combined techniques showed synergistic inhibitory activity to *L. monocytogenes*, whereas the combined techniques exerted similar effects on *S. aureus* compared to the X-ray irradiation treatment alone [89]. Pulsed light (PL) is a technology that applies short bursts of intense light to inactive microorganism in liquid and solid substances. PL was able to inhibit the following bacteria: *L. innocua, L. monocytogenes, Ps. fluorescens, E. coli* through UV-transparent packaging films [90-91]. After treating with PL and citric acid-starch film, sliced cheddar cheese showed altered pH and moisture level, but the authors suggested that the altered parameters were caused by addition of citric acid [92]. Furthermore, a recent study showed that the light-emitting diode 460-470nm (LED 460-470nm) treatment significantly reduced the amount of *L. monocytogenes* and *Ps. fluorescens* on sliced cheese surface stored in polypropylene, moreover, the treatment did not alter cheese color. As observed by the same authors, the microbial reduction effect was enhanced at refrigerated temperature (4°C) than at room temperature (25°C). The TEM images of bacteria revealed that the LED460-470nm mainly damaged cell membrane, intracellular components (RNA, proteins), leading to cell rupture and death [93].

2.4.6 | Advantages and limitations

The use of plant-derived compounds is of several advantages, such as their “GRAS” status, potent antimicrobial and antioxidant capacities. Essential oil extracted from pink pepper tree

fruit (*Schinus terebinthifolius* Raddi) demonstrated the protective role in limiting the lipid peroxidation process that is essential for the sensory quality of cheese [94]. The reuse of bioactive compounds extracted from agro-wastes have attracted many attentions as they are rich in fiber contents, phenolic compounds, flavoring substances, organic acids, proteins and enzymes [95]. Therefore, the recycle of low-cost food waste materials is of great benefits in food, pharmaceutical and chemical industrials and environmental protection. However, the strong color, flavor and tendency to form complexed with food components limit the application of plant derivatives. The coating film of chitosan/guar gum/zinc oxide bio-nanocomposites with incorporation of Roselle's calyx extract revealed red and non-transparent color, which limited its use on cheese [66]. The wrapping films of bitter vetch protein showed dark brown color, which hindered the direct observation/cover cheese original color, confusing consumers' perceptions. Furthermore, Nabulsi cheese stored in chitosan/bitter vetch protein films showed increased hardness and chewiness compared to cheese stored in PE film, demonstrating the barrier properties of active film need to be improved [96]. The yerba mate incorporated film showed a darker color (yellowish and greenish) due to the presence of natural pigments found in *Ilex paraguariensis* leaves [56].

2.7 | CONCLUSIONS

The increased concerns of less processed food and health demand the abandon of synthetic preservatives. To date, research focused on exploration of natural preservatives are still increasing. The present chapter shed light on the applications of plant-derived antimicrobials; their chemical compositions, delivery methods, their potential interactions and impacts on cheese. However, the direct application is limited due to the interaction with cheese components and possible sensorial variations. The development of nanotechnology and encapsulation techniques can help to solve these problems, maintaining its bioactivities during storage, minimizing cheese-antimicrobials impacts, and extending cheese shelf-life. Cheese properties such as moisture level, pH value also can affect the effectiveness of natural antimicrobials. Therefore, the selection of the right cheese model is important to obtain desired protection throughout storage. Future research is needed to take into consideration the selection of active compound, proper cheese model to verify their efficiency *in situ*, effects on both pathogenic and spoilage microorganisms, their preservation efficiency at different storage conditions. In addition, the variation of organoleptic attributes is equally important to verify in short and longer period as these quality changes may strongly affect consumers perceptions.

Table 1. Plant-derived antimicrobials for cheese preservation

Natural ingredients	Major chemical compounds	Matrix/ In combination with	Packaging technique	Cheese model	Target microorganisms (*in situ*)	Main results (*in situ*)	Sensory evaluation	Ref.
Oregano (*Oreganum vulgare* subsp. *hirtum*)and thyme (*Thymus vulgaris* L) essential oil	Carvacrol p-cymene, thymol, γ-terpinene	Modified atmosphere packaging (50%CO_2+50%N_2)	Spray	Feta cheese	*L. monocytogenes* *E. coli* O157:H7	0.1 ml/100g thyme or 0.1 ml/100g oregano EO limited the survival of *L. monocytogenes* and *E. coli* O157:H7 for 22 and 18 days, respectively. 0.2ml/100g oregano EO reduced the survival of *L. monocytogenes* to 14 days and *E. coli* for 16 days.	The current doses of EOs were acceptable by panelists	[45]
Cinnamaldehyde (α,β-unsaturated aldehyde)	NO	Gliadin film	Wrap by film	Cheese spread	*P. expansum* *A. niger*	Cinnamaldehyde-gliadin film inhibited the fungal growth on cheese spread for 30 days at 4°C., control sample (without cinnamaldehyde) sample showed fungal growth after 16 days.	NO	[61]
Catechin and gallic acid	NO	Zein/zein-wax composite films	active film + commercial stretch plastic film + aluminum foil	Fresh Kashar cheese	*L. monocytogenes* ATCC 7644	Active films had no antimicrobial effect on inoculated cheese (probably phenolic compounds interacted with cheese proteins). However, their antioxidant properties prevented cheese from lipid oxidation.	NO	[72]
Perilla oil (P)	Thymoquinone, 2-hexanoylfuran, trans-Bergamotene,	Mung bean starch-chitosan films (MSC)	Coated by brushing	Mongolia n cheese	Total bacteria fungi	Water chestnut starch chitosan-perilla oil film delayed the bacterial and fungal growth compared uncoated samples, reduced water loss and extended cheese shelf-life.	NO	[62]

	1,3,6-Tetramethyl-3-vinyl-8-hexahydro-1H-isochromene	Water chestnut starch chitosan film(WSC)						
Origanum vulgare essential oil (OREO) and Ethyl Lauroyl Arginate HCl (LAE)	NO	polypropylene (PP) polyethylene terephthalate (PET)	Coating	Zamorano (raw sheep milk cheese)	Two *E. coli* O157:H7 strains	PET films coated with LAE concentrations greater than 6% were effective in reducing *E. coli* counts on cheese.	LAE-PP/PET, OREO-PET showed no effect on the cheese sensorial characteristics	[97]
Thymus vulgaris L. essential oil (TVEO)	Thymol, *p*-cymene, γ-terpinene, linalool, carvacrol	At 10°C	TVEO +bacterial suspension inoculate on cheese	Coalho cheese	Pathogenic strains: *L.monocytogenes*, *S. aureus* Starter co-culture: *Lact. lactis* subsp. *lactis* *Lact. lactis* subsp. *cremoris*	1.25 mL/g TVEO showed no effect on the growth of tested strains on semi-solid cheese. At 2.5 mL/g, TVEO slightly decrease the counts of *S. aureus*, *L. monocytogenes*, and *Lactococcus* spp. However, under the same concentration of TVEO, the starter culture bacteria were more sensitive than pathogenic bacteria.	NO	[53]
Pink pepper tree fruit essential oil (*Schinus terebinthifolius* Raddi) Mature (MEO)	β-myrcene, β-cubebene, limonene, α-pinene	NO	Pre-inoculated cheese dipped in MEO, further sealed by PVC film	Fresh Minas cheese	*L.monocytogenes*	2% MEO treatment reduced the growth of *L. monocytogenes* (1.5 log CFU/g increment), compared to 2.6 log CFU/g increased in the control sample. While 1.4% MEO and 0.7% MEO showed less impacts than 2% MEO, but still possess anti-Listeria activity.	Undesirable effects on cheese due to direct application	[94]

Thyme natural extracts(NAs)	Carvacrol, thymol, γ-terpinene, *p*-cymene	High pressure processing (HPP) (200-300 MPa)	Coculture with *L. monocytogenes*	"Béja Sicilian cheese" fresh cheese	*L. monocytogenes*	NAs + HPP showed an accelerated and enhanced inactivation of *L. monocytogenes*, with 1.68log CFU/g more than NAs alone.	Acceptable sensorial threshold of thyme extract in cheese was 0.2% (v/w), lower than MIC	[98]
Lemongrass oil (*Cymbopogon citratus*)	Linalyl acetate /geranial, neral, limonene	Listerolysin O-triggered release	Spray with LO liposomes, packed in sterile bag	Kerrygold cheddar cheese	*L. monocytogenes* ATCC 19115	Liposomes containing lemongrass oil (40%) reduced the growth of *L. monocytogenes* in cheese over the storage at 4°C for 15 days.	No impact on sample surface color, texture and sensorial parameters	[67]
Pink pepper EO (*Schinus terebinthifolius* Raddi)	β-myrcene, β-cubebene, limonene, α-pinene	Cellulose acetate	Innoculated cheese placed on active film	Sliced mozzarella cheese	*S. aureus, L.monocytogenes, E. coli, S. typhimurium*	The antimicrobial efficiency of active films (2%, 4%, 6%) showed a concentration-dependent way. While for gram negative bacteria (*E. coli* and *S. typhimurium*), only CA film with 6% (highest) showed a reduction in their cell counts after 12 days storage.	NO	[99]
Oregano essential oil (OEO)	NO	Nanoemulsion (sodium alginate, mandarin fiber, Tween 80, OEO)	Edible film	Low-fat cut cheese	Psychrophilic bacteria Molds and yeasts	2.5% (w/w) OEO inhibited cheese psychrophilic bacteria growth for 6 days, molds and yeasts were inhibited for 24 days storage.	The appearance of cheese pieces was preserved. Mandarin fiber brought additional nutritional	[57]

							properties for cheese	
Ginger essential oil	α-zingiberene, geranial, (Z)-citral, β-cedrene, geranyl acetate, (1R)-α-pinene, α-curcumene, α-farnesene	Protein ultrafine fibers (soy protein isolate + polyethylene oxide + Zein 1:1:1 v/v/v)	Micro-atmosphere volatilization	Fresh Minas cheese	*L. monocytogenes*	Significant reduction of *L. monocytogenes* was observed on 3rd and 9th day of storage.	NO	[100]
Oregano essential oil	Carvacrol p-cymene, thymol, γ-terpinene	Modified atmosphere packaging (50%CO_2+50%N_2)	Nanoemulsions encapsulated on cheese surface	Minas Padrão cheese slices	*Cladosporium* sp. *Fusarium* sp. *Penicillium* sp. (isolated from cheese)	Nanoencapsulated oregano EO inhibited the selected fungi. The inhibition effect can be improved by controlling temperature and water activity.	NO	[63]
Thymus algeriensis oil	Carvacrol, p-cymene, γ-terpinene, β-caryophyllene	Emulsion	EO emulsion was incubated with conidial suspension in cheese wells	Soft cheese	*P. aurantiogriseum*	Antifungal activity depend on EO concentration, the contamination was completely eliminated by using the highest concentration (25 μL/well) of EO added.	Cheese texture and color were preserved by EO spraying	[64]
Pomegranate peel extract (PPE)	NO	Zein film	Film	fresh Himalayan cheese (Kakari)	Spoilage microorganisms, lactic acid bacteria, yeasts and molds	The 75mg/ml of PPE incorporated in zein films showed the highest reduction of total bacterial counts, completely inhibited yeast and mold growth while promoted the growth of lactic acid bacteria.	75 mg PPE film packed cheese showed high scores in overall acceptability, appearance, aroma, flavor.	[54]

Monolaurin(ML)	NO	Cellulose-chitosan films (CC)	Further stored in a sterile polyethylene bag	ultrafiltered cheese	*L. monocytogenes* ATCC 19115	The addition of 0.5% and 1% ML into CC films reduced 2.3-2.4 log *L. monocytogenes* counts on cheese after 14 days.	NO	[101]
Citrus lemon var *Pompia Gamarda* leaf essential oil (PLEO)	Limonene, linalool, neral, linalyl acetate/geraniol, geranial	Refrigeration temperature (5 °C)	EO vapor treatment and sealed by parafilm	Ricotta salata cheese	*L. monocytogenes* 20600 DSMZ / the mix of the three strains of *L. monocytogenes* Natural cheese flora: total mesophilic bacteria, LAB)	Gaseous PLEO showed a bactericidal effect on *L. monocytogenes* 20600 DSMZ and a bacteriostatic effect on the mixture of *L. monocytogenes*. A synergic anti-listerial effect was found between refrigeration temperature and PLEO No effect on the counts of total mesophiles and LAB of cheese at 30 days storage.	NO	[40]
A polyphenolic extract from olive oil by-product	Hydroxytyrosol, tyrosol, secoiridoids derivatives such as 3, 4-DHPEA-EDA	Added into cheese governing liquid	Cheese stored in governing liquid containing polyphenolic extract	"Fior di latte" cheese	Cheese spoilage bacteria: *Ps. fluorescens* Enterobacteriaceae	The addition of polyphenols (500 μg/mL) extended lag phase and reduced μ_{max} of *P. fluorecsens* growth. For the growth of Enterobacteriaceae, polyphenols (250 μg/mL and 500 μg/mL) added into governing liquid extended their lag phase without altering μ_{max}. 250 μg/mL polyphenols extended cheese shelf life by 2 days and 4 days for 500 μg/mL of polyphenols.	250 μg/mL polyphenols addition had no negative impact on cheese sensorial characteristics.	[58]
Pimpinella saxifrage essential oil (PSEO)	Anethole, pseudoisoeugeno, p-anisaldehyde	Sodium alginate (SA)	Coating	"Béja Sicilian cheese"	Total mesophilic bacteria, LAB	3% PSEO in SA coating reduced the counts of cheese mesophilic bacteria from 5.44 to 4.03 log CFU/g at 7 days of chilled storage, also reduced the counts of lactic acid	PSEO addition was more appreciated by panelists as	[55]

				fresh cheese		bacteria from 4.07 to 3.33 log CFU/g at day 7.	anethole is normally used as a sweetener	
Thymol	NO	Loaded on nanofiber mats (polyvinyl alcohl PVA/)	Coating	Kashar cheese	*A.parasiticus*	TLNs coating on cheese prevented the growth of *A. parasiticus* by visual inspections during 7th day storage.	NO	[65]
Moringa oil	Palmitic acid, phytol, ethyl palmitate, hexadecanal, heptacosane	Loaded on chitosan nanoparticles (MO@CNPs) MO@CNPs embedded gelatin nanofibers	Coating	(Kerrygol d cheddar	*L. monocytogenes ATCC 19115* *S. aureus ATCC 25923*	Under 4°C, the coating of MO@CNPs nanofibers reduced the counts of *L. monocytogenes* and *S. aureus* after 10 days for 1.5 log CFU/g and 3 log CFU/g, respectively. Under 25°C, MO@CNPs stopt the growth of *L. monocytogenes*, whereas the active coating decreased 1 log CFU/g of inoculated *S. aureus*.	Minor effects cheese surface color, no effect on cheese sensory attributes at 25°C and 4°C for 4 days.	[51]
Santolina chamaecyparissus L. solid by-product (SISRE)	1.8-cineole, 8-methylene-3-oxatricyclo[5.2.0.0(2.4)]nonane, ledol, santolina triene, β-pinene+myrcene	Chitosan solution	Edible coating	*'Manchego'* cheese	Fungi	The active coating showed enhanced antioxidant, antifungal and physical properties. SISRE coated cheese showed lowest amount of fungal colonies compared to control and uncoated cheese.	NO	[102]
Yerba mate (YM) and white tea extracts(WPI)	**YM:** Chlorogenic acid, caffeine,	Furcellaran/ Whey protein films (FUR/WPI)	Package	Fresh soft rennet-	Total bacteria, yeast and mold, total coliforms,	Total bacterial counts in edible film packed cheese decreased, the majority of TBC are lactic acid bacteria.	Edible film coated sample improved	[56]

	theobromine, caffeic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid (Bastos, 2007. Medina Jaramillo, 2016) **WPI:** epigallocatechingall-ate			curd cheese		FUR/WPI+WT film maintained the level of yeast and mold. No coliform bacteria was detected after 1 week in FUR/WPI+YM and FUR/WPI+WT.	cheese organoleptic properties	
Chitosan(CH)-/bitter vetch protein(BVPC)-based film		NO	Wrapping film	Nabulsi cheese	Total aerobic mesophiles	Chitosan films was the most effective wrapping material in hindering microorganism growth in unsalted cheese. CH and BVPC-based wrappings were able to counteract unsalted cheese spoilage more efficient than PE wrapping.	Unsalted cheese stored in CH and BVPC films showed increased hardness, chewiness, and water loss	[96]
Laurus nobilis and *Rosmarinus officinalis* EOs	*Laurus nobilis:* 1, 8-cineole, (+)-limonene, 1-terpinen-4-ol, β-pinene, α-pinene. *Rosmarinus officinalis:* 1,8-cineole, (−)-camphor, α-pinene,	Zein matrix	Edible electrospun coating and in sterile polyethylene zip bags	Fresh semi-hard cheese	*L.monocytogenes*, *S. aureus*, total mesophilic bacteria	Both EOs showed stronger activity on Gram-positive than Gram-egative bacteria. Active films reduced the counts of all tested strains, including total mesophilic bacteria. EO of *Laurus nobilis* showed higher antibacterial activity than *Rosmarinus officinalis*. Electrospinning technique extended its antimicrobial efficiency.	NO	[44]

	beta-phellandrene, 1,4-cyclohexadiene .							
Roselle calyx (RE) extract		chitosan/guar gum/zinc oxide bionanocomposites based on Roselle calyx extract (CS/ GG/RE-ZnO)	coating	Ras cheese (hard)	Cheese native microflora: total bacteria, psychotropic bacteria, Streptococci, Lactobacilli, yeasts and molds, coliforms	No impact on cheese microbiota. The decrement in bacterial counts within sample was ascribed to the moisture losses and proteolysis during ripening. Control sample showed the appearance of molds and yeasts in the first month. While for coated samples, molds and yeasts appeared only at the end of third month.	CS/ GG/RE-ZnO coated cheese exhibited higher scores in sensorial properties.	[66]
Curcumin solution		X-ray	Curcumin-treatment followed by X-ray of different doses	Cheddar cheese (slice)	*L. monocytogenes*	The combination treatment (0.4 kGy) with curcumin (0.5 mg/L) showed the highest reduction of *L. monocytogenes* (3.65 log CFU/g) compared to single treatment.	No impact	[89]
Antimicrobial starch films (ASF) (sodium benzoate SB, citric acid CA, both CASB)		Pulsed light	Inoculated cheese covered by films and treated by PL	Cheddar cheese	*L. innocua*	ASF containing CA with PL treatment showed 4.5 log cell reduction after 3 days storage indicating an additive effect between CA and PL. SB added films did not show antimicrobial activity.	Significant changes in physicochemical properties (pH, moisture level, mechanical)	[92]

2.8 | REFERENCES

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CHAPTER III

SHELF-LIFE EVALUATION OF RICOTTA CHEESE STORED IN FOUR TYPES OF COMMERCIAL POLYETHYLENE PACKAGES

3.1 | ABSTRACT

Ricotta cheese is a white fresh cheese that has a quite short shelf-life about one week under fridge condition. The aim of the present study was to characterize the impacts of four type of in-use European cheese packages (Italy, Spain, Slovenia and Romania) on fresh ricotta cheese shelf-life parameters at 4 °C. Italian (IT) and Spain (SP) packages are made of low-density polyethylene, whereas the packages used in Romania (RO) and Slovenia (SLO) were composed by both polyethylene and polyamide. Microbiological and sensorial attributes were monitored as well as the pH values. Moreover, the level of cheese volatile compounds-free fatty acids that represent the metabolic activities of cheese microbiota was examined by gas chromatography/mass spectroscopy (GC/MS). Results showed that different packages had different impacts on cheese shelf-life parameters. The microbial analysis revealed that the SP package was able to inhibit the proliferation of cheese microbes with lowest increment in analyzed bacterial groups, while IT demonstrated the highest increment in microbial counts. The amount of free fatty acids reached the peak after 3 days in IT and SP packages and 6 days in SLO and RO packages. The sensorial quality of cheese remained acceptable by panellists for 7 days in IT, RO and SP and 6 days for SLO sample. Overall, according to the safety microbial level guidance and sensorial acceptable limit, packages of RO, IT, SP showed better preservation efficiency on cheese than SLO package.

3.2 | INTRODUCTION

Ricotta cheese is a type of soft white whey cheese with high moisture, low fat/protein ratio. The manufacture of cheese involves heat and acid-coagulation followed by precipitation. The commonly used milk sources to produce ricotta cheese include sheep, goat, cow or mixture of them. Due to the soft and high moisture nature, the shelf-life of ricotta cheese is relatively short, around 2-7 days at 4°C. However, intrinsic factors related to milk microbiota and nutrient composition to extrinsic factors, such as processing techniques, transportation and storage conditions can affect considerably the shelf-life and safety of ricotta cheese.

Ricotta cheese create ideal environment for the growth of microorganisms due to its high water, neutral pH [2]. In general, the fresh ricotta cheese is highly perishable and susceptible to microbial attack, the excessive proliferation of contaminants can negatively impact cheese organoleptic and nutrition qualities leading to economic loss, even poses health risks to consumers. To extend cheese shelf-life, commonly used methods are packaging with modified atmosphere; processing with high pressure, pulsed light, x-ray irradiation; adding preservatives [3, 4]. Food packaging is material that aim to maintain food quality and safety, transport and protect food from environmental contaminations originate from physical, chemical and microbial sources. In addition, it also provides basic information of the food product to consumers. Commercial ricotta cheese is normally packed inside plastic trays with perforated holders that maintain the shape of cheese. In general, the modified atmosphere used to pack ricotta cheese is composed by CO_2 and N_2 with 30% and 70%, respectively [5]. Recently, various bioactive substances, for example, protective bacterial culture [5], plant oils [6], chitosan-based coatings [7], have been investigated to enhance ricotta cheese safety and quality.

The aim of the present study is to evaluate the variation of ricotta cheese shelf-life parameters – microbial counts, pH values, organoleptic characteristics, volatile compounds levels - during the cold storage inside four European cheese packages at 4°C.

3.3 | MATERIALS AND METHODS

3.3.1 | Packaging types

Of the currently in-use packaging in Italy (IT), Spain (SP), Slovenia (SLO) and Romania (RO), one from each country was randomly selected for the investigations in the present study. Two low-density polyethylene packages are from IT and SP, another two packages from SLO and RO are composed by polyethylene and polyamide. All the packages are transparent, IT and SP demonstrate blue color while the other two with white color. Label only presents on IT packaging bag (Figure 1).

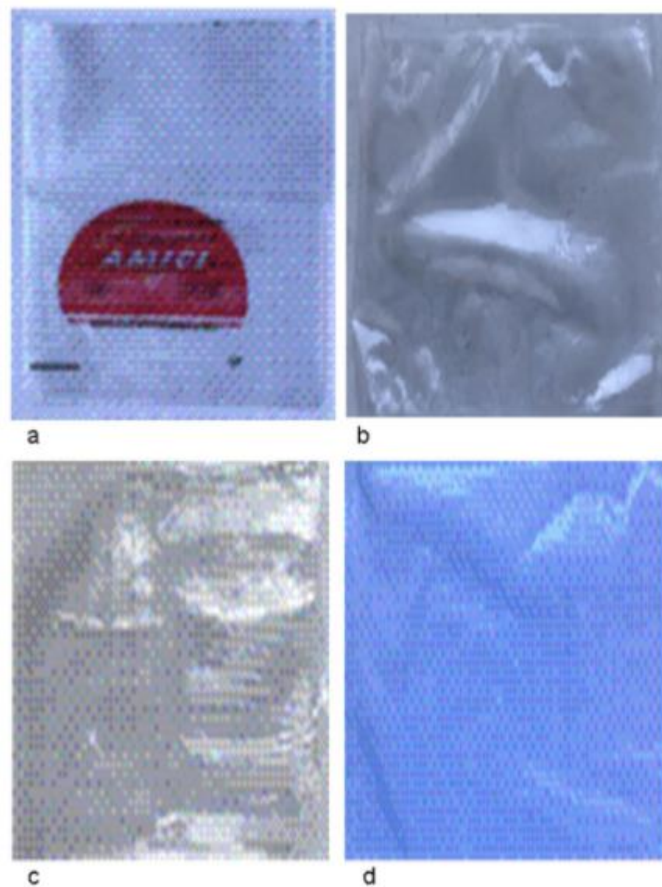


Figure 1. Type of packages: a-Italy (IT), b-Romania (RO), c-Slovenia (SLO), d-Spain (SP).

3.3.2 | Shelf-life assessment of ricotta cheese stored in plastic packages

3.3.2.1 | Samples preparation

Fresh ricotta cheese was chosen as a model cheese to study the shelf-life. The dairy products, made mainly from cow milk, were collected from a local artisanal dairy factory (Caseificio Artigianale AMICI, Camerino, IT). The batch was produced and immediately transferred into the laboratory for analyzing within the same day. Cheese samples, around 200g of cheese, were randomly allocated to four types of plastic packaging bags (IT, SLO, RO, SP) and air-packed using iron rope to mimic home storage conditions. After packaging and labelling, all samples were stored in the refrigerator at 4°C with relative humidity of 55% in dark condition. At each sampling time, samples were taken from refrigerator and examined for for cheese microorganisms counts, pH value, volatile compounds level, as well as sensorial characteristics.

3.3.2.2 | Microbiological analysis

To determine the cheese microbial profile, the counts of the following microorganisms was monitored: aerobic mesophiles, lactic acid bacteria, *Pseudomonas* spp., staphylococci, yeasts and molds, Enterobacteriaceae from the day of production (0) and after 3, 6, 9, 14 of cold storage. For the microbiological procedures we followed the official technical protocols for microbiological tests in food [8].

Briefly, 10 g of cheese sample was sterilely weighted and taken from each packaging bag (IT, SP, SLO, RO), followed by transferring into a sterile stomacher bag (Whirl-Pak®, Seward, UK) and 90 ml of sterile 0.9% saline solution (NaCl) (Sigma-Aldrich, Co., St. Louis, USA) was added into sample bags and homogenized for 2 minutes. Then, ten-fold serial dilutions were prepared using the saline solution, and 0.1 ml of the corresponding dilutions were spread onto selective agar media. The aerobic mesophilic bacteria total count was performed on Plate Count Agar (PCA - Oxoid, Basinstoke, UK) under aerobic conditions at 30 °C for 72h, following the ISO 4833 guidelines. For the enumeration of mesophilic lactic acid bacteria (LAB), de Man, Rogosa, Sharpe agar (MRS Agar at pH 5.7, - VWR, Leuven, Belgium) was used, in accordance with the ISO 15214:1998 guidelines. For the detection and enumeration of Enterobacteriaceae, violet red bile glucose agar (VRBGA - VWR) was inoculated with sample and incubated at 37°C for 24h, following the ISO 21528-2 guidelines. The enumeration of presumptive *Pseudomonas* spp. was carried out by aerobically inoculating sample on Pseudomonas Selective Agar (CFC, Liofilchem s.r.l., Roseto degli Abruzzi, Italy) at 25°C for 44h ± 4h, following the ISO 13720: 2010 (E) guidelines. The presence of staphylococci was checked through the aerobic inoculation on Baird-Parker agar medium (VWR) at 35-37ºC after 24 to 48 hours of incubation, following the guidelines of ISO 6888-1:1999 (E). For enumerating yeasts and molds, Sabouraud agar (SAB - Oxoid) was inoculated with the tested samples and incubated at 25°C for 5 days (ISO 6611:2012). After incubation, the number of colonies were counted and expressed as $\log_{10}$ CFU/g of ricotta cheese for each studied bacterial group.

3.3.2.3 | pH measurement

The cheese pH values were measured on day 0 and after 3, 6, 9, 14 days of storage. At the sampling day, 20 g of ricotta cheese samples from the IT, SP, RO, SLO packaging were measured in triplicate using an electronic pH meter (Mettler Toledo, Columbus, UK) equipped with a probe for food through direct penetration in cheese.

3.3.2.4 | Sensorial analysis

A carefully conducted sensory evaluation can represent consumers opinion about a food product. Sensorial analysis was performed by 10 trained panelists from the food laboratory of the University of Camerino. All the panelists were trained to familiarize with attributes and grading before conducting the sensory test, regarding ricotta cheese color, odor, taste, texture and overall acceptability. The detailed information of each major attributes is listed in Table 1. To evaluate each attribute, a modified hedonic scale from 0 to 5 was used (where 0 = unacceptable, 1= very bad, 2 = poor, 3 = fair, 4 = good, 5 = excellent) [4, 9,10].

Table 1. Sensorial attributes and hedonic scale of ricotta cheese.

Attributes		**Hedonic scale: 0-5**
Color	white	
	pale	
	beige	
Odour	butter	
	fresh curd	
	fresh milk	
	yogurt	
	goat	
	smoked	
Taste	sweet	
	salted	
	bitter	
	acid	
Texture	gritty	
	granular	
	soft	
	creamy	
	greasy	
	adhesive	
	spreadability	
Overall acceptability		

3.3.2.5 | Free fatty acid analysis

Five saturated free fatty acids FFA, namely butanoic acid (butyric acid C4:0), isovaleric acid (3-methylbutanoic acid), hexanoic acid (caproic acid C6:0), octanoic acid (caprylic acid C8:0) and decanoic acid (capric acid C10:0) and one aldehyde (hexanal) selected as chemical markers for the quality of Ricotta cheese were monitored throughout storage. Chemical reagents 2-Methylpentanal (CAS 123-15-9, MW 100.16 g mol^{-1}), hexanal (CAS 66-25-1, MW 100.16 g/mol), butanoic acid (CAS 107-92-6, MW 88.11 g mol^{-1}), isovaleric acid (CAS 503-74-2, MW 102.13 g mol^{-1}), hexanoic acid (CAS 142-62-1, MW 116.16 g/mol), decanoic acid (CAS 334-48-5, MW 172.26 g mol^{-1}) and monobasic sodium phosphate (CAS 10049-25-5, MW 137.99 g mol^{-1}) were supplied by Sigma–Aldrich (Milan, Italy). Octanoic acid (MW 144.2 g/mol) was purchased from Fluka (Germany).

The extraction of the chemical compounds was performed by HS-SPME (headspace - solid phase micro extraction) method. Briefly, 0.5g of ricotta cheese were placed in a 10 ml vial, then 0.75 g of monobasic sodium phosphate, 1.8 ml of deionized water and 200 µl of an aqueous solution of internal standard (containing 10 mg/l of 2-methylpentanal) were added. The vial was then sealed with a Teflon-lined septum and screw cap and immersed in a water bath at 60°C. After 30 min of equilibrium a Carboxen/PDMS fiber (Supelco, Bellofonte, PA, USA) with a film diameter of 75µm was exposed to the sample headspace under continuous slow agitation with a magnetic stirrer to promote the transfer of the compounds from the sample to the headspace. After 20 min of extraction, the fiber was removed and directly inserted into the GC injection port for analysis. A desorption time of 5 min was enough to recover most of the volatiles, which were then transferred to the analytical column. After each analysis the fibers were kept in the injector 15 min more to prevent contamination.

GC-MS analysis conducted on Agilent 6890N gas chromatograph equipped with an Agilent 5973N mass selective detector (GC-MSD) (Santa Clara, CA, USA) was employed for fatty acids quantification. The separation of the volatiles compounds was carried out using a DB – Wax column (60 m, 0.25 mm i.d., 0.25 µm film thickness) (J&W Scientific, Folsom, CA, USA). The flow rate (He) was 1 ml/min under splitless mode. The injector temperature was 260°C. The oven temperature was kept at 50°C for 3 min, then raised to 150°C at a rate of 5°C/min and to 250°C at a rate of 10°C/min and held there for 7 min. Data were acquired in the electron impact (EI) mode with an ionization voltage of 70 eV. Single ion monitoring (SIM) was used as data acquisition mode. The analytes were initially identified by matching their retention times and mass spectra with their respective standards. Whereupon specific ions were selected for each compound and monitored in specific time windows. The concentrations of the analytes were determined as relative area (R.A = analyte peak area/ internal standard peak area)

using respective calibration curves. The results are expressed as mean (mg/Kg acid and μg/Kg hexanal) ± standard deviation.

3.3.2.6 | Statistical analysis

All parameters were measured in triplicate, and data are expressed as mean ± standard deviation. The statistical significance of the differences obtained between packaging was evaluated using the Student's *t* test. Statistical significance was considered when the probability value $P < 0.05$.

3.4 | RESULTS

3.4.1 | Assessment of ricotta cheese shelf-life parameters during storage

3.4.1.1 | Microbial profile

The results of microbial counts of ricotta cheese during storage in four types of commercial packages are shown in Figure 2 (A-F). The counts of analyzed bacterial groups increased under refrigerated temperature, with relatively higher level of total mesophiles, Enterobacteriaceae, and *Pseudomonas* spp. than other bacterial groups. This increment was ascribed to the tolerance of survivability of these microorganisms under cold environment. Moreover, the presence of *Listeria* spp., *Salmonella* and *Shigella* spp. was not detected during study period, indicating no pathogen contamination of ricotta cheese.

Ricotta cheese is a fresh type that need no ageing process, therefore, the growth of yeasts and molds was detected on cheese samples only after 4 days of cold storage. Regarding the amounts of aerobic mesophiles (Figure 2A), the value was slightly over 3 $\log_{10}$ $CFUg^{-1}$ at T0 which was similar to the results reported by other studies [5, 11]. The amount gradually increased to the acceptable limit (7 $\log_{10}$ $CFUg^{-1}$) at 7 days for RO samples, 11 days for IT, SP, and SLO samples. In the present study, we used air condition for the storage of ricotta cheese, whereas under modified atmosphere - 30% CO_2 and 70% N_2 - the proliferation of total aerobic mesophiles was inhibited for longer time [5, 11]. As shown in figure 1B, the counts of LAB were under detection during the first 6 days of storage, which implied the coagulation process was done by acids - no presence of starter culture. As the fresh nature of ricotta cheese, the presence of lactic acid bacteria is often negligible. During later period of cold storage, the amount of LAB increased around 1.5 $\log_{10}$ $CFUg^{-1}$ in IT and SP samples, whereas 2 $\log_{10}$ $CFUg^{-1}$ increment was found in RO cheese. Regarding the SLO sample, the presence of LAB was under detection (<100 $CFUg^{-1}$) during first 9 days storage, following around 1 $\log_{10}$ $CFUg^{-1}$ at the end of the study. The proliferation of LAB can impact cheese pH value as their capacity to

produce organic acids. In our study, the delayed growth of LAB and the strong buffering capacity of ricotta cheese (high moisture and high protein/fat ratio) resulted stabilized pH value during study period. Similar to the trend of LAB, molds and yeasts (Figure 1C) were not detected in the early storage time, the growth occurred only after 4 days in SP, 6 days in IT and RO, 9 days in SLO samples. The amounts reached slightly above 4 log_{10} $CFUg^{-1}$ (acceptable limit) after 7 days almost all samples, with exception of SLO sample that kept lower value than acceptable for at least 14 days. Enterobacteriaceae represents the hygiene conditions of the dairy factory, this family contains many familiar pathogens - *Salmonella* spp., *E. coli*, *Klebsiella* spp. and *Shigella* spp. The presence of these pathogens or over - proliferation not only cause foods spoilage but pose healthy risks to consumers [12]. In our study, the count of Enterobacteriaceae (Figure 2D) was around 2 log_{10} $CFUg^{-1}$ at T0, which demonstrated good hygiene condition of the dairy factory, but, due to the air-packed condition, the amount of Enterobacteriaceae gradually increased and reached the acceptable limit level (5 log_{10} $CFUg^{-1}$) at 5 days for RO and SLO, at 6 days for IT, at 8 days for SP. The counts of Enterobacteriaceae detected in RO and SLO cheese samples were generally higher than in IT and SP samples. The modified packaging condition can strongly delay the proliferation of Enterobacteriaceae [5]. Many species of *Staphylococcus* are part of skin microbiota that normally are not harmful species. However, the transmission of enterotoxin-producing staphylococcal strains from animal to milk may cause staphylococcal food poisoning [13]. The count of *Staphylococcus* spp. (Figure 2E) in ricotta cheese gives us indications of possible transmission from animal skin to milk, it started to become detectable after 3 days in IT, SP, RO samples while after 6 days in SLO sample. Its growth occurred at later stage of storage but remained below the threshold of endotoxin production limit for entire study period. *Pseudomonas* spp. are generally considered as spoilage indicators in many food matrixes such as meat and fresh cheese [14]. As Figure 2F shows, during the first 6 days of cold storage, SLO and RO samples demonstrated higher amount of *Pseudomonas* spp. than other two samples. At the following period, the level of SLO kept increasing and exceed the threshold after 11 days, whereas other samples remained below the limit. A study revealed that the refrigerated temperature had no impact on the proliferation of Enterobacteriaceae and yeasts, while other bacteria groups, such as aerobic mesophiles and lactobacilli were inhibited by low temperature on Himalayan cheese [15].

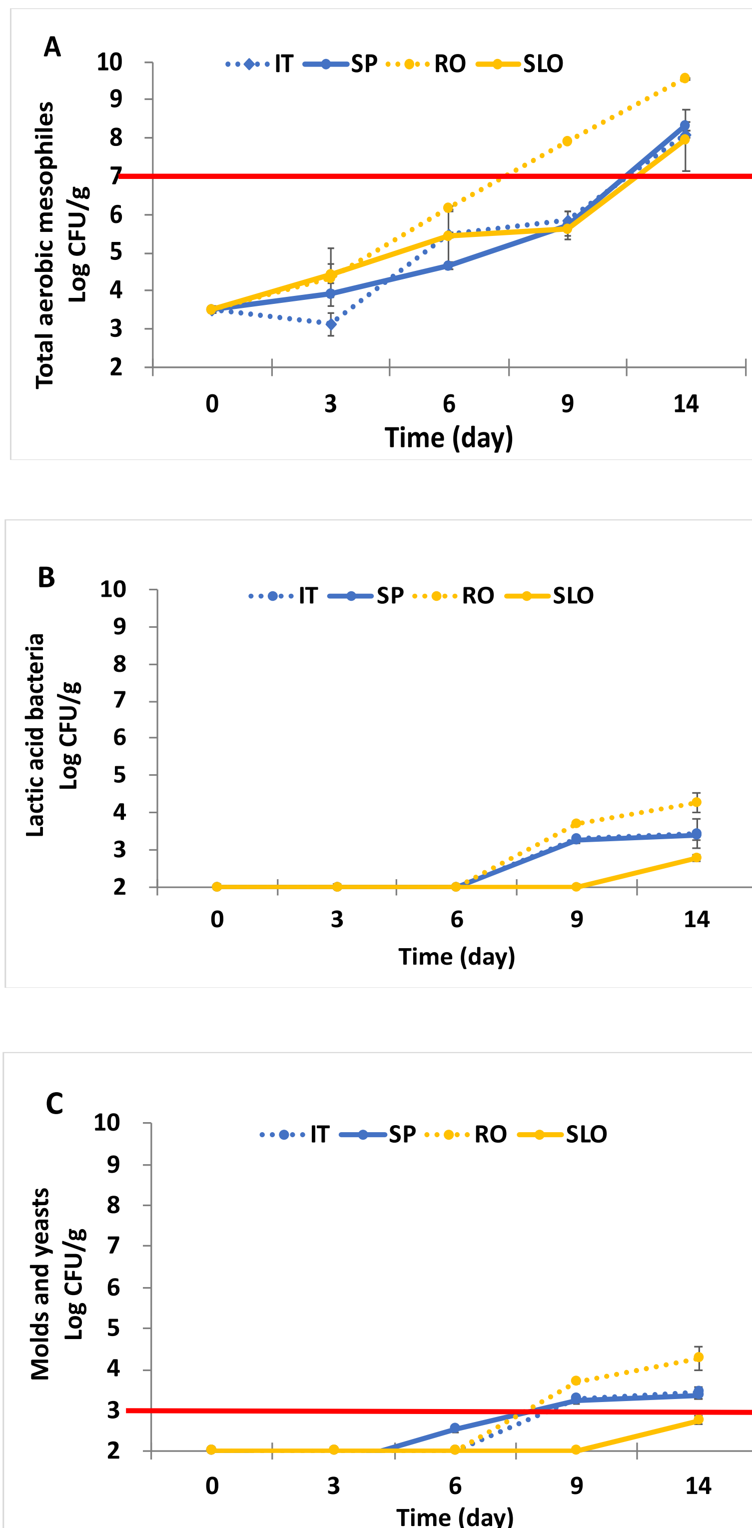
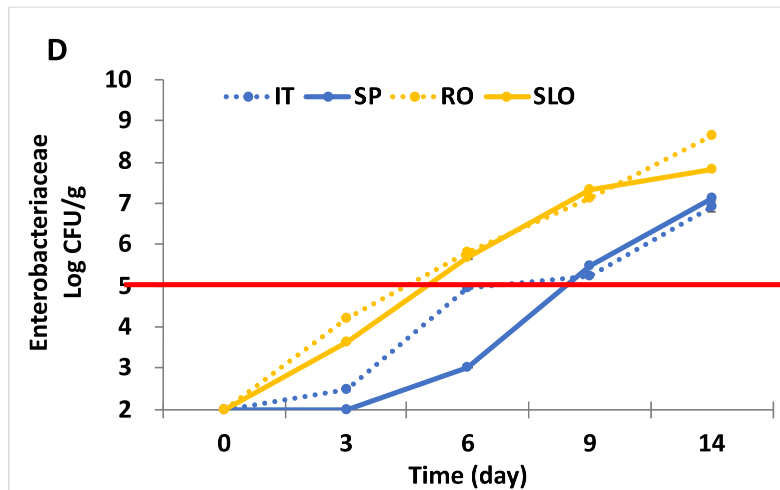
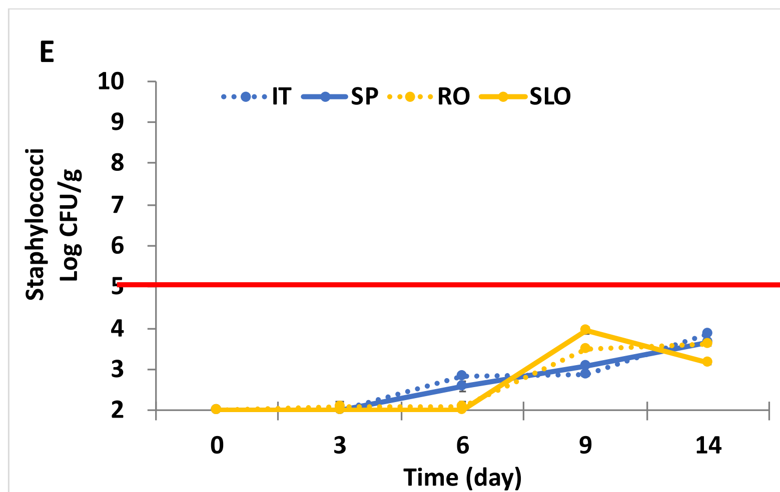


Figure 2. Microbial counts in the ricotta cheese stored in Italian (IT), Spanish (SP), Romanian (RO) and Slovenian (SLO) packages during 14 days storage at 4 °C. **A**, total aerobic mesophiles; **B**, lactic acid bacteria; **C,** molds and yeasts. The values are expressed as log CFUg^{-1}. Note: red line represents the suggested reference value of each bacterial group [8].





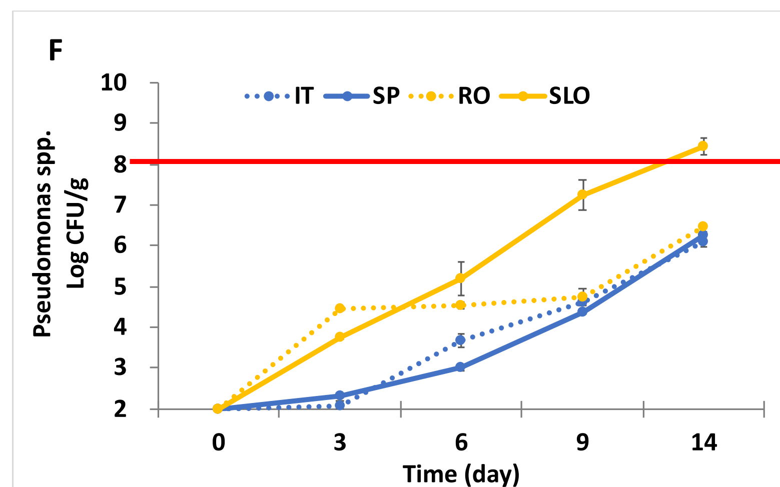


Figure 2a. Microbial counts in the ricotta cheese stored in Italian (IT), Spanish (SP), Romanian (RO) and Slovenian (SLO) packages during 14 days storage at 4 °C. **D**, Enterobacteriaceae; **E**, *Staphylococcus* spp.; **F**, *Pseudomonas* spp.. The values are expressed as log CFUg^{-1}. Note: red line represents the suggested reference value of each bacterial group [8].

3.4.1.2 | pH value

The variation of ricotta pH values is mainly explained by the metabolic activities of microorganisms, especially lactic acid bacteria, as their number increased their metabolites-organic acids and free fatty acids that create acidic environment and thus lowering the pH value of cheese [16]. Table 2 shows that the initial pH values of ricotta cheese are ranged from 5.78 to 6.35 which fell into the criteria for ricotta cheese - around 6, which was similar as described by Mancuso *et al*. [1]. Whereas as reported by Pizzillo *et al.* [9] and Di Pierro *et al.* [10] the initial pH values of ricotta cheese were slightly higher, around 6.51 and 6.65. In general, after 14 days storage at 4°C, the values of all samples slightly increased, except for SLO sample which decreased around 0.3. RO and SLO samples showed general higher values than other two samples even at the initial time point. Although the values varied during time, all the packages were able to maintain cheese acidity for 14 days under the current storage conditions.

Table 2. pH value of ricotta cheese samples.

pH value	**0**	**3**	**6**	**9**	**14**
RO	6.07±0.009	6.05±0.075	6.22±0.038	6.23±0.000	6.17±0.026
SLO	6.35±0.014	6.18±0.019	6.04±0.115	6.17±0.045	6.02±0.054
IT	5.84±0.000	5.77±0.042	5.97±0.038	5.90±0.005	5.90±0.078
SP	5.78±0.000	5.91±0.002	5.93±0.021	5.96±0.054	5.98±0.035

3.4.1.3 | Sensory profile

Figure 3 shows the organoleptic characteristics of ricotta cheese sample stored in the four investigated packages. Related to the initial cheese, the highest score of 5 was attributed to specific white color, uniformity and smell, whilst the lowest score of 0 was attributed to some unpleasant tastes (mouldy, etheric, balsamic, spicy) or appearance (fustiness, foreign colors). The microbial enzymatic activities (proteolysis, lipolysis, glycolysis) and chemical reactions (oxidation) can breakdown macromolecules and generate aromatic chemicals that cause alterations in cheese flavors during storage [17]. As shown in Figure 3, all samples showed a decrement in all sensorial attributes as the storage period increased. In general, the aspect and typical white colour of ricotta cheese deteriorated, and the scores decreased from 5 to around 2.5 in IT and SP samples, while SLO and RO cheese were a slightly whiter, with scores around 2.8. To be noticed that as the storage time increased, the colour of cheese transformed from white to beige, as a result of proliferation of pigments producing microorganisms (yeast, lactobacilli). The colour alteration was also a result of the oxidation reactions occurred under air-packed condition. Moreover, although the scores of odour attributes (butter, fresh curd,

fresh milk, yogurt, goat, smoked) decreased in all samples, SLO samples remained acceptable up to the end (around 3) of the study. However, the sensation on salty and sweet tastes is highly subjective. As some panellists perceived more sweet than salty or vice versa. Generally, cheese texture was preserved by all type of packages, RO and IT samples remained quite soft even after 14 days. Bitterness also has been perceived and reported during the last days, as the increased number of peptides catalyzed by microorganisms proteolytic enzymes [18]. Also, the reduced granular and creamy texture was noticed by panellists. It was noteworthy that ricotta cheese stored in SLO package was harder in texture than other samples at the beginning time point. The score for the overall acceptability of ricotta drops below 2.8 considered as the threshold value for overall cheese acceptability [19] after 7 days in the IT, RO, and SP packages and 6 days in the SLO package.

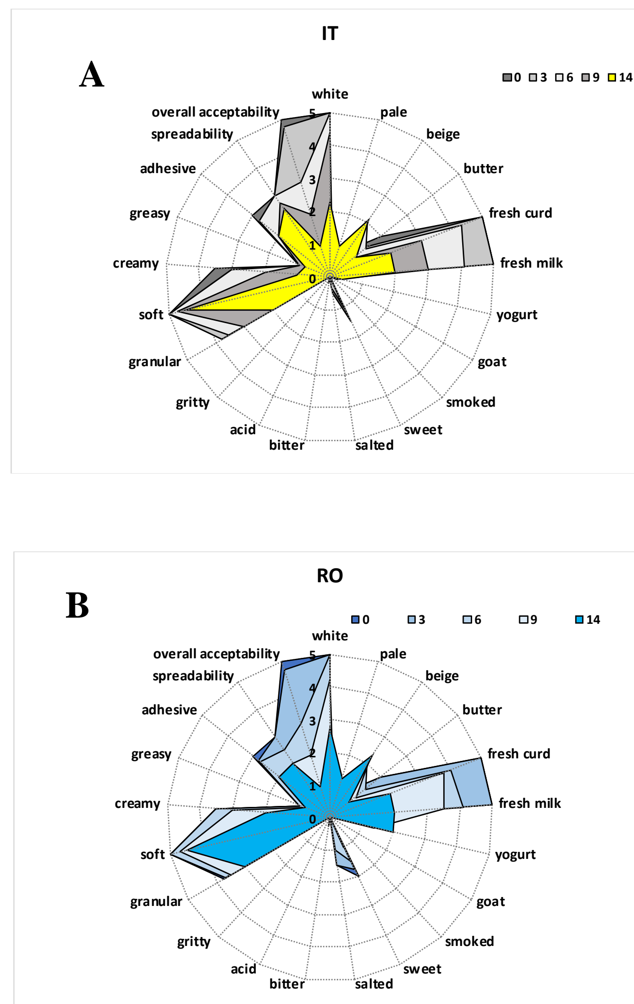


Figure 3. Sensorial profile of ricotta cheese during storage in the four packages at 0, 3, 6, 9, 14 days. A, Italy (IT), B, Romania.

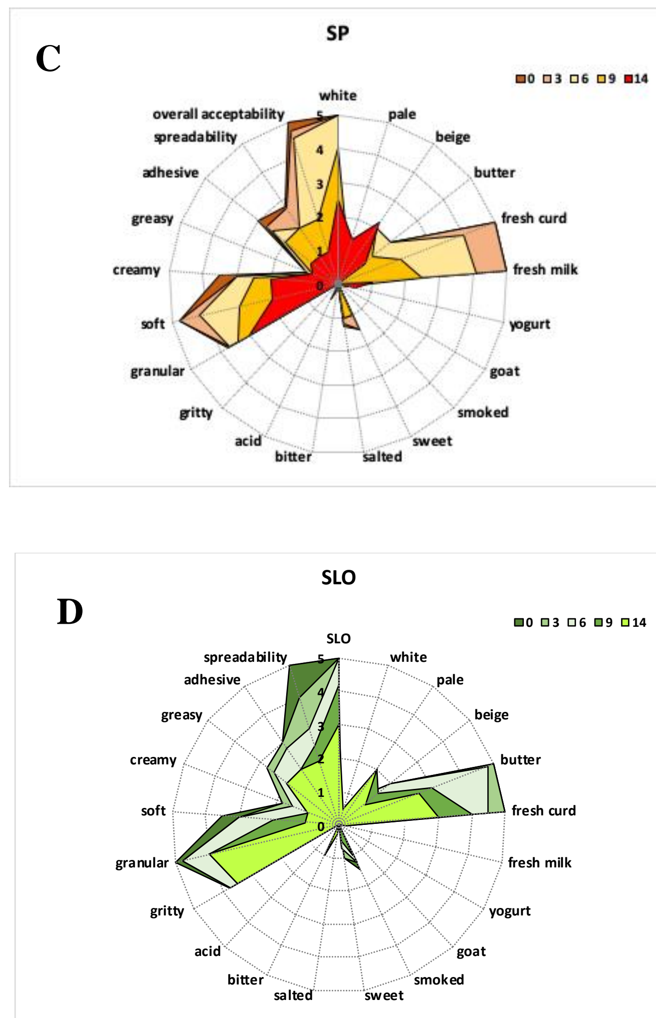


Figure 3a. Sensorial profile of ricotta cheese during storage in the four packages at 0, 3, 6, 9, 14 days. C, Spain (SP), D, Slovenia (SLO).

3.4.1.4 | Volatile compounds of ricotta cheese

FFA arises in cheese by three pathways namely proteolysis, lipolysis and lactose fermentation and can be converted to other volatile compounds [20], which gives rancid and grassy odour notes. In the IT and SP samples the total FFA amount increased in the first three days of storage of 1.40-fold and 1.35-fold, respectively (Figure 4). Their maximum level was reached in the cheese in the RO (1.38-fold) and SLO (1.27-fold) after six days of storage (Figure 4). Overall, no pronounced increment was observed for total FFA. It can be explained based on the high temperature required during the Ricotta production process (80-90°C for 25-30 minutes) which inactivated microbial lipases [21]. At the end of storage, the reduction in the amounts of total FFA occurred in the cheese in all packages due to their metabolization to other

highly flavoured compounds (aldehydes, methyl ketones, lactones) [22] but the final values remain above the initial ones.

The initial hexanal level in Ricotta cheese was 2.81-fold higher than the value reported by Bergamaschi and Bittante [20]. Its profile followed an ascending trend in the first six days of storage in all cheese samples being generated by peroxidation/hydrolysis of unsaturated fatty acids followed by the decrease due to its conversion in the corresponding alcohol or carboxylic acid according to the oxydo-reduction conditions [18]. Hexanal producing is promoted by the high temperature, light-exposure, mild heat treatment and high relative humidity [23]. High temperature required for the Ricotta production, storage in the darkness at low temperature and relative humidity as well as poor PWV of packages resulted in low amounts of hexanal in all cheese samples.

Regarding the concentration of investigated FFA (Figures 5A-5D) of each sample, the most abundant FFA present at the initial time point was butanoic acid (27.71 mg Kg^{-1}) followed by hexanoic acid (4.65 mg Kg^{-1}) and octanoic acid (3.62 mg Kg^{-1}). Decanoic acid and isovaleric acid were poorly represented at 0.39 mg Kg^{-1} and 0.01 mg Kg^{-1}, respectively. Packaging influenced the fatty acid profiles to different extent with maintaining the gaps between the fatty acids level. Both ascending and descending trends in the evolution of each FFA can be noticed in all packages, but among all kinds of FFA, only the profile of butanoic acid corresponds to that of the total FFA. In the first three days of storage the amount of butanoic acid increased 1.42-fold and 1.32-fold in the cheese in IT and SP samples, respectively. While in the RO and SLO samples the maximum concentration was reached after 6 days being 1.36-fold and 1.17-fold higher than that at starting point. After reaching the maximum concentrations the amount of butanoic acid decreased, but at the end of storage its level did not fall below the initial values. Hexanoic, octanoic and decanoic acids reached their maximum amount after 6 days of storage, their production could be associated with the activity of lipases and esterases [20]. Isovaleric acid maintained the initial concentration over the 14 days of storage, it low values (0.01 mg Kg^{-1}) suggesting that the propionic fermentation from which is originated [24] did not occurred. As compared to the values reported by Bergamaschi and Bittante [20], the initial amount of butanoic acid in our cheese samples was significantly more consistent (1.21 mg Kg^{-1} against 27.71 mg Kg^{-1}) while the levels of hexanoic and octanoic acids were slightly higher (2.44 mg Kg^{-1} as compared to 4.65 mg Kg^{-1} against, 1.81 mg Kg^{-1} as compared to 3.62 mg Kg^{-1}). In opposition the level of decanoic acid in our initial samples (0.39 mg Kg^{-1}) was lower than the value (1.73 mg Kg^{-1}) reported by Bergamaschi and Bittante [20].

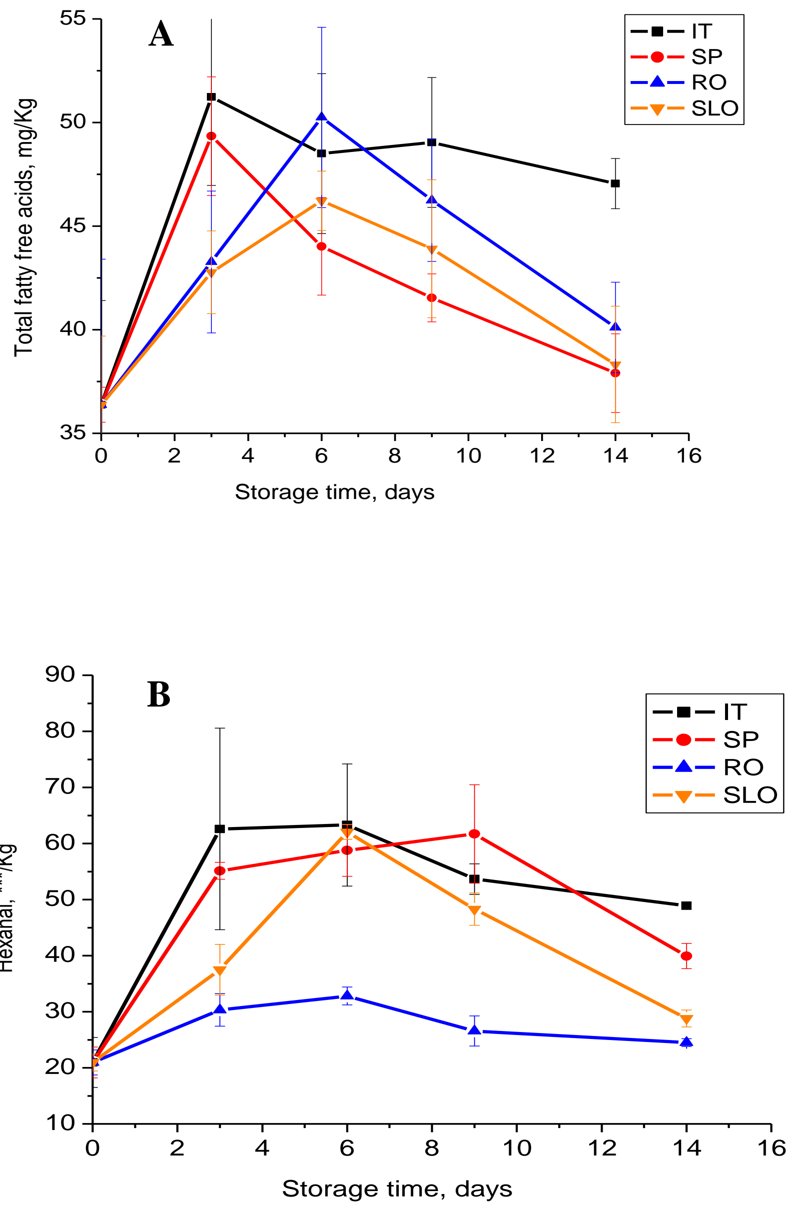


Figure 4. The amount of total fatty acids (A) and hexanal (B) of IT, SP, RO, SLO samples during 14 days storage.

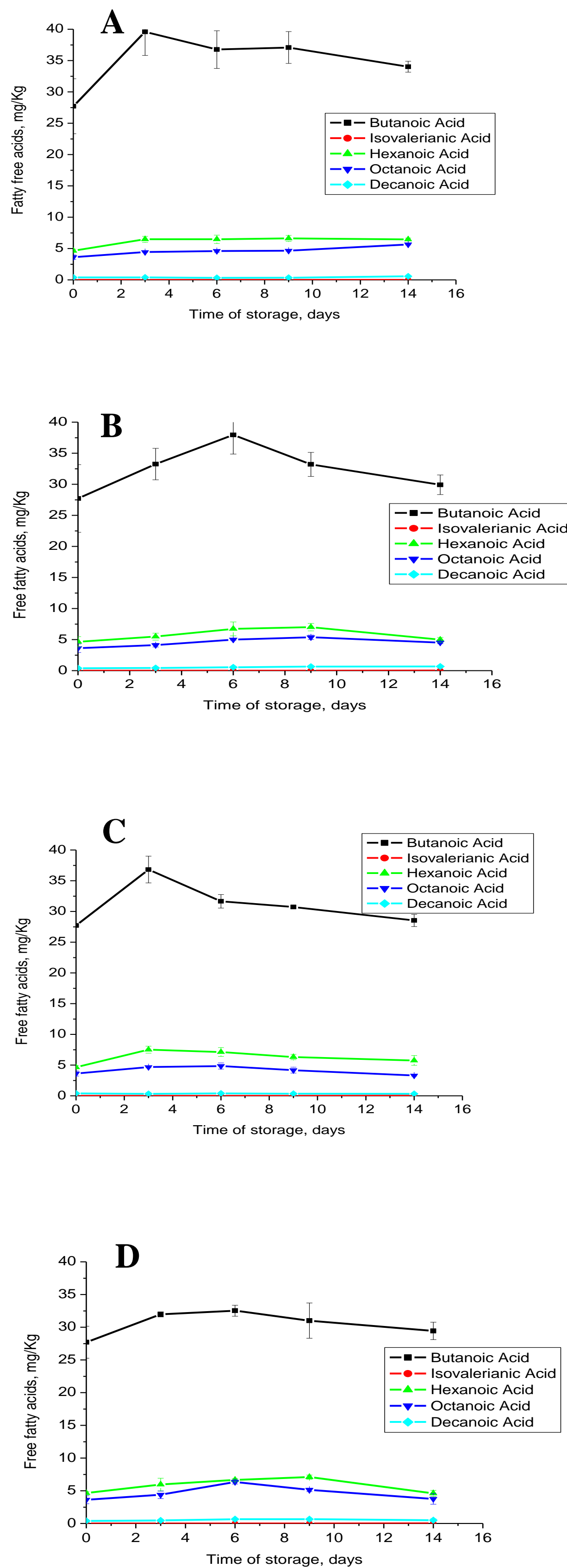


Figure 5. The concentration and kind of free fatty acids of IT (A), RO (B), SP (C), SLO (D) samples during 14 days storage.

3.4.1.5 | Correlation between packaging properties and cheese parameters

Apart from the above-mentioned food shelf-life parameters, our team also characterized the intrinsic properties of the different commercial packages, such as water vapours permeability (WVP), oxygen transmission rate, degradation markers of polyethylene and crystallinity degree [16]. SLO revealed higher permeability to water vapor ($2.705 \cdot 10^{-11} \pm 0.17_a$) than other packages, which leading to increased level moisture in packaging headspace and further facilitated the growth of aerobic bacteria, such as Enterobacteriaceae and *Pseudomonas* spp. (Figure 2D and 2F). Like SLO, the WVP value of IT ($2977 \cdot 10^{-11} \pm 0.11_a$) was also higher than other packages, contributing to relatively higher growth rate of total aerobic mesophiles (Figure 2A), especially from day 3 to day 6. However, SLO package demonstrated lower contact angle values (24.14°) that allowed more close contact between cheese surface and packaging, leading to delayed growth of LAB (Figure 2B), moulds and yeasts (Figure 2C). Moreover, IT and SP package showed relatively high oxygen transmission rate with 3452 and 6860, respectively. The proliferation of moulds and yeasts (Figure 2C), as well as staphylococci (Figure 2E) of IT and SP samples were probably enhanced by the increased oxygen in headspace gas.

3.5 | DISCUSSION AND CONCLUSIONS

The shelf-life of fresh ricotta cheese is highly dependent on the initial microbial content, the packaging properties as well as the storage conditions. Modified atmosphere in combination with hurdle technologies, for instance, refrigerated temperature are normally used to control the microbial, chemical and organoleptical quality of fresh cheese. In the present study, due to the neutral pH value of ricotta cheese and normal atmosphere packaging conditions that could not completely prevent the chemical reactions and proliferation of cheese microbiota (total aerobic mesophiles, *Pseudomonas* spp., *Staphylococcus* spp., moulds and yeasts) and thus leading to altered physical and sensorial charactertistics during storage.

Regarding the sensory analysis of cheese, although the characteristic attributes - white colour, texture - reduced during storage, the ricotta cheese stored in SP package was acceptable for 8 days, and 6 days for other samples. However, in the current study, the major factor that affected the shelf-life of ricotta cheese was the microbial level during storage, such as Enterobacteriaceae, which exceeded the threshold before 6 days storage.

In summary, the investigated packages - IT, RO, SLO, SP - showed preservation capacities in delaying the proliferation of microbes, maintaining cheese pH value and organoleptic characteristics, although the shelf-life stored in analyzed packages was slightly shorter than the suggested period (around 7 days) that mainly result from microbial overgrowth. The simultaneous compliance of microbiological safety level and overall acceptability score

indicates a shelf-life for ricotta cheese of 3 days in IT, SP, and RO packages and 1 day in SLO package. Therefore, for a longer shelf life of ricotta, attention should be paid on applying hygiene practices during cheese production and using an appropriate packaging system.

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CHAPTER IV

CHICKEN BREAST MEAT STORED IN COMMERCIAL PAPER PACKAGING SYSTEMS

4.1 | ABSTRACT

The surface of poultry is highly susceptible to microbial attack as poultry meat contain high level of nutrients (proteins and carbohydrates), moisture, and nearly neutral pH. Preservation measures are proposed to eliminate and control the microbial and chemical spoilage of meat, including the use of low temperature, modified atmosphere, use of irradiation and addition of preservatives. The present study aimed to evaluate the efficacy of four types of paper-based packages, commonly used in four different countries (Romania, Spain, Italy and Slovenia), on preserving raw chicken breast meat through monitoring the variation of meat microbial population, acidity and sensorial attributes during 14 days of storage at 4ºC. The packages of Romania (RO) and Italy (IT) are composed by paper laminated with polyethylene film, whereas those from Spain (SP) and from Slovenia (SLO) are double-side waxed paper and coated with hydrophobized starch, respectively. The results of microbiological analysis showed that under studied storage conditions, RO package was considered more efficient in delaying the growth rate of microbes than other packages. The meat pH values increased during storage due to the metabolic activities of microorganisms. The sensorial characteristics revealed that the waxed surface of SP package transformed the pink colour of raw meat into dark red appearance of dried meat. On the contrary, RO package was considered the best in preserving meat sensorial quality.

4.2 | INTRODUCTION

According to FAO, by 2014, poultry meat was the second most consumed meat in the market [1]. Poultry meat is rich in high quality proteins; the protein content of roasted breast meat without skin is around 31% of meat [2]. In addition, the low amounts of connective tissue protein-collagen brings tenderness to poultry meat [3]. Compared to red meat, poultry meat has relatively low fat and cholesterol content that mainly found in skin, and the low fat is mainly polyunsaturated fatty acids (long-chain n-3 polyunsaturated fatty acids) that may positively

modulate immune activities [4]. Moreover, poultry meat also contains various minerals, vitamins, and bioactive compounds.

The manufacturing and processing methods may bring environmental contaminants to raw meat, microorganisms, mainly *Campylobacter* spp., *Salmonella* spp. have been reported to cause foodborne diseases due to the ingestion of contaminated poultry meat [5]. Commonly identified spoilage species include *Pseudomonas* spp., Enterobacteriaceae, *Moraxella* spp., *Staphylococcus* spp. and some species of lactic acid bacteria that can cause spoilage and produce undesirable sensory changes, causing huge economic loss [6]. Moreover, the microbial metabolic activities such as amino acids decarboxylation can produce harmful chemicals, such as biogenic amines. Even at low concentrations, biogenic amines can induce toxicological effects on human immune, gastrointestinal and neuron systems [7].

The most used preservation methods include low temperature, irradiation and high pressure, atmosphere modification, addition of antimicrobials [8]. Recently, the advances in nanotechnology have focused on using nanoparticles incorporated bioactive substances (essential oils) to extend meat shelf-life [9, 10]. Other technology, such as cold plasma with rosemary extract has also been investigated to reduce the bacterial functional diversity in poultry ground meat [11].

In the present study, we aimed to evaluate the efficiency of four types packaging material that are currently used for meat storage by four European countries, Italy (IT), Spain (SP), Slovenia (SLO), Romania (RO). The efficiency of packages on fresh chicken breast meat parameters was established by analyzing the microbial counts, pH value, organoleptic characteristics, and biogenic amine concentration for 14 days storage at 4ºC.

4.3 | MATERIALS AND METHODS

4.3.1 | Packages preparation

The composition and image of the packages commonly used in the four countries (RO, IT, SP, SLO) are listed in Table 1 and represented in Figure 1.

Table 1. Composition of the packages.

Country	Composition
Romania Italy	Paper laminated with polyethylene (PE surface in contact with meat)
Spain	Waxed paper package on both surfaces
Slovenia	Paper modified with oxidized starch (starch surface in contact with meat)

A

B

C

D

Figure 1. Type of the packages: A-Romania (RO), B-Spain (SP), C-Slovenia (SLO), D-Italy (IT).

4.3.2 | Storage of chicken breast in the packages and experimental design

Slices of fresh chicken breast meat (cut from a single chicken breast) were purchased from local market in Camerino (Italy) and immediately transferred into laboratory. The average size of each packaging film was about 30 cm - 40 cm, which is commonly used size for wrapping and transferring fresh meat slices. Around 40 g of chicken meat (2 slices) was placed on the inner surface of the packaging (polyethylene surface/hydrophobized starch/waxed surface), then the meat was wrapped, and two ends of the packaging folded in such a way that similar to the wrapping method commonly used by supermarkets (Figure 2). All samples were labelled with different sampling time and packaging codes, respecting the countries (IT, SP, SLO, RO) and stored at 4°C for 14 days. At each sampling time - 0, 3rd, 6th, 9th and 14th day - samples were taken from the fridge and the following parameters were measured: counts of the main microbes groups as markers of meat hygienic condition and spoilage, pH values, sensorial profile and biogenic amines content.

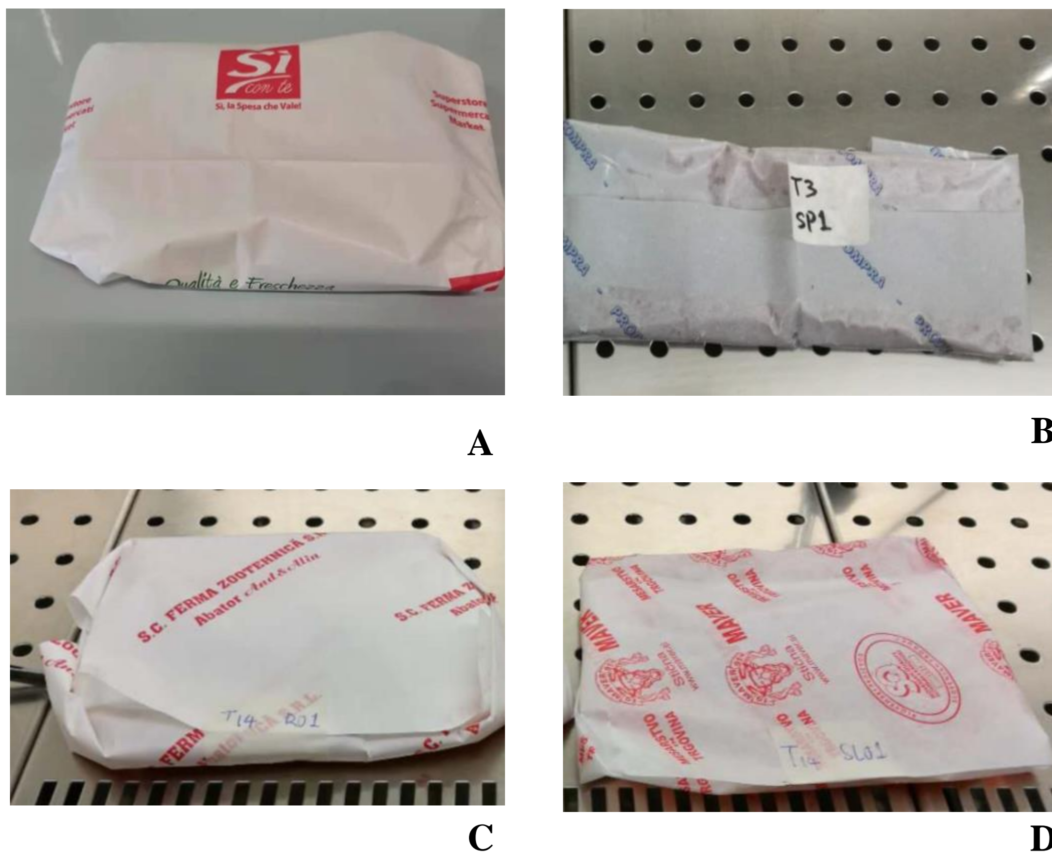


Figure 2. IT (A), SP (B), RO (C), and SLO (D) packages with chicken breast meat.

4.3.3 | Microbiological assay

An amount of 10 g (wet weight) of chicken breast meat was homogenized into 90 ml of peptone water (Liofilchem, Roseto degli Abruzzi, Italy) for 4 min in a Stomacher Lab Blender Model 80-BA 7020 (Seward Medical, London, UK). The meat homogenate was serially diluted thus preparing a series of 10-fold dilutions (10^{-1} to 10^{-8}). A given amount of each dilution (50 μl) was plated in each half of the different agar plates. The aerobic mesophilic bacteria were counted on Plate Count Agar (PCA - Oxoid, Basinstoke, UK) under aerobic conditions at 30°C for 72h, following the ISO 4833 guidelines. For the enumeration of mesophilic lactic acid bacteria (LAB), de Man, Rogosa, Sharpe agar (MRS Agar at pH 5.7, VWR, Leuven, Belgium) was used (ISO 15214:1998 guidelines). For the detection and enumeration of Enterobacteriaceae, violet red bile glucose agar (VRBGA - VWR) was inoculated with sample at 37°C for 24h, following the ISO 21528-2 guidelines. The enumeration of presumptive *Pseudomonas* spp. was carried out by aerobically inoculating sample on Pseudomonas Selective Agar (CFC - Liofilchem) at 25°C for 44h ± 4h, following the ISO 13720: 2010 (E) guidelines. The presence of staphylococci was checked through the aerobic inoculation on Baird-Parker agar medium (VWR) at 35-37ºC after 24 to 48 hours of incubation (ISO 6888-1:1999(E). According to the guideline ISO 15213:2003(E), the detection of sulphite-reducing bacteria was carried out on iron sulphite agar (VWR) with sample inoculum incubated in an anaerobic jar at 37°C for 24h to 48h. After incubation, the number of colonies were counted and expressed as $\log_{10}$ CFU/g of meat for each studied bacterial group.

4.3.4 | pH measurement

An amount of 20 g meat sample was chopped into smaller pieces by using a sterile knife, and subsequently transferred into a sterile stomacher bag. The pH values of each sample at every time point were measured by an electronic pH meter (Mettler Toledo, Columbus, UK) equipped with a probe for foods (Mettler Toledo).

4.3.5 | Sensorial analysis

The sensorial panel was composed by 10 assessors - 8 females and 2 males. An instructive session was conducted before the analysis in order to develop a consensus vocabulary for aspect, colour, odour, texture and taste attributes for chicken meat. The sensorial attributes were determined at 0, 3rd, 6th, 9th and 14th day of refrigerated storage. At each analysis time point, all samples were taken out of fridge and cut into 2 cm^2 pieces and placed inside glass plate. Each meat sample was marked with a 3-digital code. The sensory evaluation on aspect, odour, colour and elasticity was performed on skin off raw chicken breast meat, after raw meat evaluation,

the same piece of meat was cooked by using microwave (Whirlpool) at 750W for 2 minutes. The cooking method was based on the method described by Baston and Barna [12]. After that, attributes such as juiciness, tenderness, firmness, flavour and overall palatability were evaluated on the cooked meat. The evaluation was conducted individually. Table 2 shows the hedonic scale composed by scores from 0 (worst perception) – 3 (optimum).

Table 2. Attributes considered in the sensory evaluation of raw chicken breast meat.

Attributes	Description	Values
Aspect	All surface covered with slime	0
	Slime present in most parts	1
	Slime present in some parts	2
	No slime (chicken characteristic)	3
Odor	Foreign odors	0
	Off-odor	1
	Slightly off-odor	2
	Characteristic	3
Color	Yellow/other colors	0
	Dark pink	1
	Pale pink/yellow	2
	Pink to red	3
Elasticity	No return	0
	Slightly return	1
	Slow return	2
	Return	3

4.3.6 | Biogenic amines analysis

The biogenic amines were determined by SPE-HPLC-DAD method previously developed [13, 14]. Before analysis, the samples were processed in the following stages:

Extraction. Each slice of meat (chicken) was grinded with a blender, then 5 g of sample were homogenized for two minutes by using an Ultra-Turrax S 18N-10 G (IKA-Werke Gmbh & Co., Staufen, Germany) with 25 ml of 5% trichloro-acetic acid (TCA), centrifuged at 5000 rpm for 10 min.

Derivatization. An aliquot of supernatant acid solution (1 ml) was mixed with 300 µl of a saturated $NaHCO_3$ solution, 200 µl of NaOH 2M and 2 ml of dansyl chloride (10 mg/ml acetone). The dansylation reaction was conducted in the dark at 45 ºC for 45 min.

Concentration. After that, the residual dansyl chloride was destroyed by adding 100 µl of 28% NH_4OH. The mixture was evaporated to 1.5 ml under flow of N_2.

Purification SPE C18. The aqueous residue was purified on a Strata C18-E cartridge (6 ml, 1 g). The cartridge was activated with 2x2 ml of acetonitrile, conditioned with 2x2 ml of water, then the aqueous residue was loaded onto the cartridge at a flow rate lower than 0.5 ml min^{-1};

the cartridge was then washed with 2x2 ml of water, thoroughly dried, and then elution was performed using 4 ml of acetonitrile. The elute was filtered on 0.45-µm PTFE filter and injected in HPLC-DAD. Following Sirocchi *et al.* [14] methodology, *HPLC-DAD analyses* were performed using a Hewlett Packard (Palo Alto, CA) HP-1090 Series II, made of an autosampler, a binary solvent pump, and a diode-array detector (DAD). The separation was performed on a C18 Gemini column (5 mm, 250 x 3 mm) equipped with a Gemini C18 guard column (5 mm, 4 x 3 mm), both from Phenomenex (Torrance, CA). The flow rate was 0.5 ml min^-1, and the column temperature was set at 40 ºC. The mobile phase for the HPLC-DAD analysis was water (A) and methanol/acetonitrile (70:30, v/v) (B). The gradient program was. 0 min 60% B, 0–10 min 70% B, 10–20 min 90% B, 20–26 min 100% B, 26–30 min 100% B. Finally, phase B was decreased to 60% from 30 to 35 min and held at 60% until the end of the run at 50 min. The peaks were detected at 254 nm. The injection volume was 20 µl. BAs were identified by their correspondence with UV spectra and retention-time of analyte standards. The quantification of each targeted BA was performed using the respective calibration curves.

Considering the concentration of detected biogenic amines, the following parameters, i.e., Biogenic Amine Index (BAI), Chemical Quality Index (CQI), ratio Spermidine/Spermine (SPD/SPM), total Biogenic Amines (BAs) were determined:

$$\text{BAI} = \text{putrescine} + \text{cadaverine} + \text{histamine} + \text{tyramine} \tag{1}$$

$$CQI = \frac{putrescine + cadaverine + hista\min e}{sper\min e + spermidine + 1} \tag{2}$$

$$\text{BAs} = \text{putrescine} + \text{cadaverine} + \text{histamine} + \text{tyramine} + \text{spermine} + \text{spermidine} + \text{tryptamine} + \text{phenyl-ethyl-amine} \tag{3}$$

The calibration curves used for determination of BAI, BAs, CQI and SPD/SPM parameters, characterizing the level of biogenic amines generated in the chicken breast meat during storage were in the range 0.5-25 mg kg^{-1} on five points and had the coefficients of correlation higher than 0.996. BAI and SPD/SPM are especially used for fresh meat, while CQI is relevant for fish and seafood [15-17].

4.3.7 | Statistical analysis

All assays were performed in triplicate. Student's t test was used to detect differences among mean values of chicken breast meat properties in all the test intervals.

4.4 | RESULTS

4.4.1 | Microbiological analysis of breast meat during storage

The results of the microbiological analysis on the chicken breast meat stored in the packages are presented in Figure 3, in general, the counts of bacterial groups significantly increased during storage. During the study, the presence of sulphite-reducing, anaerobic bacteria, *Listeria* spp., *Salmonella* spp. was not detected.

The amount of total aerobic mesophiles is given in Figure 3A. The initial value was 3.5 log_{10} CFU g^{-1} of meat, falling within the acceptable limit [18]. After 2 days storage, IT reached the acceptable limit for total aerobic mesophiles, followed by SP and SLO, reaching the limit after 3 days storage. To be noticed that the RO sample contained lowest number of mesophilic bacteria than other samples and it reached the limit around 7 days. The counts of mesophilic bacteria in RO sample were significantly lower than that of SP sample ($p<0.05$). Some psychrotrophic lactic acid bacteria (LAB) are one of the potential spoilers found in chilled poultry that packed either with air or modified conditions [19, 20], causing sliminess, souring on raw meat [21]. In our study, the air and chilled storage condition did not affect the growth of LAB. Although SP showed higher amounts of LAB at day 14, the overall amount of LAB did not exceed the acceptable limit after 14 days in all samples. The counts of LAB of SP, RO and SLO increased continuously from 0-14 days, with 5 log_{10} $CFUg^{-1}$ increment in SP, 3.54 log_{10} $CFUg^{-1}$ increment in RO, and 3.79 log_{10} $CFUg^{-1}$ increment in SLO. However, IT increased 2 log_{10} $CFUg^{-1}$ at the first period of storage (0-6 days), the amount of LAB was kept for another three days and increased to 5 log_{10} $CFUg^{-1}$ in the end. The count of Enterobacteriaceae correlates the level of contamination during processing, handling and storing as well as the hygiene conditions in poultry slaughterhouses [22]. As shown in figure 3D, Enterobacteriaceae grew quickly during the first 6 days of storage for IT, SP and SLO samples and reached the limit (6 log_{10} $CFUg^{-1}$) for around 3 days. While Enterobacteriaceae of RO sample proliferated in lower growth rate and reached the limit at 6 days. Similar to LAB, *Pseudomonas* spp. can tolerate temperature less than 7 °C and is considered as the dominant spoilage genera on meat [19, 23]. *Pseudomonas* spp. grew similarly in all samples during the first 3 days of storage (Figure 3C), while after that, the counts of *Pseudomonas* spp. in IT continuously increased, with around 9 log_{10} $CFUg^{-1}$ increment at the end. While SP and SLO increased to 8.6 log_{10} $CFUg^{-1}$ at day 9 and slightly decreased at day 14. The level of *Pseudomonas* spp. detected in RO was increased 3 log_{10} $CFUg^{-1}$ during the first 3 days storage and maintained for another 3 days, followed by another increase and finally dropped to the same level of SLO and SP. *Staphylococcus* were detected in all samples, IT and SLO reached the limit around 2 days, followed by 3 days for SP, RO showed the longest preservation period that it reached the limit

around 6 days storage. In summary, although each packaging showed different behaviour in controlling the content of microorganisms in meat stored inside. Regarding the microbial growth pattern of each bacteria group, RO packaging demonstrated better preservation efficiency in delaying the spoilage process compared to other packages.

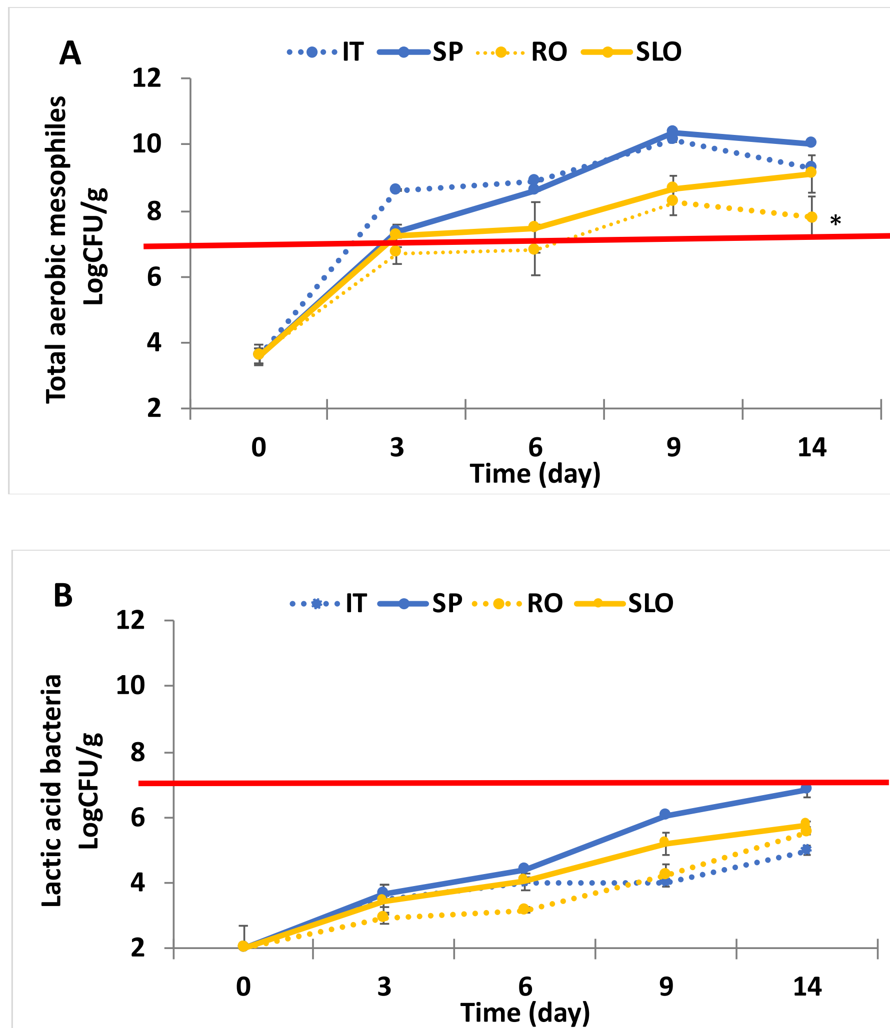


Figure 3. Microbial profile of chicken breast meat - total aerobic mesophiles (A), lactic acid bacteria (B) - stored in IT, SP, SLO, RO packages during 14 days at 4 ºC. Red line represents the suggested reference value of each bacterial group [24].

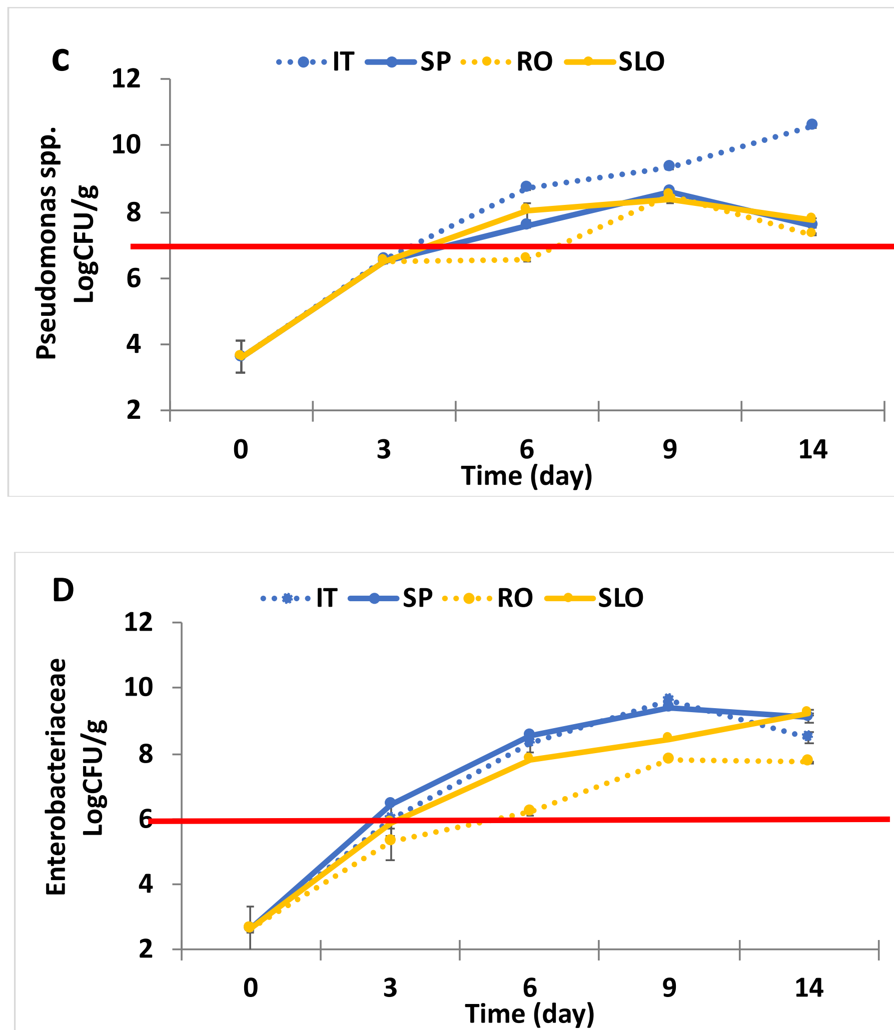


Figure 3a. Microbial profile of chicken breast meat - *Pseudomonas* spp. (C), Enterobacteriaceae (D), *Staphylococcus* spp. (E) - stored in IT, SP, SLO, RO packages during 14 days at 4 ºC. Red line represents the suggested reference value of each bacterial group [24].

4.4.2 | pH measurement

The pH values of chicken breast meat samples that deposited in the packages for 14 days at 4 ºC (Table 3) showed an increment in the case of all packages. The initial pH value of each sample was around 6.1-6.4 which were slightly higher than what reported by Chmiel *et al.* (around 5.9) [25].

During the first 3 days of storage, the pH value was slightly increased in all samples. However, a significant rise was noticed from T3 up to T14 for the meat stored in IT, SLO and SP, but the pH value of RO sample slightly dropped from T9 to T14. During whole study period,

the biggest variation was found in IT and SLO samples that increased for more than 1 value. The increasing trend in chicken meat pH during storage is attributable to the microbial metabolic activities - proteolysis that cause the accumulation of metabolites in meat [26].

Table 3. pH value of chicken breast meat.

pH value	**0**	**3**	**6**	**9**	**14**
IT	6.43±0.09	6.38±0.03	6.83±0.17	6.85±0.03	7.58±0.25
SP	6.17±0.03	6.30±0.00	6.61±0.10	6.90±0.10	7.09±0.06
RO	6.22±0.04	6.24±0.02	6.60±0.11	7.10±0.35	6.96±0.08
SLO	6.18±0.00	6.27±0.09	6.63±0.16	7.10±0.14	7.52±0.32

4.4.3 | Sensorial analysis

Figures 4A - 4D show that the sensorial attributes of meat samples deteriorated with increasing storage time. During the first 3 days of storage, the scores of IT, RO and SLO slightly decreased. Sensorial characteristics, such as odour, colour, elasticity, of SP meat (Figure 4B) was decreased after 3 days, except the meat aspect. It is interesting to notice that in our study, the lost of good aspect was considered as increased presence of slime. As mentioned before, SP package is paper coated with wax. During the assessment, panellists reported that meat stored in SP package showed a dryness on meat surface that demonstrated darker colour compared to other samples. Therefore, the high scores of SP sample aspect were ascribed by the absence of slime. However, due to the drying property of SP paper, the meat that stored inside greatly changed its colour, from fresh pink (t0) to dark pink/red (t4). Also, the characteristic meat odour of SP was not perceived by any panellists at the end of storage.

As for the IT sample (Figure 4A), the meat general characteristics were preserved until 6 days of storage, with slightly decrement. From day 9 to day 14, there was a significantly reduction in all attributes and a lost of freshness in the end. The same trend was also observed in SLO sample (Figure 4D), a noticeable reduction was found during the last storage period. However, SLO showed better elasticity than other samples. Compared to other samples, RO showed generally higher scores during each study time point. Even at day 14, the meat colour and elasticity were preserved.

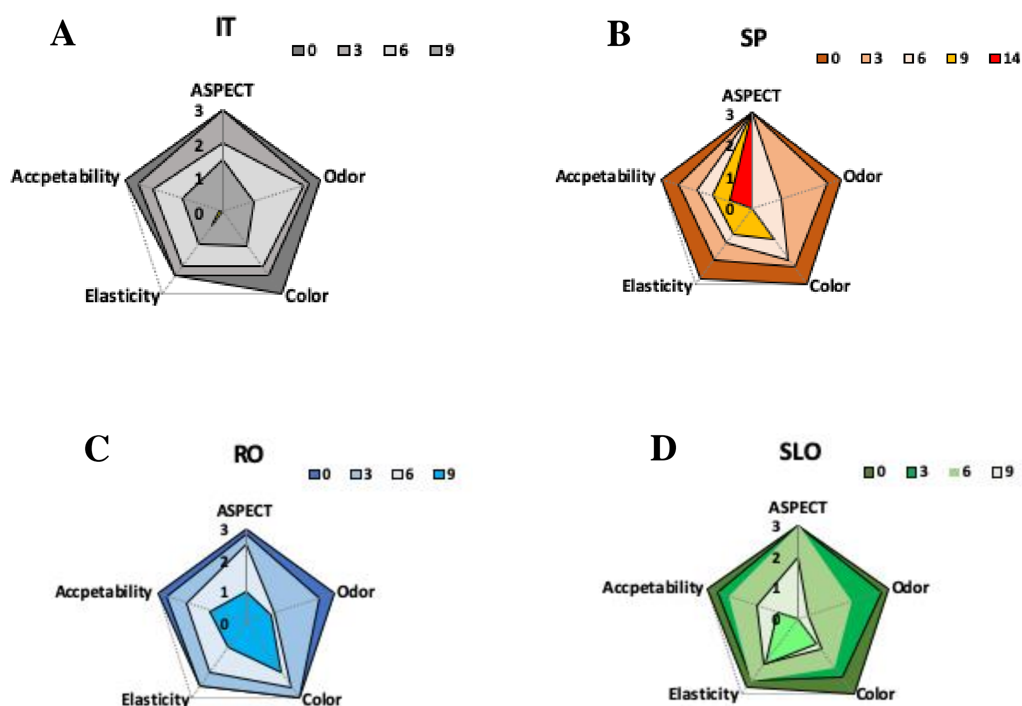
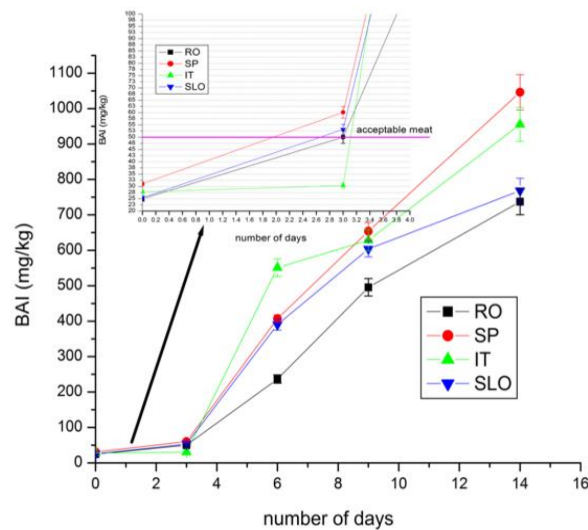


Figure 4. Sensorial spiderweb chart of IT (A), SP (B), RO (C), SLO (D) meat at 0, 3, 6, 9 days of storage.

4.4.4 | Biogenic amines analysis

During 14 days of storage, the values of biogenic amine index (BAI) and the level of total biogenic amines (BAs) increased in all packages (Figures 4 A-E). The lowest value of BAI and BAs was formed in the meat deposited in SLO and RO paper and the highest in the SP paper. The chemical quality index (CQI) parameter rides up as well during the 14 days of storage in all packages (Figure 4C). The slowest intensity was obtained in the case of RO paper at T14 and, contrary, the most accentuated degradation process occurred in the chicken breast meat deposited in the IT paper. Spermidine (SPD)/spermine (SPM) ratio (Figure 4D) decreased in the first 3 days in the case of all packages, suggesting that the level of spermidine decreased (Figure 4C). The most accentuated decrease of SPD/SPM was measured for IT sample and the lowest for SP paper. This ratio continued to drop until day 6 for SP and RO but increased for IT and SLO. From day 6 until day 14, the ratio increased again for all packages. Levels of putrescine and cadaverine increased in the case of all packages (Figures 4A and 4B). The general trend of the spermidine evolution is declining in the case of all packages, the most accentuated being recorded for IT, followed by SLO, RO and SP. Histamine level raised up during storage in all packages (Figure 4D).

The most accentuated increase was noticed for SLO and IT while the most reduced for SP and RO. According to the Commission Regulation (EU) No 1019/2013 that modifies the (EC) No 2073/2005, the only biogenic amine regulated is histamine whose maximum level in fish and fishery products can be 200 or 400 mg kg^{-1} depending on the species and type of fishery product (EU No 1019/2013). The level of histamine is below 200 mg kg^{-1} in meat stored in all four packages.



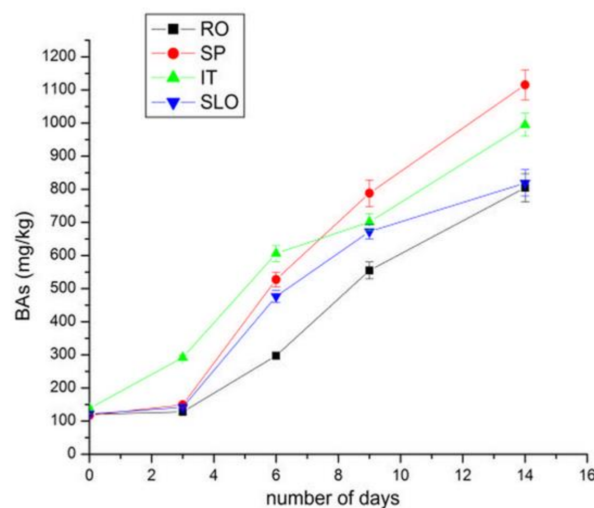
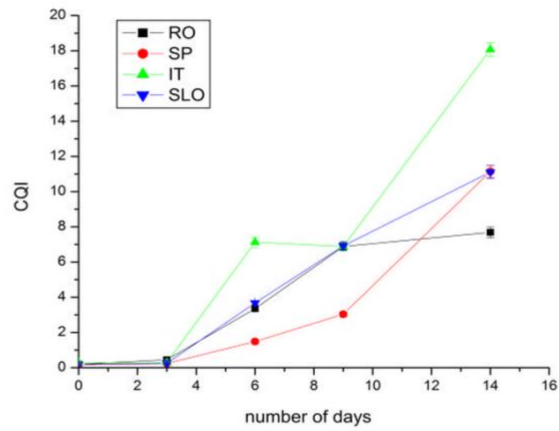


Figure 5. Biogenic amine index (BAI) - **A**, total biogenic amines (BAs) - **B** of the chicken breast meat stored in RO, SP, IT, SLO packages.



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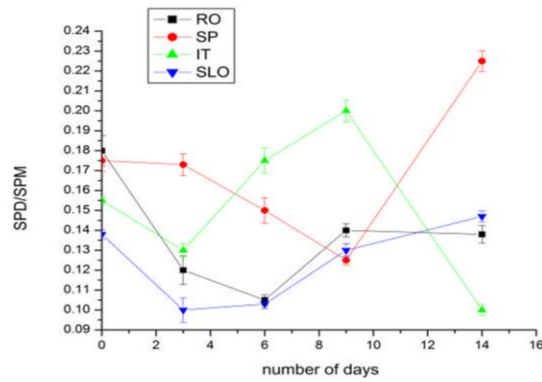

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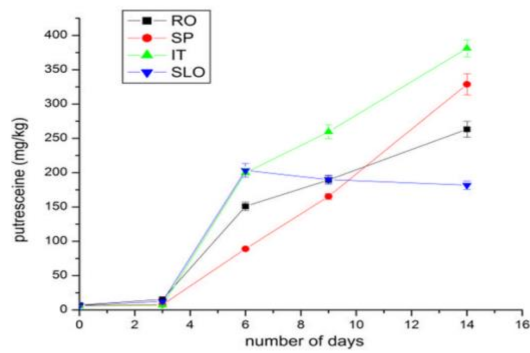

E

Figure 5a. Chemical quality index (CQI) – **C**, spermidine (SPD)/spermine (SPM) ratio – **D**, putrescine – **E,** of the chicken breast meat stored in RO, SP, IT, SLO packages.

4.4.5 | Correlation between packaging properties and chicken meat parameters

Taking into considerations of the packaging characteristics (density, roughness, breaking length, bending resistance) and packaging properties (capillary-hydroscopic, barrier - water vapor permeability, antimicrobial, wettability - contact angle, migration to food stimulants), we found that RO package (polyethylene-paper) possessed good food packaging characteristics, barrier properties against water vapor, low solubility, high contact angle, which were suitable for high-moisture and acidic food storage. These results were further confirmed by the shelf-life assessment on raw chicken meat, RO-stored meat showed minimum changes in microbial count, pH value, chemical indexes (BAs, BAI) and sensorial attributes, demonstrating its preservation efficiency in maintaining meat quality and safety under current storage conditions. SLO package (hydrophobized starch-paper) was slightly weaker than RO package in preserving meat parameters, but it was still better than IT and SP package and it was the only package that demonstrated antimicrobial ability through modified agar test. However, due to poor stability of the hydrophobized starch under acidic conditions, SLO package was not suitable for acidic food storage. On the contrary, SP package (wax-paper) failed the packaging requirements due to its poor barrier property, antioxidant capacity, recyclability, stability in high-moisture and acidic conditions. In addition, the high content of metal in SP package caused high electrical conductivity and possible migration from packaging to food matrix.

4.5 | DISCUSSION AND CONCLUSIONS

The study characterized the effects of four paper-based packages on the microbial counts, pH values, sensorial characteristics, chemical parameters of chicken breast meat during chilled storage for 14 days. For the packaging materials, RO and IT packages are composed by paper coated with polyethylene film, SP is double-waxed paper, and SLO is paper modified with hydrophobized starch.

The microbiological analysis revealed that different packaging materials, air exposure, and chilled storage acted differently. Among all samples, the microbial count of RO meat microbiota was lower than other samples during study period, whereas the amount of *Pseudomonas* spp. was higher in IT sample. *Pseudomonas* spp. are associated with the production of histamine and cadaverine [27], the results of chemical quality indicator (CQI) further confirmed the proliferation and metabolic activities of *Pseudomonas* spp. on IT sample. The pH value of meat increased, this effect was ascribed to the metabolic activities of microbes. As for the organoleptic characteristics of meat, the results showed that the SP package dried meat surface, leading to loss of smell and darker colour. On the contrary, SP, RO and SLO packages preserved better the general quality of meat. Moreover, the concentration of biogenic

amines detected among different samples revealed that poor bacteriological quality of SP sample, with highest amount of BAs, BAI, SPD/SPM indicators at the end of the storage. In the present study, we evaluated the general impacts of different packaging systems on meat microbial profile and on physio-chemical properties of meat. However, the mechanical and barrier properties of the packaging are also essential to complete the package efficiency study.

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CHAPTER V

SELECTION OF GRAPHENE COMPOSITES AND PROBIOTIC STRAIN USED FOR ACTIVE FOOD PACKAGING

5.1 | ABSTRACT

In food applications, graphene-based composites have been used as toxin/pesticide detectors and as antimicrobial packaging for the storage of food. As their wide existence in foods and health benefits associated with probiotics, *Lactobacillus* are being considered by food professionals as new bio-preservatives to ensure food safety and quality. In such circumstances, our aims are to investigate the potentials of graphene-based composites together with probiotic strains in food preservation applications. However, before building such prototype, we need to carefully characterize and select the proper composite and probiotic strain in order to obtain the desired results. Therefore, in the present study, we firstly investigated the interactions between selected graphene composites and probiotic strains (SYNBIO® and *Lactobacillus plantarum* IMC 509), both on solid agar and in liquid broth. Two pathogenic bacteria - *S. aureus* and *E. coli* – were used to examine and compare the activity of composites on bacteria. The results showed that pure graphene and graphene/titanium dioxide composites did not inhibit the growth of tested bacteria, whereas graphene composites that modified with silver particles showed antimicrobial capacity. Moreover, we noticed that probiotic bacteria - *Lactobacillus plantarum* IMC 509 - was able to tolerate the presence of 0.5% of Ag-GN-TiO_2 II 0.02 tt in liquid culture. Therefore, we aimed to continue our research with the combination of Ag-GN-TiO_2 II 0.02 tt and *L. plantarum* IMC 509 as active agents in manufacturing active food packaging.

5.2 | 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 [1]. In addition, researchers find that graphene sheets can also wrap around microorganisms and inhibit their metabolic activity and proliferation [1, 2].

However, there are controversial voices in the graphene-bacterial interactions as researchers found that the activity - either inhibition or enhancement - on microbial growth is highly dependent on the particle size, stability, concentration in solution as well as the properties of the target bacteria. For example, one study revealed that under low concentration, graphene oxide (GO) showed bactericidal activity on tested pathogens (*E. coli* and *S. aureus*), whereas the high concentration facilitated the GO-bacteria complex formation and inhibit/enhance bacteria growth [3]. In addition, graphene composites may act as a scaffold that enhance the growth of microbes. GO could act as growth-enhancer for *E. coli* [4]. Although the bactericidal activity of graphene composites has been widely conducted on pathogenic microbes, the effects on beneficial bacteria - *Lactobacillus* and *Bifidobacterium* - remained scarce. One study revealed that in bacterial media, GO was able to form anaerobic scaffold, which promoted the growth of key human gut microbiota - *Bifidobacterium adolescentis,* and also enhanced its antagonistic activity against pathogens [5]. However, due to the variances among different studies, it is difficult to compare and define the graphene composites – microbial interactions.

Based on the interests and requirements of the project, in the present study, we firstly examined the interactions between graphene-based composites and selected bacteria, including *Lactobacillus* and two pathogenic bacteria on solid agar surface. Then, after comparing the results of probiotics with that of pathogens, we further examined the bacterial viability of probiotic bacteria in broth media with addition graphene-based composites and the growth curve of each strain was plotted using spectrophotometer values. According to the bacterial growth curve under the different concentration of graphene-based composites, the tested *Lactobacillus* strain showed the highest tolerance to graphene composites and was selected as potential food bio-preservative. Moreover, the graphene-based composite that had least effect on *Lactobacillus* strain proliferation was considered as potential active compound for designing active packaging prototype.

5.3 | MATERIALS AND METHODS

5.3.1 | Graphene composites

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 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 1. Examples of graphene-based composites

5.3.2 | Bacterial strains and culture conditions

5.3.2.1 | Pathogenic strains

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℃ 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.

5.3.2.2 | Probiotic strains

Two probiotic products - SYNBIO® and *L. plantarum* IMC 509 - were chosen based on their human-origin, well-characterized functionalities and safety [6]. SYNBIO® is composed by two *Lactobacillus* strains with 1:1 ratio: *L. rhamnosus* IMC 501® and *L. paracasei* IMC 502®. 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. To obtain the single colony of each probiotic strain, broth culture was further streaked on de Man Rogosa and Sharpe (MRS) agar (Oxoid).

5.3.2.3 | Bacterial suspension preparation

Tested bacteria include: SYNBIO® strains, *L. plantarum* IMC 509, *E. coli* DSM 1103, and *S. aureus* ATCC 25923. Briefly, 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^{-1}.

5.3.3 | Agar well diffusion test and disc diffusion test

In this test, due to the size and dispersibility of the composites, the composites were either loaded on sterile filter paper disc: GN, Ag-GN, Ag-GN-TiO_2 II 0.02 nett (7) or deposited directly in the well of the inoculated agar plate (composites listed in Table 1). To prepare the graphene-loaded filter paper disc, 0.02g, 0.015g, 0.01g, 0.005g of the graphene-based composites were deposited on each filter paper discs (5mm diameter) and one plain filter paper disc was used as negative control.

Table 1. Graphene-based composites and their preparation procedures.

Composites	Test	Description
GN	disc and well	Graphene
Ag-GN	disc and well	Immersion of GN powder in silver nitrate solution, follo by reduction of silver ions in the presence of sodium cit followed by evaporation and drying at 60°C for 24h
GN-TiO_2 0.02 tt	well	Including the GN powder (0.02g) in the TiO_2 solution (2 followed by aging 3 weeks, drying at 60°C for 24h, the treatment at 500°C, 2h
Ag-GN-TiO_2 II 0.02 nett	disc and well	Immersion of GN-TiO_2 0.02 gel in silver nitrate solu 0.015M for 24h, followed by drying at 60°C, 24h
Ag-GN-TiO_2 II 0.02 tt	disc and well	Immersion of GN-TiO_2 0.02 gel in silver nitrate solu 0.015M for 24h, followed by drying at 60°C, 24h, the treatment at 500°C, 2h

5.3.3.1 | Disc diffusion method (DDM)

Briefly, Muller-Hinton agar (MH agar, Oxoid) was inoculated with tested bacterial suspension using sterile cotton swabs. Then the filter paper discs either with composites (different amount) or not (negative control) were placed on the agar surface using sterile forceps in the following manner (Figure 2), in which the composite-loading surface of the disc was in touch with agar. After incubation at 37℃ for 24h, the inhibition zone appeared around the filter paper disc, and it has been measured.

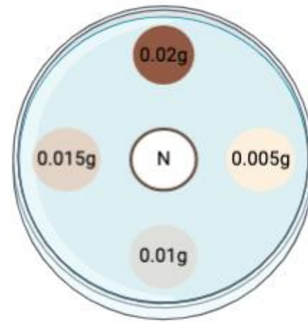


Figure 2. Scheme of the filter paper discs loaded on MH agar with different amount of composites (0.02g, 0.015g, 0.01g, 0.005g), N: negative control.

5.3.3.2 | Agar well diffusion method (WDM)

The same amount of graphene-based composites (0.02g, 0.015g, 0.01g, 0.005g) was deposited in 5mm diameter wells of the agar plate. Similar to disc diffusion test, the surface of MH agar was inoculated with tested bacterial strains, then wells were filled with graphene-based composites. The well in the centre was loaded with sterile distilled water as negative control. All the plates were incubated at 37℃ for 24h. The inhibition zone around the wells was measured.

5.3.3.3| Cell viability test

Five ml of bacterial cells (10^8 CFU ml^{-1}, SYNBIO® or *L. plantarum* IMC 509) were incubated with the listed graphene-based composites (Table 1) with concentration of 0.02g (0.4 mg ml^{-1}), 0.015g (0. 3 mg ml^{-1}), 0.01g (0. 2 mg ml^{-1}), 0.005g (0.1 mg ml^{-1}) in flasks with 45 ml of sterile MRS broth. A flask contained 45 ml sterile MRS with addition of 5 ml bacterial suspension was used as positive control (Figure 3). All the flasks were incubated in water bath at 37 ℃ under 130 rpm shaking speed for 24h. The growth curve of each strain was plotted

by recoding the optical density value of each sample at λ_{max} =560 nm with one-hour interval up to 9 hours using the spectrophotometer (UV-1601, Shimadzu). Also, 100 µl of each sample was taken for diluting and plating on MRS agar plate to further confirm the presence of live bacteria.

Later, only *L. plantarum* IMC 509 (10^8 CFU ml^{-1}) was inoculated with higher concentration (30 mg ml^{-1} and 0.5 mg ml^{-1}) of selected graphene-based composites (Table 3), the procedure was the same as described above.

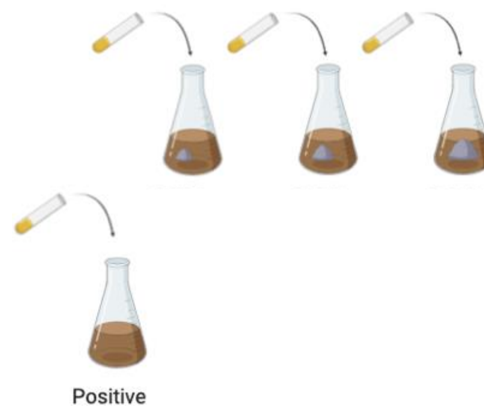


Figure 3. Scheme of inoculation of probiotic bacteria with graphene-based composites in co-culture tests (lower concentration: 0.02%-0.04% and higher concentration).

Table 2. Coculture of new graphene-based composites with and *L. plantarum* IMC 509 at higher concentrations (30 mg ml^{-1} and 0.5 mg ml^{-1})

Composites	Descriptions
Ag-GN-TiO_2 0.02 II ALA nett	GN powder was included in silver nitrate solution, sonication 60 min. Alanine solution 1% (wt) was included in TiO_2 solution during the preparation of TiO_2 sol, followed by aging, immersion in $AgNO_3$ 0.015M 24h, finally drying.
Ag-GN-TiO_2 0.02 II ALA tt	GN powder was included in silver nitrate solution, sonication 60 min. Alanine solution 1% (wt) was included in TiO_2 solution during the preparation of TiO_2 sol, followed by aging, immersion in $AgNO_3$ 0.015M 24h, finally drying thermal treated at 500°C, 2h.
Ag-GO-TiO_2 0.02 II ALA tt	GO flakes was included in silver nitrate solution, sonication 60 min. Alanine solution 1% (wt) was included in TiO_2 solution during the preparation of TiO_2 sol, followed by aging, immersion in $AgNO_3$ 0.015M 24h, finally drying thermal treated at 500°C, 2h.
Ag-GO-TiO_2 III 0.02 tt	GO-TiO_2 is prepared and immersed in $AgNO_3$ 0.015M 24h, drying and thermal treated at 500°C, 2h.
Ag-GN-TiO_2 II 0.02 tt	Immersion of GN-TiO_2 0.02 gel in silver nitrate solution 0.015M for 24h, followed by drying at 60°C, 24h, thermal treatment at 500°C, 2h

5.3.4 | 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.

5.4 | RESULTS

5.4.1 | Graphene-based composites preparation and characterization

The major characteristics were summarized as below: thermal treatment on graphene composites resulted higher λ irradiation activation. Through the wastewater decontamination test, Ag-GN-TiO2 I 0,04 nett showed the best photocatalytic activities. The Ag content in composite did not alter the photoactivity of the composites. Sonification process improved the dispersibility and homogeneity of GO flasks in ethanolic phase during composite

manufacturing. As a biomolecule, alanine was used in green synthesis method to reduce graphene oxide. The presence of alanine showed no activity on composite photoactivities. Overall, the characterization studies carried out by partners from UTCN, CEPRO and NIC groups considered Ag-GN-TiO_2 II 0.02 tt as the best graphene composites to be incorporated in active packaging.

5.4.2 | Disc diffusion method

Disc diffusion method aims to evaluate the inhibitory activity measured by appearance of inhibition halo around antimicrobial-containing discs. Pure graphene composite (GN) and GN-TiO_2 (Table 3) showed no activity on the growth of tested probiotic and pathogenic strains: SYNBIO®, *Escherichia coli* DSM 1103, *Staphylococcus aureus* ATCC 25923, whereas Ag-GN and Ag-GN-TiO_2 composites was affecting the growth of all tested bacteria (Tables 4-5). This result was presumed by Ag+ modification that enhanced the antimicrobial ability of the composites.

Regarding the antimicrobial efficiency on pathogenic strain - *S. aureus* and *E. coli*, Ag-GN and Ag-GN-TiO_2 acted differently. Composite, Ag-GN, acted stronger against the growth of *S. aureus* than *E. coli*. Also, the composite exerted anti-*S. aureus* activity in a concentration-dependent way. Probably, due to the non-homogenous distribution of composite on *E. coli* loaded agar, similar inhibitory effect was not observed. On the contrary, the modified TiO_2 composite (Ag-GN-TiO_2) showed stronger inhibitory activity on *E. coli* than on *S. aureus*. Different from our study, rGO-TiO_2 inhibited the growth of both *E. coli* and *S. aureus* with stronger activity on *S. aureus* (larger zone of inhibition). The author presumed that the photocatalytic property of rGO-TiO_2 enhanced its antimicrobial efficiency [7].

However, due to the differences in composite composition and preparation method among studied, it is hard to compare the results. In comparison to pathogenic bacteria, composites of Ag-GN and Ag-GN-TiO_2 had less ability in limiting the growth of SYNBIO® with less inhibition diameter observed. The difference was statistically significant especially at higher concentration used, 0.02g and 0.015g for Ag-GN, 0.02g for Ag-GN-TiO_2. It is interesting to notice that higher amount of Ag-GN-TiO_2 II 0.02 tt (0.02g and 0.015g) had less impact than lower concentration on SYNBIO®, suggesting probably due to the cell wall differences of *Lactobacillus* strains tested. Other techniques, such as scanning electron microscope, may needed to visualize their interactions and further confirm the obtained result.

Table 3. Inhibition zone diameter (mm) of GN and GN-TiO_2 on SYNBIO®, *Escherichia coli* DSM 1103 and *Staphylococcus aureus* ATCC 25923 by DDM.

GN/GN-TiO_2	0.02g	0.015g	0.01g	0.005g
SYNBIO®	-	-	-	-
E. coli DSM 1103	-	-	-	-
S. aureus ATCC 25923	-	-	-	-

Table 4. Inhibition zone diameter (mm) of Ag-GN on SYNBIO®, *Escherichia coli* DSM 1103 and *Staphylococcus aureus* ATCC 25923 by DDM.

Ag-GN	0.02g	0.015g	0.01g	0.005g
SYNBIO®	9.00±0.00	8.95±0.07	9.00±0.00	8.10±0.14
E. coli DSM 1103	11.20±0.04*	11.20±0.02*	11.20±0.14	9.30±0.05
S. aureus ATCC 25923	13.15±0.64 *	11.95±1.06*	11.45±1.63	10.95±2.33

* Significantly different from SYNBIO® by Student's *t* test.

Table 5. Inhibition zone diameter (mm) of Ag-GN-TiO_2 II 0.02 nett on SYNBIO® , *Escherichia coli* DSM 1103 and *Staphylococcus aureus* ATCC 25923 by DDM.

Ag-GN-TiO_2 II 0.02 nett	0.02g	0.015g	0.01g	0.005g
SYNBIO®	8.80±0.00	9.15±1.77	9.90±0.71	9.35±0.78
E. coli DSM 1103	12.00±0.04*	11.00±0.02	11.00±0.14	10.90±0.05
S. aureus ATCC 25923	10.90±0.99*	10.15±1.20	9.00±1.13	8.05±0.07

* Significantly different from SYNBIO® by Student's *t* test.

5.4.3 | Well diffusion test

Similar to disc diffusion test, pure graphene composite (GN) and GN-TiO_2 showed no activity on the growth of tested probiotic and pathogenic strains: SYNBIO®, *Escherichia coli* DSM 1103 and *Staphylococcus aureus* ATCC 25923 (data not shown). On the contrary to our study, Karimi *et al.* [8] showed that graphene/titanium dioxide nanocomposites loaded cotton completely inhibited the growth of *S. aureus*, *E. coli* and *Candida albicans*. The presence of $Ag+$ ions, such as in Ag-GN, Ag-GN-TiO_2 II 0.02 nett, and Ag-GN-TiO_2 II 0.02 tt (Table 6-8), exhibited antimicrobial effect on tested bacteria. In general, the inhibition zone diameter obtained by disc-diffusion method was larger than that of well-diffusion method. The difference was ascribed to the composites that deposited in wells could not diffuse, whereas graphene-based composites disc was in direct contact with loaded bacteria, resulting higher antimicrobial

ability. According to the results listed in Table 9, Ag-GN was more active against *E. coli* than *S. aureus* inhibitory activity of composites deposited in well, which was contrary to results obtained by disc diffusion test, but like the effect of rGO-TiO_2 [7]. Moreover, *S. aureus* was more susceptible to the composites of Ag-GN-TiO_2 II 0.02 nett, and Ag-GN-TiO_2 II 0.02 tt. Due to the non-homogenous and nondiffusible properties of composites, there was no concentration-dependent effect observed in well-diffusion method, only bacteria that close to the well were inhibited. Studies have shown that silver/graphene oxide exhibited bactericidal activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* at pH 5.6 [9].

Table 6. Inhibition zone diameter (mm) of Ag-GN on SYNBIO®, *Escherichia coli* DSM 1103 and *Staphylococcus aureus* ATCC 25923 by WDM.

Ag-GN	0.02g	0.015g	0.01g	0.005g
SYNBIO®	8.10±1.41	6.55±0.49	6.00±0.14	7.50±0.00
***E. coli* DSM 1103**	7.40±0.04	8.00±0.02	7.10±0.14	6.00±0.05
***S. aureus* ATCC 25923**	6.40±0.85	5.20±1.13	5.20±1.13	5.50±0.14

* Significantly different from SYNBIO® by student's test.

Table 7. Inhibition zone diameter (mm) of Ag-GN-TiO_2 II 0.02 nett on SYNBIO® , *Escherichia coli* DSM 1103 and *Staphylococcus aureus* ATCC 25923 by WDM.

Ag-GN-TiO_2 II 0.02 nett	0.02g	0.015g	0.01g	0.005g
SYNBIO®	6.00±0.28	6.05±0.35	6.15±0.21	5.70±0.14
***E. coli* DSM 1103**	6.00±0.04	6.00±0.02	4.70±0.14	4.70±0.05
***S. aureus* ATCC 25923**	7.90±0.14	6.90±1.56	7.50±1.84	7.40±1.70

Table 8. Inhibition zone diameter (mm) of Ag-GN-TiO_2 II 0.02 tt on SYNBIO®, *Escherichia coli* DSM 1103 and *Staphylococcus aureus* ATCC 25923 by WDM.

Ag-GN-TiO_2 II 0.02 tt	0.02g	0.015g	0.01g	0.005g
SYNBIO®	6.05±0.49	4.60±1.27	4.05±1.91	3.35±0.92
***E. coli* DSM 1103**	6.60±0.04	8.10±0.02	5.10±0.14	5.00±0.05
***S. aureus* ATCC 25923**	8.80±0.28	8.30±0.42	5.00±1.41	3.50±0.71

5.4.4 | Cell viability test

The preliminary studies were conducted using the lower concentrations (0.02g, 0.015g, 0.01g, 0.005g) of the graphene-based composites (Table 1 and Table 2). All of them had no effect on the growth of tested probiotic strains - SYNBIO® and *L. plantarum* IMC 509 (data not shown). The growth of the multi-strains of SYNBIO® was difficult to monitor in broth, and the growth curves of *L. plantarum* IMC 509 with presence of composites had a similar trend to the positive control (data not shown). Therefore, we focused on *L. plantarum* IMC 509 with increased concentration of graphene composites, with 30 mg ml^{-1} and 0.5 mg ml^{-1} that have been co-incubated with *L. plantarum* IMC 509 (Figures 4A-E). Higher concentrations of composites inhibited the growth of *L. plantarum* IMC 509 (Figures 4A-D), with exception of Ag-GN-TiO_2 II 0.02 tt (Figure 4E). The plate count method further confirmed that the OD values were ascribed to the presence of live bacteria of *L. plantarum* IMC 509 (data not shown).

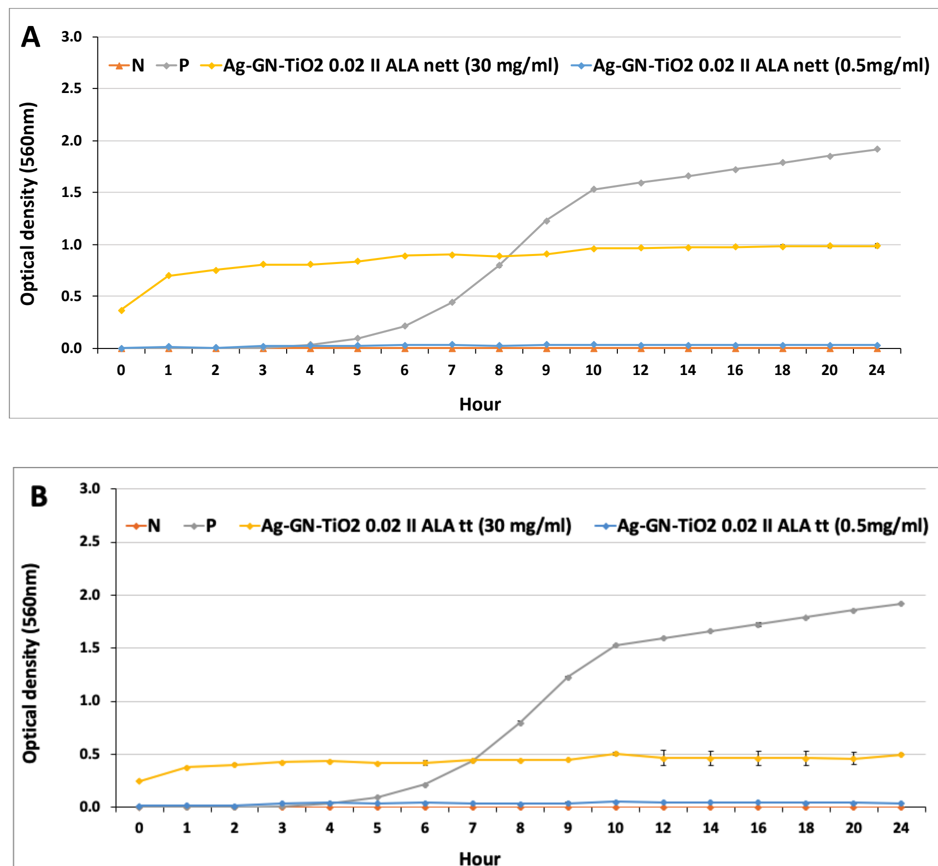
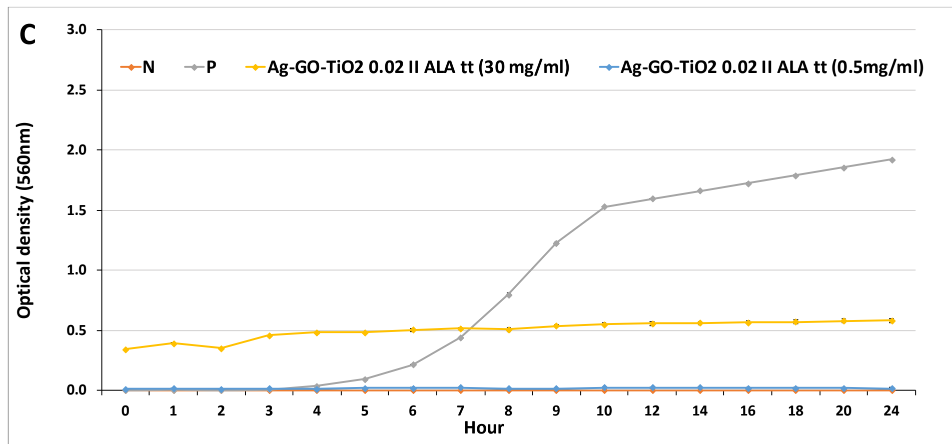
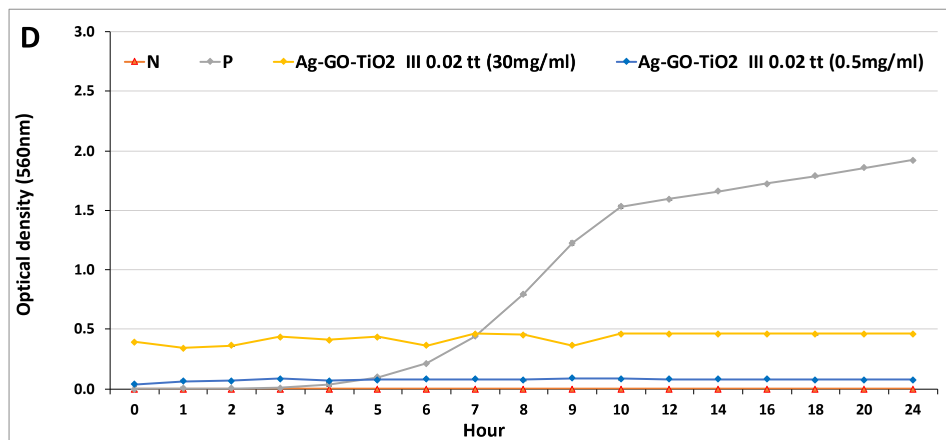


Figure 4. The growth curve of *L. plantarum* IMC 509 with the presence of Ag-GN-TiO_2 0.02 II ALA nett (A), Ag-GN-TiO2 0.02 II ALA tt (B).





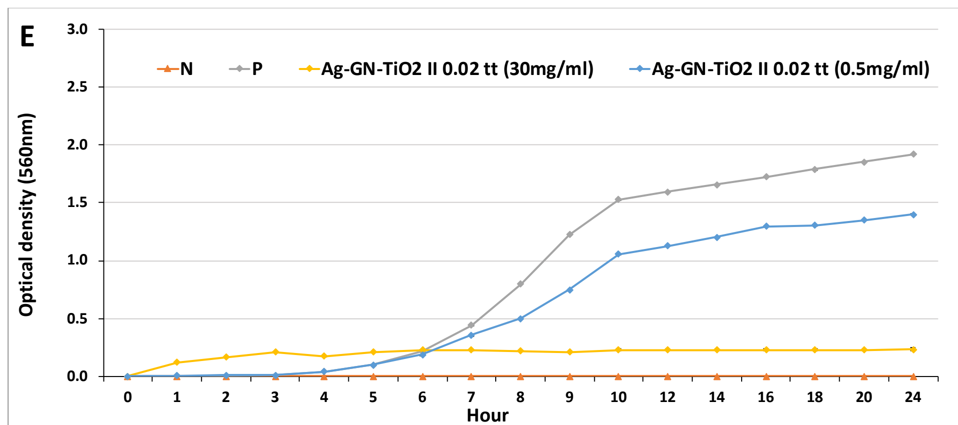


Figure 4a. The growth curve of *L. plantarum* IMC 509 with the presence of Ag-GO-TiO_2 0.02 II ALA tt (C), Ag-GO-TiO_2 III 0.02 tt (D), Ag-GN-TiO_2 II 0.02 tt (E).

5.5 | DISCUSSSION AND CONCLUSIONS

In the present study, we evaluated the impacts of graphene-based composites on the growth of Gram-positive and Gram-negative strains both on solid agar through disc diffusion method and well diffusion method. Moreover, we also monitored the growth of *Lactobacillus* in liquid media with addition of graphene composites. Pure GN and GN-TiO_2 composites exhibited no antimicrobial activity on selected bacterial strains. On the contrary, composites with Ag+ functional groups showed inhibitory activity to all tested strains in all tests. In general, *Lactobacillus* strains demonstrated higher tolerance to graphene-based composites than pathogenic strains used in the present study. Comparing the results of disc diffusion and well diffusion methods, we found that the disc diffusion method seemed more proper as it allowed direct contact between non-diffusible composites with bacteria that inoculated on agar surface. The different antimicrobial behaviours were presumed by the differences in chemical structures, light activation, the number of tested microorganisms, pH value. The zone of inhibition appeared onto inoculated agar when the composites applied containing silver ions, demonstrating the antimicrobial activities in our study are mainly ascribed to the silver ions. In broth media, although the growth of *L. plantarum* IMC 509 was inhibited by majority of composites under tested concentrations, it can tolerate the composite-Ag-GN-TiO_2 II 0.02 tt-at 0.5mg ml^{-1}. Therefore, based on the above-mentioned findings and agreements from the data coming from the other partners, we considered to use the composite-Ag-GN-TiO_2 II 0.02 tt - as active composite in manufacturing graphene-based food packaging. Current study gave a preliminary insight into the interactions between selected graphene-based composites and microbes on agar surface or in liquid broth. Although the combination of graphene composites and probiotic bacteria is mainly applied on packaging material, the *in situ* interactions with packaging materials and food components are factors need to be examined. Further research is needed to quantify the effective amount as well as to investigate better the antimicrobial mechanisms of interest composites possibly by visualization methods, such as using scanning electron microscope. Moreover, the different preparation method, type of composites, final concentration tested, and target microorganisms make the comparison extremely hard between different study. Standardizing the production procedure and using proper methodology are also essential for fully understand the mechanisms behind graphene-based composites.

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CHAPTER IV

LACTOBACILLUS STRAINS TREATMENT ON COMMERCIAL PACKAGING PAPER AS PRELIMINARY STUDY FOR EXTENDING THE SHELF-LIFE OF CHICKEN MEAT

The content of this chapter corresponds to what it has been published as article by
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6.1 | ABSTRACT

Lactobacillus plantarum IMC 509 and SYNBIO® (1:1 combination of *Lactobacillus rhamnosus* IMC 501® and *Lactobacillus paracasei* IMC 502®), were investigated as natural anti-spoilage agents sprinkled on commercial packaging, polyethylene (PE) laminated paper sheet, to extend raw chicken breast meat shelf-life at 4°C. Slices of chicken breast meat, wrapped in commercial packaging papers sheet sprayed by *Lactobacillus* cell suspensions, were analyzed at 0, 2, 5, 7 days of storage. Total aerobic mesophilic bacteria, pH value, sensory changes and biogenic amines (BAs) production were checked. *Lactobacillus* strains viability on paper packaging was also monitored. The best ability of preserving meat parameters was observed in Lactobacillus sprayed PE laminated papers compared to control paper. The BAs extracted from meat preserved into *L. plantarum* IMC 509 sprayed PE laminated paper sheet were significantly less than other samples. *L. plantarum* IMC 509 showed a high stability, keeping its viability in all paper surfaces. It reduced both spoilage microbial growth and BAs accumulation, providing further evidence for its suitability to be used in packaging application. *Lactobacillus* strains may be assumed as bio-preservatives applied on papers sheet to extend chicken meat shelf-life without affecting the flavor.

6.2 | INTRODUCTION

Poultry meat is a valued food for nutrition due to its high contents of digestible proteins, unsaturated lipids, vitamins (especially B-group), minerals and low carbohydrate contents. Its moderate energy content has drawn a lot of attention from nutritionists, athletes, dieters and healthy eaters [1]. The typical characteristics (high moisture content, moderate pH value, nutrient-rich) [2] of poultry meat make it an easy spoiling matrix by certain microbes, such as psychrotrophic bacteria [3]. Microorganisms, which are introduced into meat from the natural environment or by improper handling of market operators, air or contaminated water, may survive during handling and processing and multiply quickly under high temperature or temperature fluctuation conditions [4,5]. The effect of all these factors may lead to the appearance of undesirable colour, texture, flavour, odour and slime on meat surface. The 58.5 % of all food spoilage bacteria species affecting meat surface is composed by *Pseudomonas* spp. [6] some bacteria strains, especially, *Escherichia* spp., *Enterobacter* spp., *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp., *Clostridium perfringens*, *Streptococcus* spp., *Lactobacillus* spp. and *Leuconostoc* spp. have been shown to be linked to histamine production [7]. Histamine is one of the biogenic amines (BAs) described as a group of low molecular weight, heat stable, non-volatile, basic nitrogenous compounds with biological activity [8]. BAs are mainly created by microbial decarboxylation of amino acids in foodstuffs. Therefore, knowing the levels of BAs in foods is an important aspect to assess the health hazard arising from food consumption and can be used as food quality markers [9].

Lactic Acid Bacteria (LAB) have been isolated from various food and human sources, many strains are defined as probiotics with well-characterized molecular, genome structure and secretory systems. They are widely applied as natural food bio-preservatives [10] or starter cultures in various kinds of food products since ancient times [11] The main antimicrobial mechanisms involved in the preservation process are through competing nutrients and space with spoilage bacteria, the production of a wide variety of antimicrobial substances such as organic acids, hydrogen peroxide, carbon dioxide, ethanol, bacteriocins. The production of organic acids lowered the surrounding pH value, thus creating unfavourable growth environment for spoilage bacteria [12]. Most studies did focus on the application of probiotic strains as additives on/in food to enhance food safety or extend food shelf-life [13,14]. However, few of them shed light on utilizing probiotics as preservatives which being applied as a part of active packaging material.

This study aimed to evaluate the versatility of some *Lactobacillus* strains, already characterized as probiotic bacteria [15,16] being applied on commercial packaging paper in order to extend fresh chicken breast meat storage time without affecting meat quality. The choice of commercial packaging paper sheet was a preliminary approach to investigate the

feasibility of such a kind of probiotic treatment. The following parameters were used to assess the effect of PE laminated paper sheets with PE surface sprayed with probiotics, on chicken breast meat qualities: (a) the total viable count of aerobic mesophilic bacteria; (b) the levels of BAs, especially some specific indexes as chicken breast fillet freshness markers; (c) the organoleptic characteristics; (d) the viability of probiotic strains over paper during meat storage period and longer.

6.3 | MATERIALS AND METHODS

6.3.1 | Preparation of probiotic strain PE laminated paper sheet

The addition of probiotics on paper sheet was realized by spraying bacterial cells suspension onto commercial PE laminated paper sheet (40 cm x 30 cm), kindly provided by a local supermarket (Camerino, Italy). Before the spraying process, all PE surface of packaging paper sheet was sterilized by UV lamp up to 3 hours under Biohazard hood (FASTER, Milan, Italy). 0.5 grams of *L. plantarum* IMC 509 and SYNBIO® lyophilized powders (10^{11} CFU/g) (SYNBIOTEC Srl, Camerino, Italy) were dissolved in 10 ml of sterile PBS buffer. These probiotic strains [15] were chosen basing on their well-studied safeness, antimicrobial properties and adaptation capability [16]. After spraying a drying process was done in oven (Heraeus, Hanau, Germany) at 45°C until the weight reached a constant value.

6.3.2 | Meat preparation in probiotic strains PE laminated paper sheets

Slices of fresh breast meat derived from one chicken, purchased from a local supermarket, were immediately transferred to the laboratory, where they were further divided, wrapped and stored in previously prepared PE laminated paper sheet: a) sterile packaging PE laminated paper sheet, as control sample (CTR); b) packaging PE laminated paper sprayed with *L. plantarum* IMC 509, labelled as LP sample; c) packaging PE laminated paper sprayed with SYNBIO®, as SYN sample. Each sample of two slices of breast meat (about 100g) was stored at 4 °C. At days 0, 2, 5 and 7, microbiological, chemical and sensorial analysis were conducted.

6.3.3 | Enumeration of total aerobic mesophilic bacteria

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). Serial dilutions were made for each replicate sample and were further plated on Plate count agar (PCA-Oxoid, Basinstoke, UK) for enumerating total aerobic mesophilic bacteria. Incubation process was done at 35 ± 1°C for 24-48 h in aerobic condition.

6.3.4 | Biogenic amines quantification and determination of freshness indexes

6.3.4.1 | Materials and standards

The BAs studied are reported in Table 1. All of them and 1,7-diaminoheptane used as internal standard ($C_7H_{18}N_2$, CAS No. 646-19-5) and dansyl chloride ($C_{12}H_{12}ClNO_2S$, CAS No. 605-65-2) were supplied by Sigma-Aldrich (Milano, Italy).

Table 1. Biogenic amines monitored in the study.

Biogenic amine	Code	Formula	CAS No.
Spermine tetrahydrochloride	SPE	$C_{10}H_{26}N_4$ 4HCl	306-67-2
Spermidine trihydrochloride	SPD	$C_7H_{17}N_3$ 3HCl	334-50-9
Cadaverine dihydrochloride	CAD	$C_5H_{14}N_2$ 2HCl	1476-39-7
Putrescine dihydrochloride	PUT	$C_4H_{12}N_2$ 2HCl	333-93-7
Histamine dihydrochloride	HIS	$C_5H_9N_3$ 2HCl	56-92-8
Tyramine hydrochloride	TYR	$C_8H_{11}NO{\cdot}HCl$	60-19-5
2-phenylethylamine hydrochloride	PHE	$C_8H_{11}N{\cdot}HCl$	156-28-5
Tryptamine hydrochloride	TRY	$C_{10}H_{12}N_2$ HCl	343-94-2

Individual stock solutions of BAs were prepared by dissolving 10 mg of each compound in 10 ml of HCl 0.1 mol l^{-1} (Merck, Darmstadt, Germany) and stored at 4 °C. Standard working solutions at various concentrations were daily prepared by appropriate dilution of different aliquots of the stock solutions with 0.1 mol l^{-1} HCl. HPLC- grade methanol and sodium sulphate >99 % were supplied by Sig- ma-Aldrich. HPLC-grade acetonitrile and methanol were supplied by Merck. All the solvents and solutions were filtered through a 0.45 µm PTFE filter from Supelco (Bellefonte, PA) before use. Cartridges Discovery SPE DSC-18 Silica Tube (6 ml, 1 g) were from Supelco.

6.3.4.2 | Biogenic amines extraction and analysis

The extraction and analysis of BAs was performed following the methods of [17], with slight modifications. Slices of chicken samples were grinded with a blender and then 5 g of sample were homoge- nized with 25 ml of 5 % TCA for 2 minutes using an Ultra-Turrax S 18N-10 G (IKA-Werke Gmbh & Co., Staufen, Germany). The ob- tained homogenate was centrifuged at 2500 rpm for 10 min. Due to their lack of chromophores, BA once extracted needed to be derivat- ized before analysis by liquid chromatography. Briefly, 1 ml of the supernatant TCA extract was derivatized with 300 µl of a saturated $NaHCO_3$ solution, 200 µl of a NaOH solution (2 mol l^{-1}) and 2 ml of dansyl chloride solution (10 mg ml^{-1} acetone). Dansylating reaction was conducted under magnetic stirring, in the dark at 45 °C for 45 min.

After derivatization, the residual dansyl chloride was neutralized by adding 100 µl of 28 % NH_4OH. The mixture was evaporated to 1.5 ml under flow of N_2. The aqueous residue was purified by solid phase extraction (SPE) using a SPE DSC-18 cartridge (6 ml, 1 g), which was activated with 4 ml of acetonitrile and conditioned with 4 ml of Milli-Q water using a vacuum system. The aqueous residue was then loaded onto the cartridge at a flow rate <0.5 ml min^{-1}. The cartridge was then washed with 4 ml of water and thoroughly dried under vacuum.

Analytes were finally eluted from the cartridge using 4 ml of acetonitrile. The eluting solution was filtered on 0.45 µm PTFE filter and analysed in HPLC-DAD.

The analysis of BAs was performed through HPLC-DAD from Agilent 1100 series (Agilent Technologies, Santa Clara, CA, USA). The injection volume was 20 µl. The separation of analytes was per- formed on an analytical column Gemini C18 (250 x 3.0 mm, 5 µm) preceded by a security guard column C18 (4 x 3 mm, 5 µm), both from Phenomenex (Torrance, CA, USA). The mobile phase used for analytes separation was made of water (A) and methanol/acetonitrile (70:30, v/v) (B), at a flow rate of 0.5 ml min^{-1}. The gradient program was: 0 min 60 % B, 0–10 min 70% B, 10–20 min 90 % B, 20–26 min 100 % B, 26–30 min 100 % B, 30–35 min 60 % B and finally 35-50 min 100 % B. Analytes were detected at 254 nm.

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:

$$\mathrm{BAI} = putrescine + cadaverine + histamine + tyramine$$

$$CQI = \frac{putrescine + cadaverine + hista\min e}{sper\min e + spermidine + 1}$$

$$SPD / SPM = \frac{spermidine}{sper\min e}$$

$$\text{Total BAs} = putrescine + cadaverine + histamine + tyramine + spermine + spermidine + phenylethylamine.$$

The C.Q.I was proposed by [18] to evaluate the quality of fish and seafood. The B.A.I was created by Veciana- [19] to improve the C.Q.I. The SPD/SPM ratio was proposed by [20] and is considered suitable to assess the chicken meat quality [17]. The Total BAs was used to have more ample vision on the BAs evolution in the different type of samples.

6.3.5 | pH measurement

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

6.3.6 | Chicken meat sensory characteristic evaluation

Ten members were selected and trained on meat description following the methods reported by [21] 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 2).

Table 2. Sensorial attribute values for raw chicken meat (modified from [21])

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
Colour	Pink	3
	Dark pink	2
	Pale pink/yellow	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 | Probiotic strains viability on packaging PE laminated paper sheet

To investigate the viability of probiotic bacteria on the packaging materials surface, at each time point, paper sheet of 9 cm^2 was cut from both surfaces: one that was in contact with the meat and another that was not. Sterile cotton swabs wetted with saline solution were used to

brush the cut packaging surface and subsequently the swab heads were transferred into tube with 5 ml saline solution, vortexing [22] and serial dilutions were made and plated on corresponding de Man, Rogosa, Sharpe, modified with the addition of vancomycin and gentamicin, agar plates (MRS – VWR International, Milan, Italy [23] then incubated at 36 ± 1 °C for 48 h in aerobic condition. The survival rate of each probiotic strains was also calculated by using the following formula:

$$survival\,\% = \left(\frac{CFU\ N}{CFU\ N0}\right) x\ 100\%$$

CFU N is the count (CFU ml^{-1}) of probiotic strain on packaging sheet at each sampling time; CFU N_0 is the count (CFU ml^{-1}) of probiotic strain on PE laminated paper sheet at the preparation day. The value was expressed as percentage.

6.3.8 | Statistical analysis

All the microbiological and chemical analyses were carried out in triplicate. The results are expressed as mean ± SD. The Student's t test was used to assess the statistical significance of the differences between the chicken samples wrapped in the different packaging. Differences were considered significant for $P < 0.05$.

6.4 | RESULTS

6.4.1 | Total aerobic mesophilic bacteria on preserved chicken meat

Although the initial number of aerobic mesophilic bacteria was already quite high (around 6 log CFU g^{-1}), the counts arose rapidly in the meat wrapped inside the three packaging systems during the first two days, increasing 4 log in SYN-packed meat, 3 log in CTR-packed meat and 2 log increment in LP-packed meat (Figure 1). The trend slowed down in the following storage days, with around 9.5 log CFU g^{-1} in LP-meat and around 10.7 log CFU g^{-1} in both CTR and SYN-packed meat. Chicken slices in LP-packaging had the lowest mesophiles counts vs CTR sample during time, though the difference was not significant.

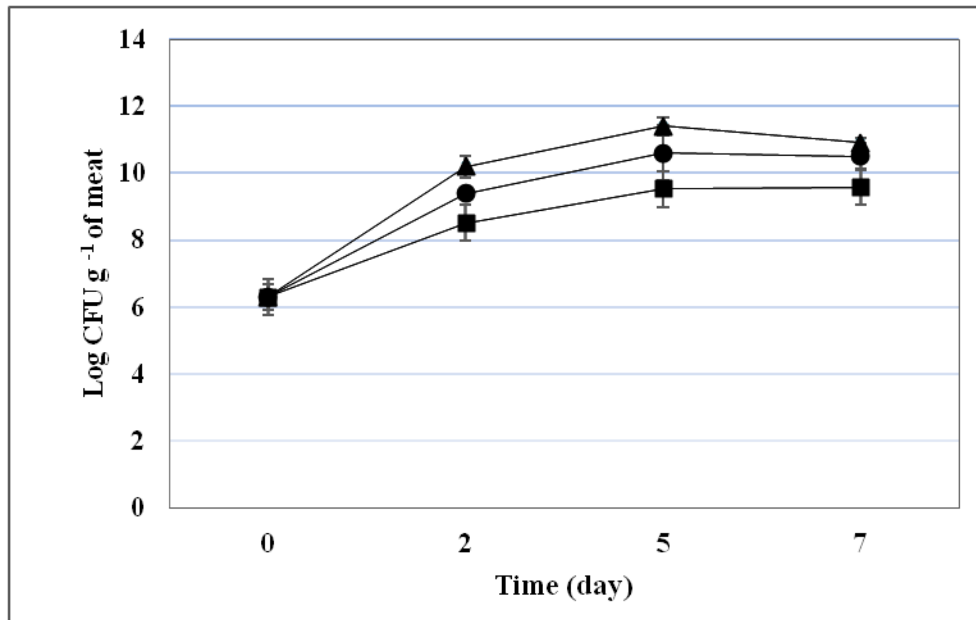

Fig. 1. Total aerobic mesophilic bacteria count in chicken breast meat samples stored in three types of packaging paper sheet. (●) CTR: control-packaging; (▲) SYN: PE laminated paper sprayed with SYNBIO®; (■) LP: PE laminated paper sprayed with *Lact. plantarum* IMC 509.

6.4.2 | BAs concentration and chemical indexes

Six of the 8 monitored BAs were detected in the chicken samples (PUT, CAD, HIS, TYR, SPD and SPM) (Table 3). SPD and SPM presented the highest concentrations at the beginning of the experiment (t0) from all types of packaging prototypes, with SPM as the most abundant BA (53.6 - 58.1 mg kg^{-1}). The levels of PUT, CAD, HIS and TYR were low from t0 to t2, but intensely increased from t5, where- as SPD and SPM levels decreased or remained constant during the chicken storage. PUT, CAD and HIS presented the highest increment, with HIS showing the highest levels (853 ± 30 and 390 ± 12 mg kg^{-1}) in chicken samples packed in SYN and LP-packaging, respectively. PUT exceeded 100 mg kg^{-1} after 7 days of storage (113 – 126 mg kg^{-1}), while CAD was in the range of 212 – 294 mg kg^{-1} at the end of the study (t7).

BA concentrations were used to determine chemical indexes assessing the effects of probiotic packaging on the Chicken quality during storage (Figure 2). Considering SYN vs CTR, the levels of BAs throughout the storage, tended to be higher in chicken samples wrapped in SYN than in CTR-packaging.

The CQI levels, starting with similar values (0.064 and 0.065) at t0, increased during chicken meat shelf-life, but tended to be higher in SYN-packaging samples respect to CTR from t2 until t7 (27.57 and 21.35). These differences were statistically significant ($P < 0.05$) at t2, with C.Q.I levels of 7.18 and 5.16 for samples in SYN and CTR-packages, respectively.

From t2, BAI levels and total BAs concentrations have the tendency to be higher in samples wrapped in SYN than in CTR-packaging. The gaps were statistically significant (P< 0.05) at the end of the study (t7) with B.A.I levels of 1294 and 1027 mg kg-1 from SYN and CTR respectively (Figure 2A). Unlike the SYN-packaging, from t2 to t7, the levels of PUT, CAD, HIS and TYR increased during the storage, but remained lower during each monitored day in chicken samples stored in LP-packaging respect to CTR.

Figure 2B reports the evolution of CQI, BAI, SPD/SPM, and Total BAs during the monitored days in chicken samples in LP and CTR-packaging. LP-packaged meat presented a reduction of the BAs increment during storage. CQI, BAI and Total BAs have the tendency to be lower in PL-packaged samples than in CTR-samples. These differences were statistically significant (P< 0.05) at t2 (CQI: 0.53 vs 1.75; B.A.I: 46.4 vs 149 mg kg-1) and t7 (CQI: 13.1 vs 20.1; B.A.I: 609 vs 970 mg kg-1).

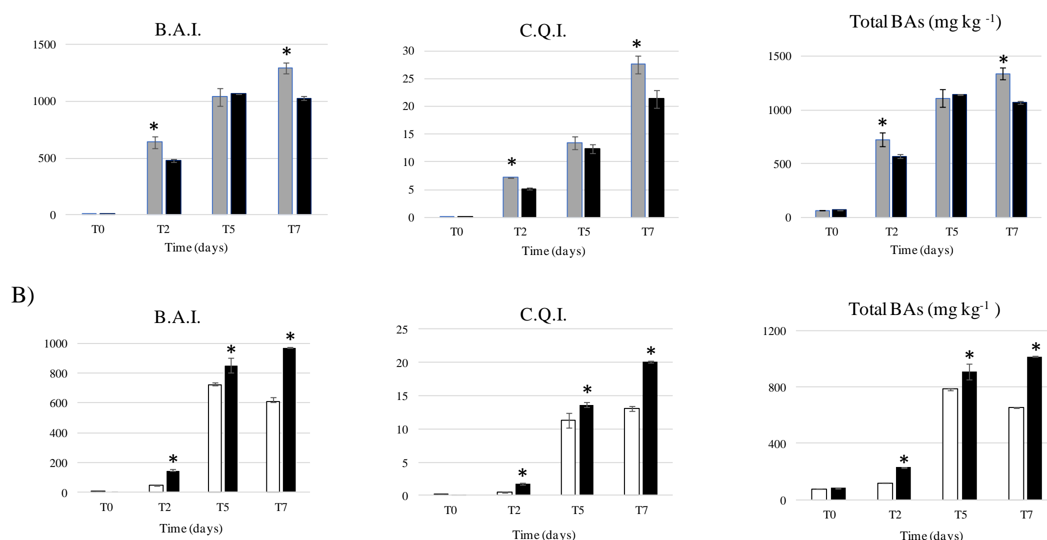

Fig. 2. Evolution of chemical marker indexes in the tested packaging during the shelf-life experiments. (A) First experiment: SYN vs CTR packaging; (B) Second experiment: LP vs CTR packaging; (n = 3) B.A.I: Biogenic amines index; C.Q.I: Chemical quality index; Total BAs: Total level of biogenic amines (mg kg^{-1}); SYN: PE laminated paper sprayed with SYNBIO®; LP: PE laminated paper sprayed with *L. plantarum* IMC 509; CTR: Control packaging. ■ SYN; ■ CTR; □ LP; *Statistically significant difference (P< 0.05).

Table 3. Levels of the monitored biogenic amines (mg kg^{-1}) in chicken meat during the storage in tested packaging

Biogenic amines	t0		t2		t5		t7	
	SYN	LP	SYN	LP	SYN	LP	SYN	LP
Putrescine	0.99 ± 0.1	0.68 ± 0.1	64.62 ± 5.1	5.45 ± 0.4	101.55 ± 2.2	122.19 ± 1.0	113.42 ± 4.6	125.96 ± 1.6
Cadaverine	0.74 ± 0.1	0.14 ± 0.0	151.94 ± 10.1	20.46 ± 2.1	237.84 ± 3.9	186.35 ± 0.5	294.48 ± 15.2	212.43 ± 3.0
Histamine	1.78 ± 0.1	3.79 ± 0.4	402.76 ± 38.1	13.41 ± 0.4	665.98 ± 78.3	390.29 ± 12.8	853.10 ± 30.2	252.58 ± 23.1
Tyramine	0.09 ± 0.0	0.02 ± 0.0	20.38 ± 1.8	6.97 ± 0.1	33.37 ± 0.3	24.69 ± 0.1	32.74 ± 0.9	17.94 ± 2.2
Spermidine	10.66 ± 0.0	14.91 ± 0.7	24.96 ± 1.9	14.99 ± 0.9	26.70 ± 3.1	6.82 ± 0.2	3.83 ± 0.2	4.45 ± 0.6
Spermine	53.60 ± 0.4	58.18 ± 1.6	60.30 ± 5.6	58.08 ± 2.0	46.90 ± 2.4	54.06 ± 4.6	41.05 ± 4.7	39.74 ± 1.3

SYN: packaging PE laminated paper sprayed with SYNBIO®

LP: packaging PE laminated paper sprayed with *L. plantarum* IMC 509.

6.4.3 | pH of meat during storage

One of the poultry meat spoilage indicators is the sour smell on meat surface which makes it utterly repellent [24]. Whereas the pH values of all samples at t0 were around 5.5 ± 0.2, the pH showed relatively different variations afterwards (Figure 3). The pH value of CTR-meat exhibited an increment after 2 days storage, from 6 ± 0.2 at t5 to 6.2 ± 0.1 at t7. However, the meat pH de- creased to 4.8 ± 0.4 after two days stored in LP-package sheet, while it restored to initial pH value after 3 days in fridge, reaching 6.0 ± 0.2 at t7. SYN-package had no effect on meat pH values for 7 days storing.

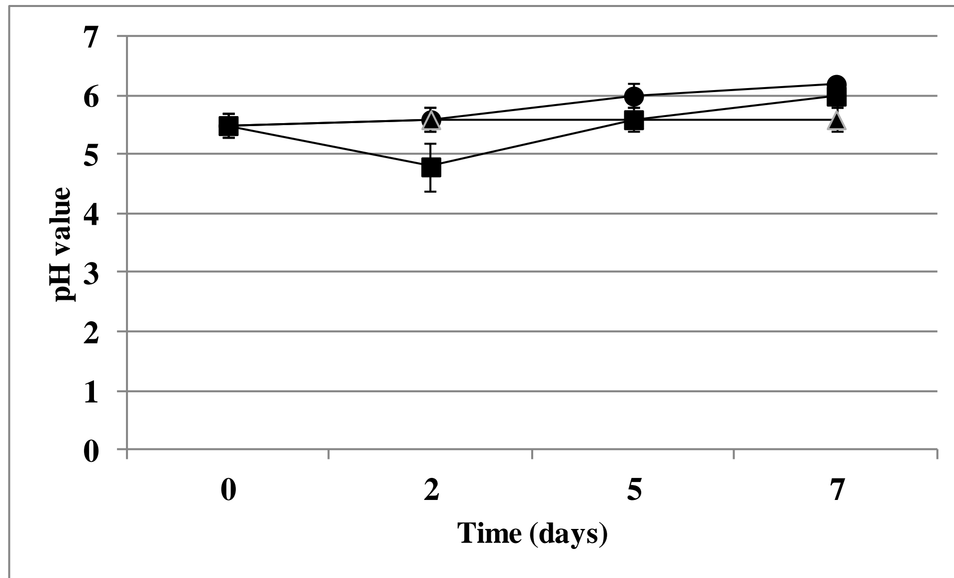

Fig. 3. pH value trend of three meat samples stored in different probiotic-paper sheets at 4°C for 7 days. (●) CTR: control-packaging; (▲) SYN: PE laminated paper sprayed with SYNBIO®; (■) LP: PE laminated paper sprayed with *Lact. plantarum* IMC 509.

6.4.4 | Changes in sensory characteristics

All samples exhibited optimal sensorial qualities, having maximum scores in all attributes at t0 (Figure 4 –A, B, C). After two days at 4°C, a slightly decrement was found in meat odour in all three samples, in addition, meat stored in CTR PE laminated paper sheet also lost a bit at its aspect. At t5, all meat samples showed reduced scores in all characters, notably, a larger decrement was found in meat colour, when it was in LP-packaging. Figure 4-D shows all the attributes in the three meat samples at the last day of storage. Compared to CTR, meat in both probiotic-packaging paper sheets showed a better preservation of main meat characteristics. Furthermore, SYN-pack- aging seemed to be better more efficient than LP-packaging in preserving aspect, odour, colour and elasticity. Looking at the overall acceptability, after 7 days, meat preserved inside SYN-package was still positively accepted by all panelists, while meat in LP-package had less scores.

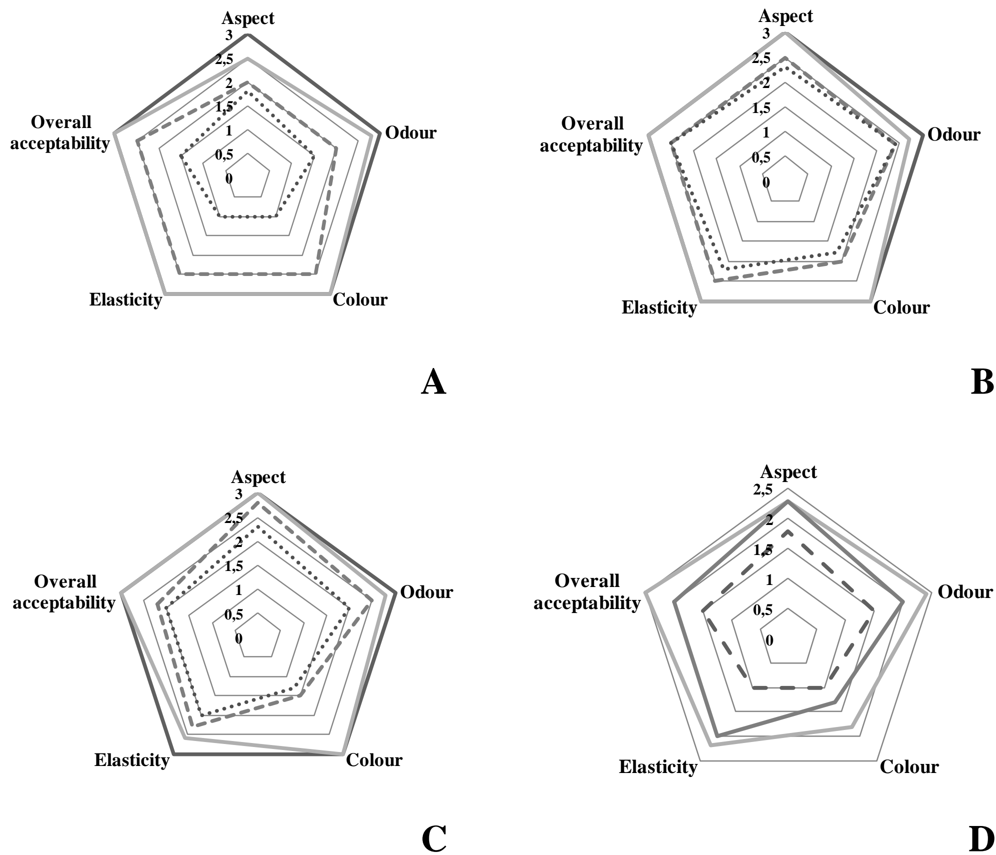

Fig. 4. Changes of five main characters of chicken meat during 7 days under refrigerator conditions, including aspect, odour, colour and elasticity, as well as overall acceptability. — t0; — t2; - - t5; ...... t7. (A) CTR: Control packaging; (B) SYN: PE laminated paper sprayed with SYNBIO®; (C) LP: PE laminated paper sprayed with *Lact. plantarum* IMC 509. (D) Comparison of all attributes among the three meat samples at the last day of storage. — CTR; —SYN; - -LP.

6.4.5 | Probiotics viability on packaging paper sheet

Table 4 shows the viability of probiotic strains and the survival rate (%), which was calculated after recovering from both paper sheet area touched with meat and area not in touch with meat during storage period. The SYNBIO® combination and the *L. plantarum* IMC 509 strain showed excellent survival rate during the study period. SYNBIO®, showed an updrawn trend in its cell counts after 5 days (18 times over 100 %), and further increased after 7 days of its initial value on areas which were not in touch with meat surface (30 times over 100 %). On the other hand, from the area, which is in contact with meat, its survival rates were firstly increased 15 times over 100 % after 2 days of storage and this percentage was nearly kept up to day 5. At day 7, a 40 time over 100 % survival rate was observed. *L. plantarum* IMC 509

strain demonstrated a lower but a more stable viability from beginning time point to the end, in particular on the sampling area in contact with meat (Table 4).

Table 4: Cell count values (expressed as CFU ml^{-1}) of probiotic bacterial strains, monitored at 0, 2, 5 and 7 days on paper sheets, from both paper sheet area touched with meat and area not in touch with meat. The respective survival rate (%) is also reported.

Bacterial strains	Time point (days)	Bacterial cell counts (CFU ml^{-1})*	Survival rate (%)
Not in contact with meat			
SYNBIO®	0	(1.0 ± 0.0) x 10^2	100
	2	(1.0 ± 0.0) x 10^2	100
	5	(1.8 ± 0.1) x 10^3	>> 100
	7	(3.0 ± 0.2) x 10^5	>>> 100
Lact. plantarum IMC 509	0	(2.0 ± 0.0) x 10^8	100
	2	(1.7 ± 0.4) x 10^8	85
	5	(3.7 ± 0.1) x 10^7	20
	7	(5.8 ± 0.0) x 10^6	3
In contact with meat			
SYNBIO®	0	(1.0 ± 0.0) x 10^2	100
	2	(1.5 ± 0.1) x 10^5	>> 100
	5	(0.5 ± 0.2) x 10^5	>> 100
	7	(1.0 ± 0.0) x 10^6	>>> 100
Lact. plantarum IMC 509	0	(3.5 ± 0.7) x 10^6	100
	2	(3.4 ± 0.3) x 10^6	97
	5	(1.6 ± 0.3) x 10^6	46
	7	(2.3 ± 1.3) x 10^6	66

*mean value ± SD

6.5 | DISCUSSION

The packaging materials sprayed with the two selected probiotic suspensions showed different effect on the growth of total aerobic mesophilic bacteria in the meat. Differences were observed among three meat samples, with the higher number of mesophiles in SYN- wrapped meat compared to CTR sample, while lower amounts of mesophiles were found in LP-wrapped meat. This trend put in evidence a sort of inhibitory activity of *L. plantarum* IMC 509 toward common spoilage bacteria, while the probiotic combination SYNBIO® showed promoting effects on the growth of meat microbes compared to CTR sample.

The synthesis and accumulation of BAs during chicken storage are in accordance with related studies [25] and are associated to the presence of microorganisms with decarboxylating activity. Enterobacteriaceae have been associated principally with the production of PUT and CAD [26] while LAB strains of *Enterococcus* spp. and *Lactobacillus* spp. are the main producers of TYR [27]. Enterobacteriaceae and LAB strains are reported to be histamine-producing bacteria [28].

High oral intake of BAs in food can be hazardous to human health inducing adverse reactions such as nausea, headaches, rashes and change in blood pressure [29] However, the only BA for which maximum levels have been regulated by the EU and the USA is histamine. FDA limits HIS levels to 500 mg kg^{-1} [30] while the Commission Regulation (EU) setup the maximum levels of HIS to 200 and up to 400 mg kg^{-1} in fish and fishery products, respectively, [31-33] suggested the maximum tolerable levels of PUT and CAD in Austrian fish products to be respectively 170 and 510 mg kg^{-1}.

Our results from chemical indexes were contrary to the effects expected proving by this fact that the probiotic strains chosen in the SYN-packaging were not able to prevent the formation of high levels of BAs. Indeed, they have rather increased the BAs synthesis in chicken during storage. *L. rhamnosus* IMC 501® and *L. paracasei* IMC 502®, which were combined in SYN-packaging, possess inhibitory activity towards the multiplication of pathogenic microorganisms and are highly competitive due to their production of several antimicrobial compounds such as bacteriocins [34] Moreover, other studies reported that *L. rhamnosus* was suitable for use as starter cultures in fermenting dry sausage allowing the BAs levels to remain low during the ripening [35] However, although having antimicrobial activities, these strains caused a higher increment of BAs levels in chicken during the experiment. This could be explained by the fact they possess also BA-producing properties. Indeed, *L. paracasei* has been reported as a BAs producing microorganism especially for PUT, CAD and TYR [36, 37] also reported the production of BAs by *L. rhamnosus*. These results could imply that the BAs accumulation in chicken is not determined by the antimicrobial activity of the probiotic strains but by their positive amino-acid decarboxylase activity.

According to Mietz and Karmas (1997) [18] formula, a CQI < 1 indicates a good tuna quality, between 1-10, tuna quality is borderline, while a CQI > 10 indicates a decomposed tuna [38]. Although these ranges have been defined for fish, they could be applied on chicken to comment the effect of the LP- packaging on chicken quality during storage. Indeed, at t2, LP-packed samples were still in good quality with a CQI < 1 (0.53) while CTR samples were already in a borderline state (CQI = 1.75). According to the same CQI ranges, at the end of the study, the LP-packed samples were closed to the borderline state (CQI = 13.1) while the CTR samples were in advanced state of decomposition (CQI = 20.1).

Considering the Total BAs, at the end of the study, the LP-packaging allowed a 35.8 % reduction of BAs levels (mg kg^{-1}) compared o the CTR-packaging. This reduction seems to be clearly caused by the probiotic strain used during LP-packaging preparation. Indeed, besides its antimicrobial activities, *L. plantarum* also demonstrated to possess the ability to reduce the BAs accumulation in chicken during storage. This ability can be explained by the amino-oxidase activity, which is reported in different LAB strains such as *L. sakei* and *L. pentosus* [39]. The amino-oxidase activity of these strains allows them to degrade BAs once they have been synthesized in the food matrix and thus, they can prevent the formation of high BAs levels in chicken. Different authors have reported the amino-oxidase activity of *L. plantarum* [40]. Moreover, it has been used in various studies to reduce the BAs accumulation in different food matrix such as wine and sausage [41, 42] similar results were observed in the present study; however, it is important to note that it is the first time that *L. plantarum* is used simultaneously as an antimicrobial agent and also as BA-degrading microorganism in active packaging for chicken. The variations of meat samples pH value demonstrated the effects of different Lactobacillus species on fresh chicken meat acidity. A sharp decrease in the pH value of LP-package stored chicken meat was observed after 2 days. This decrement was also observed by [43].

The sensory evaluations of the chicken meat samples were positive, considering the packaging materials with both probiotic strains; especially the combination product seemed to greatly maintain the sensorial characteristics of meat.

Both probiotics have a good capacity to maintain their viability on paper sheets, but *L. plantarum* IMC 509 had a higher stability, maintaining its count during time, even if showed a decrease in the area without contact. SYNBIO® had a high increase in the meat contact area. It may be explained by the fact that SYNBIO® strains, when touch meat surface, use meat nutrients not only to survive, but also to grow, facilitating meat spoilage process. A further investigation showed that both probiotic strains were still viable on the packaging paper sheets after 50 days (data not shown).

6.6 | CONCLUSION

In summary, between the different probiotic PE laminated paper sheets in this preliminary study, both the SYN and LP-packaging showed some promising characteristics for extending the shelf-life of chicken meat, using a commercial packaging PE laminated paper sheet.

In particular, when taking into account the total aerobic mesophiles, the lower bacterial counts demonstrated the inhibition capability of *L. plantarum* IMC 509 toward common spoilage bacteria, while the probiotic product SYNBIO® showed promoting effects on meat

microbes when compared to control. In addition, the determination of the BAs levels put in evidence that *L. plantarum* IMC 509 possess an interesting activity as BA-degrading microorganism.

The high bacteria count on meat at the beginning of the study may hamper the inhibition effects exerted by probiotic strains. Thus, to have a better understanding of the predicted effects, chicken samples with low microbial counts should be applied for further studies.

At last, *L. plantarum* IMC 509 had a high stability on the paper maintaining its survival rate during time and not affecting the microbial count of the meat. Keeping viable for such a high value during the whole study period is noteworthy, in this way they can work as a preservative for food.

Therefore, the differences generated from SYN and LP-packaging, highlighting the fact that the choice of an appropriate probiotic strain in meat active packaging development, not only depends on its antimicrobial activity, but also on its amino-oxidase activity. These properties could work synergistically to implement bio-preserve packaging to maintaining chicken meat quality for longer period of time, by reducing both the pathological microbial growth and the BAs accumulation during chicken meat shelf-life.

6.7 | AKNOWLEDGEMENTS

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CHAPTER VII

ASSESSMENT OF RICOTTA CHEESE SHELF-LIFE STORED IN PLA-BASED COMPOSITE PACKAGING BAGS AND COMPARISON WITH COMMERCIAL PACKAGE

7.1 | ABSTRACT

Ricotta cheese is a white fresh cheese that has a quite short shelf-life around 7-11 days at 4°C. Polylactic acid is a “GRAS” biodegradable thermoplastic that commonly used for food packaging material. Various active additives are used to mix with PLA polymer for developing active food packaging with reinforced packaging performances. Among additives, graphene composites have been widely investigated as their excellent thermal, physiochemical, conductivity, packaging as well as antimicrobial properties. In the present study, firstly, we characterized the packaging properties of graphene-based composite PLA film and probiotic (*L. plantarum* IMC 509)-composite-PLA on cheese shelf-life parameters include microbial content, pH values, and free fatty acids. Secondly, we tried to compare the preservation efficiency of active packages with commercial packages in term of their effects on cheese microbial and physiochemical properties. The film characterization showed that the addition of composites in PLA film improved the pH, redox potential and conductivity of PLA film. PLA composites (1% and 3%) showed inhibitory activity against the growth of *Listeria monocytogenes* and *Pseudomonas aeruginosa* through shaking flask method. Although the modification with graphene composite helped to enhance the permeability to water, the active PLA films were weaker in fat permeability than polyethylene film. Regarding their preservation efficiency on ricotta cheese, probiotic-modified packaging was effective in limiting the growth of *Bacillus cereu*s and less effective in inhibiting the proliferation of total mesophiles, *Pseudomonas* spp., and coliforms in cheese. The lipolysis activity occurred in cheese stored in PLA composite (0.5%) was lower than cheese that stored in PLA with 3% graphene composite. Compared to package efficiency on controlling microbial growth, PLA-based packages were slightly weaker than commercial packages that demonstrated better barrier properties. However, the addition of *L. plantarum* on packaging materials slowed down the proliferation of selected microbial groups. Therefore, our present study gave ~~an~~ insights into the PLA-based packaging

with addition of low amount of graphene-based composites and probiotic bacteria in maintaining ricotta cheese quality and safety.

7.2 | INTRODUCTION

Polylactic acid is a biopolymer material derived from microbial fermentation and chemical synthesis with "GRAS" status. As packaging applications, PLA possess several functional properties, including barrier, thermal, mechanical, biodegradable, elasticity and polarity [1]. The incorporation of active substances in PLA for manufacturing food packaging material has been widely investigated, especially nanoparticles that due to their advantageous property, such as high surface to volume ratio. The incorporation of nanocomposites into PLA film, on one hand, helps to improve the physiochemical structures. On the other hand, PLA serve as matrix for designing various applications and helps to improve the biodegradability of the packaging. More recently, montmorillonite, organocaly, nanodiamond with surface modifications, metal ions have been used as nanofillers for developing bionanocomposite packaging film, the developed packaging showed improved thermal, mechanical as well as antimicrobial properties [2-5]. PLA matrix with incorporation of graphene composites demonstrated improved tensile strength, thermal properties. Regarding the antimicrobial capacity of the PLA/graphene film, the graphene particle composition-functional groups, size and concentration strongly affect its efficiency.

L. plantarum is generally considered as beneficial bacteria that can find in environment, food matrixes, animal and plant origins [6]. With the "GRAS" status and technological advantages, such as high tolerance to low pH and temperature, high salt content, *L. plantarum* is generally used as starter culture or adjunct culture in ferment foods or as protective culture [7, 8]. Moreover, apart from the food transformation role, *L. plantarum* strains can also produce various beneficial metabolites, especially plantaricins, which possess preservation potentials as their efficiency in inhibiting the proliferation of closely related bacterial species and common food spoilage and pathogens [9]. In general, for food preservation, *L. plantarum* are used in the form of live bacteria to exert antagonistic activity as well as *in situ* production of active metabolites, but the viability and metabolic activity of *L. plantarum* highly depend on the food matrix and storage conditions. Moreover, *L. plantarum* can be used with plant oil to prevent the spoilage microorganism grown in mayonnaise [10]. In addition, purified form of bacteriocins can also be applied directly on food surface as preservatives, however, as the protein nature of bacteriocins, their structure and efficiency are susceptible to proteinases. A plantaricin (Bac23)-capped silver nanoparticles had showed higher efficiency and broader inhibition spectrum against food-borne pathogens than plantaricin alone [11]. Also, studies have investigated the potential of *L. plantarum* cell extracts that containing various metabolites in maintaining food safety and shelf-life [12, 13]. The objectives of the present study were: 1)

to monitor the efficiency of pure PLA packaging film and PLA film with incorporation of different concentrations of graphene-based composites on the microbial and chemical parameter of fresh ricotta cheese; 2) to verify the PLA/*L. plantarum* IMC 509 or PLA/ composites/*L. plantarum* IMC 509 on microbial quality of ricotta cheese; 3) to compare the efficiency of active packaging (composite/PLA/probiotic) with commercial packages for cheese preservation.

7.3 | MATERIALS AND METHODS

7.3.1 | PLA packaging film preparation

Polylactic acid (PLA) film and PLA film with incorporation of composites (Ag-GN-TiO_2) of 0.5%, 1% and 3% (Figure 1) are made and characterized by other partners of the project from Andaltec and Technical University of Cluj Napoca (UTCN) in collaboration with Ceprohart Romania (CEPRO) respectively. For preparing *L. plantarum* IMC 509 modified PLA/composite films, all inner surface of PLA packaging film was sterilized by UV lamp up to 3 hours under Biohazard hood (FASTER, Milan, Italy). 0.5 grams of *L. plantarum* IMC 509 lyophilized powders (10^{11} CFU g^{-1}) (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.

Figure 1. PLA and PLA/Ag-GN-TiO_2 composite film (0.5%, 1%, 3%)

7.3.2 | PLA packaging film characterization

The physiochemical properties of the PLA films were characterized by the researchers from Technical University of Cluj Napoca (UTCN) and Ceprohart Romania (CEPRO).

7.3.3 | Ricotta cheese preparation

Fresh ricotta cheese was purchased from an artisanal cheese factory (Delizie dei Fratelli Angeli, Pieve Torina). The cheese was made by mixture of cow and sheep milk through acid coagulation, the final pH value was around 6.5. Each cheese was served in perforated plastic tray with around 100g per cheese (Figure 2). After production, cheese samples were immediately transferred to the laboratory for analyzing quality parameters. Other cheese samples were randomly distributed into different packaging films and further labeled with date and packaging type. Samples were labeled with the following letters: PLA (cheese stored in pure PLA film), PLA + LP (cheese stored in PLA film with modification of *L. plantarum* IMC 509), PG 0.5 (cheese stored in PLA film with incorporation of 0.5% composites), PG0.5 + LP (cheese stored in PLA film with incorporation of 0.5% composites and *L. plantarum* modification), PG1 (cheese stored in PLA film with incorporation of 1% composites), PG1 + LP (cheese stored in PLA film with incorporation of 1% composites with probiotic modification), PG3 (cheese stored in PLA film with incorporation of 3% composites), PG3 + LP (cheese stored in PLA film with incorporation of 3% composites with probiotic modification). All the samples were closed and stored in the refrigerator at 4°C with relative humidity of 55% in dark condition. At each sampling time, samples were taken and analyzed for microbial, pH, free fatty acids content.

Figure 2. Ricotta cheese used for the analysis

7.3.4 | Microbiological analysis

Briefly, cheese microbial profile was checked by determining aerobic mesophiles, *β*-glucuronidase positive *Escherichia coli*, coliforms, anaerobic sulfite-reducing bacteria, coagulase-positive staphylococci, yeasts and molds, *Pseudomonas* spp., presumptive *Bacillus cereus* at day of 0, 3, 6, 9, 14 of cold storage. For the incubation conditions, we followed the criteria of Centro Interdipartimentale di Ricerca e Documentazione sulla Sicurezza alimentare (CeIRSA) [14].

Briefly, 10 g of cheese sample was sterile weighted and taken from each packaging bag (PLA, PLA + LP, PG0.5, PG0.5 + LP, PG1, PG1 + LP, PG3, PG3 + LP), then transferring into a sterile stomacher bag (Whirl-Pak®, Seward, UK) and 90 ml of sterile 0.9% sodium chloride solution (NaCl) (Sigma-Aldrich, Co., St. Louis, USA) was added and homogenized for 2 minutes. Then, 10-fold serial dilutions were prepared using saline solution, and 0.1 ml of the corresponding dilutions were spread and inoculated onto selective agar media [14].

7.3.5 | pH measurement

The cheese pH values were measured on day 0 and after 3, 6, 9, 14 days of storage. At the sampling day, 20 g of cheese samples of PLA, PLA + LP, PG0.5, PG0.5 + LP, PG1, PG1 + LP, PG3, PG3+LP were measured in triplicate using an electronic pH meter (Mettler Toledo, Columbus, UK) equipped with a probe for food through direct penetration in cheese.

7.3.6 | Chemical analysis (Free fatty acids)

The evolution of free fatty acids (FFAs) in the different cheese samples during the storage was monitored. FFAs were extracted by HS-SPME method and analyzed in GC-MS [15]. Five FFAs were monitored: butanoic acid, hexanoic acid, heptanoic acid, octanoic acid and nonanoic acid. The concentrations were evaluated in Ricotta cheese samples stored in the different packaging during the shelf-life.

7.3.7 | Statistical analysis

All experiments were performed in triplicate. The results were subjected to ANOVA test for the significant difference ($p<0.05$) by IBM SPSS Statistics (version 23).

7.3.8 | Comparison of commercial packages with PLA-based graphene-LP packages

To compare the packaging activity in limiting the growth of cheese microbiota, the differences between different time point (Day_x) with Day_0 have been calculated and they were ranked by using colors, from green (lower increment) to red (higher increment). Moreover, the maximum growth rate of each sample was also calculated using Combase, DMFit, Baranyi and Roberts model (https://browser.combase.cc/DMFit.aspx).

7.4 | RESULTS

7.4.1 | Packaging characterization

The structural and mechanical properties of the PLA composite film were being analyzed by UTCN and CEPROHART (Romania). The results showed that the values of pH, redox potential, and electrical conductivity of PLA/composite film are generally higher than plain PLA packaging films. The addition of graphene-based composite in PLA film improved the water vapor permeability compared to plain PLA film. As for the fat permeability, in general, the composite film is more permeable than reference film (polyethylene) both at 20°C and 4°C. PLA with 3% of composite is significantly higher than of PLA and PLA0.5%. The antioxidant activity of PLA, PLA 0.5% and PLA 3% is comparable with that of polyethylene. Furthermore, the shaking flask method revealed that all PLA packages was able to inhibite the growth of *Pseudomonas fluorescens*, whereas *Listeria monocytogenes* was sensitive only to the presence of PLA 1% and PLA 3%.

7.4.2 | Microbial analysis

Figures 3 (A-D) show that the microbial counts of each bacterial group increased for 14 days storage at 4°C. Compared to the mean values of Sicilian ricotta cheese microbiota, reported in a study [16], the initial level of total mesophiles and coliforms detected in our samples were lower, whereas the amounts of *Pseudomonas* spp. and *Bacillus cereus* were similar [16]. In the current study, the number of total mesophiles (Figure 3A) was generally higher in samples stored in *L. plantarum* modified packaging bags as the sprayed *L. plantarum* on packaging contributed to the increased number. Therefore, the number of total mesophiles of LP samples exceeded the defined limit after 3 days storage, and that level was kept for 9 days storage, following another increment to 9 $\log_{10}$ $CFUg^{-1}$ at day 14. As for samples that stored in PLA or PLA/composite packages, the level of mesophiles increased continuously during storage. Only PLA sample was able to maintain the number of total mesophiles below the limit for 6 days.

Coliforms (Figure 3 B) indicate the general hygiene conditions of cheese manufacture. Due to their intolerance to thermal treatment in ricotta cheese manufacture, their presence is generally associated with secondary contamination caused by inadequate hygiene conditions [16]. Once the contamination occurred, the proliferation of coliforms is strongly affected by water activity of cheese [17]. Contrary to our expectation, the growth of coliforms was not inhibited by *L. plantarum*-packaging, showing higher number of coliforms than PLA-composite samples (PLA, PG0.5, PG1, PG3) at the first 9 days of storage, although the counts decreased and generally lower than graphene-PLA samples at 14 days.

Pseudomonas spp. are psychrotrophic spoilage bacteria [18]. Similar to coliforms, the presence of this bacteria group suggests the poor hygiene condition, indicating secondary contamination of cheese [16]. Figure 3C shows that the amount of *Pseudomonas* spp. in all samples were lower than the acceptable limit during storage. Similar to the trend of total mesophiles and coliforms, the samples stored in PLA films without probiotic modifications contained less amount of *Pseudomonas* spp. than that of LP modified samples. The counts of *Bacillus cereus* at T0 were like previously reported by Scatassa, *et al.* with aroud 3.5 log CFU g^{-1} [16]. *B. cereus* is of particular importance in ricotta cheese as it produces thermos-resistance spores that may survive during thermal processing and germinate during storage [19]. In our study, the amounts of *B. cereus* in PLA, PLA+LP, PG0.5+LP, PG3+LP did not over the threshold for toxin production [14] for 6 days storage. It is noteworthy that, samples in the LP modified packages showed significantly lower amount of *B. cereus*, suggesting the anti-*B. cereus* ability of *L. plantarum* inhibited the proliferation of *B. cereus* in ricotta cheese. Similar to the results obtained by Zhang *et al.* [20]., *L. plantarum* ZDY2013 showed an inhibitory activity to the pathogenic *B. cereus* mainly through acids production as well as competition during milk fermentation process [20].

β-glucuronidase positive *Escherichia coli*, anaerobic sulfite-reducing bacteria, coagulase-positive staphylococci, yeasts and molds were under detection during the study. This may be due to the heat treatment involved in cheese production that eliminated or greatly reduced these bacterial groups.

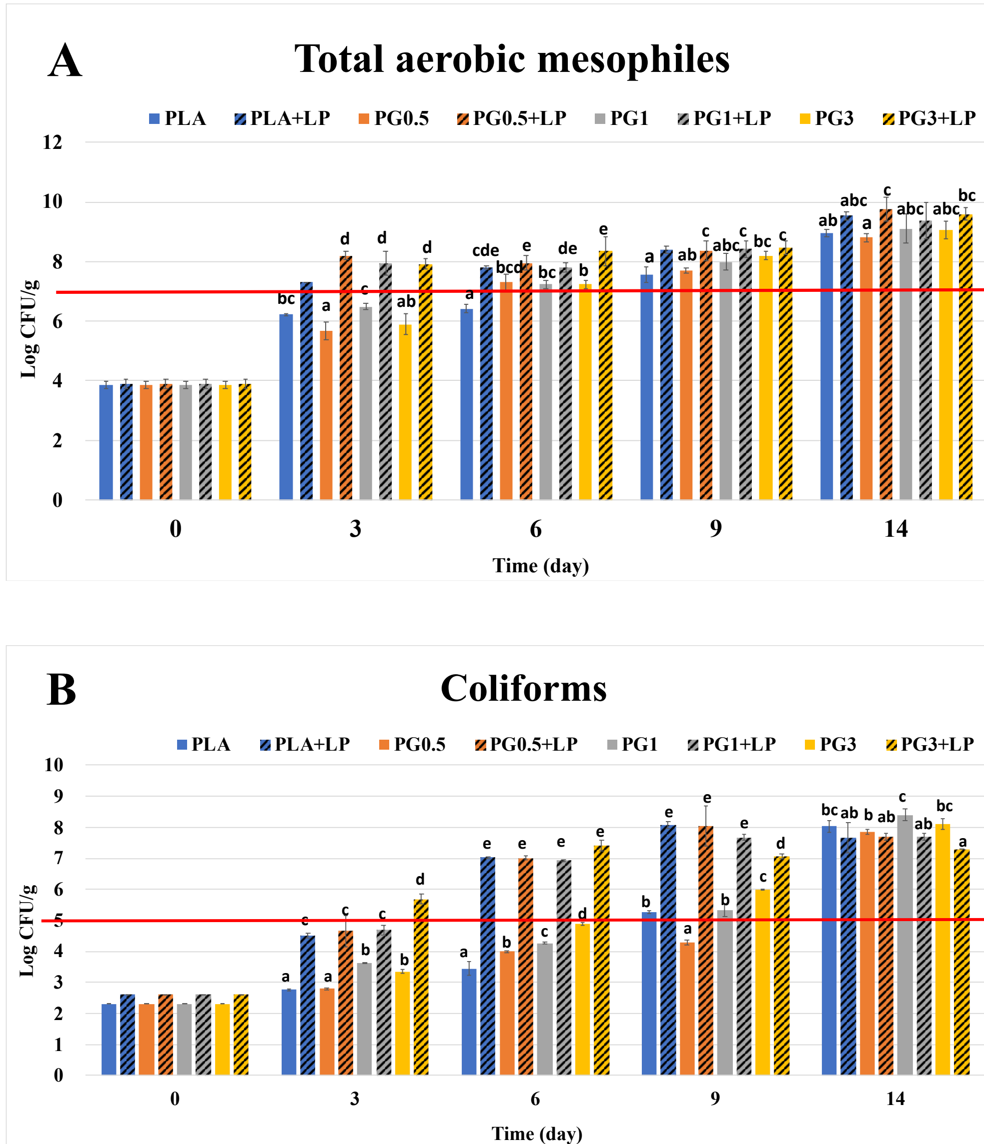

Figure 3. Microbial profile of ricotta cheese (Total mesophiles-A; Coliforms-B) stored in PLA, PLA+LP, PG0.5, PG0.5+LP, PG1, PG1+LP, PG3, PG3+LP. $^{a, b, c, d, e, f}$ statistically significant different by ANOVA test.

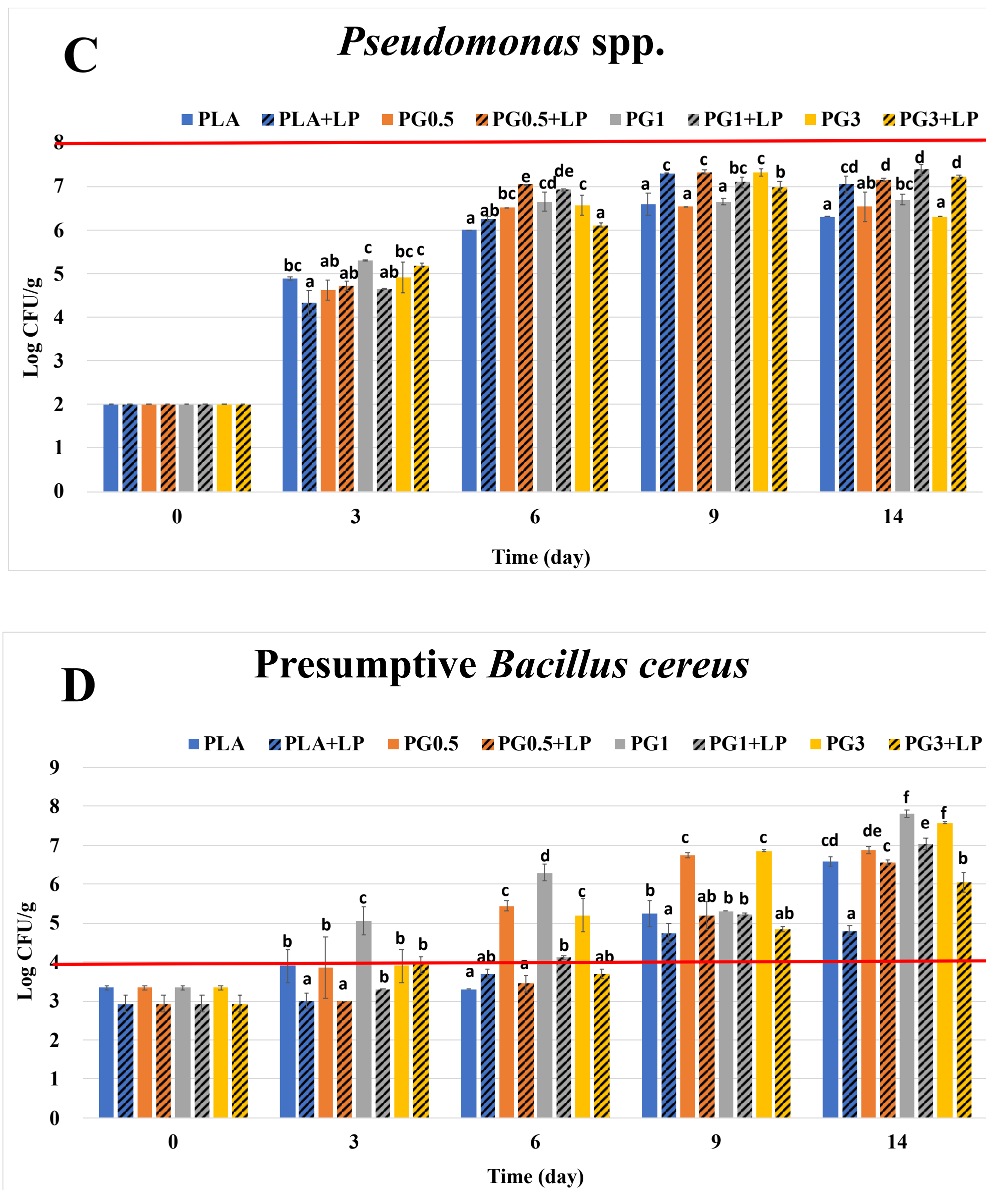

Figure 3a. Microbial profile of ricotta cheese (*Pseudomonas* spp.-C; presumptive *Bacillus cereus*-D) stored in PLA, PLA+LP, PG0.5, PG0.5+LP, PG1, PG1+LP, PG3, PG3+LP. [a, b, c, d, e, f] statistically significant different by ANOVA test.

7.4.3 | pH values

Figure 4 illustrates the pH values of each cheese sample during storage at 4°C for 14 days. The significant difference was found between LP groups and PLA/PG groups since the day of production. In general, after storage, the pH values of LP group decreased around 0.25, whereas 0.5 decrement was observed in groups store in PLA/PG packaging films. From the obtained pH values, we noticed that during the present storage conditions, the active package modified by *L. plantarum* IMC 509 had little impact on ricotta cheese acidity.

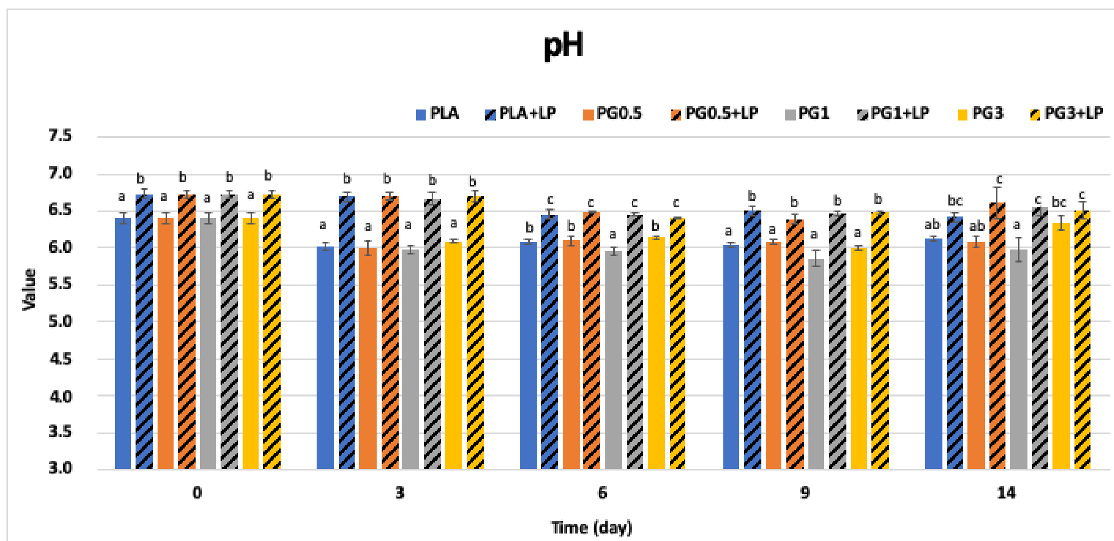

Figure 4. pH values of ricotta cheese stored in PLA, PLA+LP, PG0.5, PG0.5+LP, PG1, PG1+LP, PG3, PG3+LP. $^{a, b, c, d, e, f}$ statistically significant different by ANOVA test.

7.4.4 | Free fatty acids analysis

The amount of free fatty acids (FFA) produced mainly by lipolysis that can impact cheese organoleptic characteristics. Figure 5 shows the variation of FFA concentrations in PLA, PG0.5, PG1, and PG3 samples during storage. FFA production increased in all samples, with highest increment found in PG3 sample (78 mg kg^{-1}) and lowest in PLA sample (30 mg kg^{-1}).

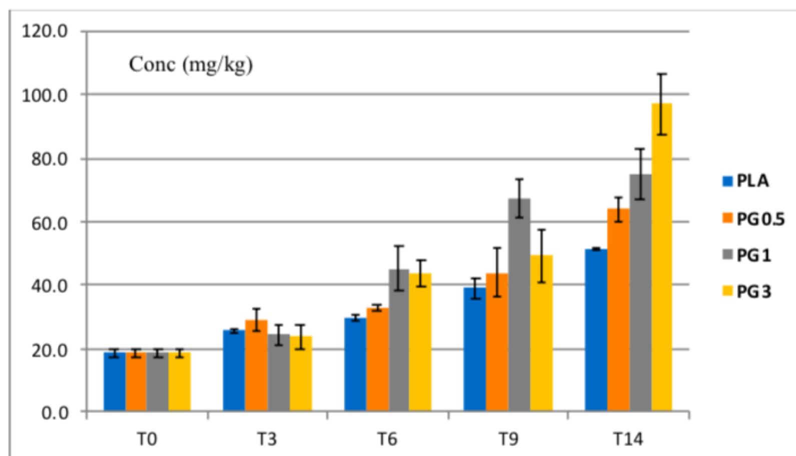

Figure 5. The concentration (mg kg^{-1}) of total free fatty acids in ricotta cheese.

7.4.5 | Comparison of commercial packages with PLA-based graphene-LP packages

7.4.5.1 | | Microbial increment and growth rates in different kind of packages

Table 1 shows that the highest increment of total mesophiles was found in samples stored in LP-modified package-suggesting the LP of package contributed part of the increased count-following by the PLA-based packaging and commercial packaging (polyethylene, IT-SLO) that demonstrated the lowest increment during storage with exception of RO. Similar to the bacterial increment trend, the rates were higher in LP-modified packages, whereas the maximum growth rates of PLA-based packaging samples were similar to commercial packaging samples, indicating the ability to control microbial growth of PLA-based packages was similar to commercial packages. The counts of coliforms at each time points were compared to starting point, as shown in Table 2, the increased amount was generally lower in IT and SP compared to other samples. For PLA-based packages (without LP modification), the level of coliforms kept increasing during the study period, but the maximum growth rates were relatively low. However, LP-modified samples (except PG3-LP) and SLO, RO showed higher increased amount and reached the peak at Day 9, following by a reduction observed in LP-containing samples and an increase in RO and SLO. Moreover, the maximum growth rates of LP samples were similar to that observed in IT and SP. The trend of coliforms indicates that *L. plantarum* may require time to adapt to cheese environment and thus exerting the antagonistic activity against the growth of other bacterial groups. Table 3 shows the increased amount of *Pseudomonas* spp. at each time, similar to Table 1, the proliferation of *Pseudomonas* spp. and the maximum growth rates were relatively low under commercial package storage, with exception of SLO that demonstrated the highest increment. Although the counts of this bacteria group were high in LP-modified PLA packages than PLA-based packages, the maximum

growth rate revealed that compared to pure PLA or PLA-GN packages, the LP-modification slowed down the proliferation of *Pseudomonas* spp. during storage period.

As we know the gas and water barrier properties of packaging highly affect the microbial growth on food, therefore, the microbial profile among packages could be ascribed to the different packaging barrier properties. To confirm our presumptions, results from partners of UTCN and CEPRO revealed that compared to commercial packages, PLA-based packages generally exhibited higher the water vapor permeability (WVP) value (Table 4), indicating the barrier properties were slightly weaker than that of polyethylene-based food packaging. Moreover, the impact of packages on cheese pH values were similar (data not shown).

Table 1. The difference of total aerobic mesophiles counts (Day_x - Day_0, as Log CFU g^{-1}) and the respective growth rate related to the different packages.

Total aerobic mesophile	Day					Maxi growth rate
Package	0	3	6	9	14	
PLA	0.00	2.36	2.56	3.71	5.09	0.382
PG0.5	0.00	1.8	3.44	3.84	4.94	0.474
PG1	0.00	2.63	3.38	4.13	5.25	0.467
PG3	0.00	2.04	3.37	4.34	5.19	0.51
PLA+LP	0.00	3.42	3.92	4.52	5.66	1.143
PG0.5+LP	0.00	4.31	4.07	4.48	5.87	1.507
PG1+LP	0.00	4.07	3.93	4.54	5.49	1.408
PG3+LP	0.00	4.04	4.47	4.59	5.72	1.376
IT	0.00	-0.38	1.96	2.34	4.59	0.456
SP	0.00	0.44	1.15	2.21	4.81	0.469
RO	0.00	0.85	2.66	4.41	6.05	0.626
SLO	0.00	0.93	1.93	2.10	4.45	0.314

Table 2. The difference of total coliforms counts (Day_x - Day_0, as Log CFU g^{-1}) and the respective growth rate related to the different packages.

Total coliforms	Day					Maxi growth rate
Package	0	3	6	9	14	
PLA	0.00	0.46	1.15	2.97	5.74	0.603
PG0.5	0.00	0.49	1.69	2.00	5.55	0.522
PG1	0.00	1.32	1.97	3.03	6.10	0.471
PG3	0.00	1.05	2.58	3.69	5.80	0.444
PLA+LP	0.00	1.90	4.42	5.47	5.05	0.893
PG0.5+LP	0.00	2.06	4.41	5.45	5.09	0.834
PG1+LP	0.00	2.09	4.33	5.08	5.09	0.791
PG3+LP	0.00	3.09	4.82	4.46	4.70	1.0406
IT	0.00	0.48	2.93	3.23	4.92	0.868
SP	0.00	0.00	1.02	3.49	5.10	0.865
RO	0.00	2.22	3.82	5.10	6.63	0.576
SLO	0.00	1.63	3.67	5.32	5.81	0.679

Table 3. The difference of *Pseudomonas* spp. counts (Day_x - Day_0, as Log CFU g^{-1}) and the respective growth rate related to the different packages.

Pseudomonas spp.	Day					Maxi growth rate
Package	0	3	6	9	14	
PLA	0.00	2.89	4.00	4.59	4.30	0.96
PG0.5	0.00	2.63	4.51	4.53	4.54	1.0671
PG1	0.00	3.31	4.65	4.65	4.69	1.112
PG3	0.00	2.91	4.57	5.32	4.30	0.961
PLA+LP	0.00	2.32	4.25	5.30	5.04	0.731
PG0.5+LP	0.00	2.73	5.04	5.33	5.15	0.971
PG1+LP	0.00	2.64	4.92	5.09	5.39	0.892
PG3+LP	0.00	3.19	4.11	4.99	5.21	1.0434
IT	0.00	0.09	1.66	2.61	4.09	0.446
SP	0.00	0.32	1.00	2.36	4.24	0.402
RO	0.00	2.45	2.54	2.75	4.48	0.282
SLO	0.00	1.76	3.19	5.26	6.45	0.601

Table 4. The water vapor permeability (WVP) of different packages

Package	WVP (g/s m Pa) 10^{11}	
	20 ^{0}C	4 ^{0}C
PLA	3.13	2.19
PG0.5	7.98	4.75
PG1	7.04	4.02
PG3	7.07	4.06
IT	6.103	1.902
SP	0.511	0.12
RO	0.512	0.52
SLO	10.31	2.705

7.5 | DISCUSSION AND CONCLUSIONS

In general, the results shown that the incorporation of graphene-based composite improved the pH, redox, conductivity, water vapor permeability, and antimicrobial properties compared to pure PLA film. However, composites modified PLA film showed higher permeability to fat, especially with higher concentration of graphene composite (3%) and at lower temperature (4°C). The counts of total mesophiles bacteria, *Pseudomonas* spp., and coliforms were higher in *L. plantarum* IMC 509 and composite-modified samples than in samples that stored in PLA or PLA/composite films, whereas the counts of *B. cereu*s revealed that *L. plantarum*-package inhibited the growth of *B. cereu*s in cheese.

The anti-spoilage activity of *L. plantarum* IMC 509 was less pronounced in our study. It was probably due to the presence of graphene composites, low temperature involved and complex matrix of cheese that limit its efficiency in lowering spoilage microorganisms during storage. pH values of cheese were slightly decreased during storage, meaning that the acid production of *L. plantarum* IMC 509 was less in our study. The chemical analysis - free fatty acids concentrations - showed that the level of lipolysis increased as the concentration of composites increased in PLA-based packages.

Through the comparison among packages, in terms of microbial growth and maximum growth rate, the results revealed that due to the good barrier properties commercial packages were efficiency in controlling the proliferation of cheese microbes. Although the *L. plantarum*-modified package increased cheese microbial counts, the growth rates confirmed that the presence of *L. plantarum* had inhibition effect on cheese microbiota developing, compared to pure PLA-based packages. In brief summary, the selection of appropriate bio-preservative strain in the right food matrix, together with the packaging conditions help to maintain longer shelf-life of food.

Therefore, our current study validates and offer some new insights in using the active packaging system of the PLA-composite-*L. plantarum* for the preservation of fresh ricotta cheese. Further research is needed to improve the barrier property of the active package as well as to deepen the knowledge of cheese microscopic variations.

7.6 | REFERENCES

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CHAPTER VIII

ASSESSMENT OF CHICKEN BREAST MEAT SHELF-LIFE STORED IN PAPER-PLA GRAPHENE PACKAGING

8.1 | ABSTRACT

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 achieve the recyclability of graphene-based composites, 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. Firstly, the efficiency of active paper-PLA active package was evaluated on meat quality parameters. Moreover, also the efficiency of active package with recycled graphene-based composites was examined, monitoring their impacts on meat parameters. Lastly, a comparison between the efficiency of commercial used meat packages, paper-PLA active package, paper-PLA active package with recycled graphene composites was made. 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.

The packages made with addition of recovered graphene showed efficiency in maintaining the meat microbial quality within acceptable limit for whole study period, and the packages had little impact on meat pH and sensorial properties.

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.

8.2 | INTRODUCTION

Packages made by renewable sources are considered as sustainable packaging trends in replacing fossil-based materials and reducing environmental impacts [1]. 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 [2]. 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 is 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 [3]. The development of active packaging with antimicrobial and antioxidant properties helps to efficiently delay the spoilage process 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 [4].

The aims of the present study are: 1) to characterize the preservation efficiency (meat microbial profile, pH value, chemical parameters) of active paper-PLA based (modified with composite and probiotic strain - L. plantarum IMC 509) packaging on raw chicken meat; 2) to evaluate the efficiency of active paper-PLA packages (made with recovered graphene-based composite and L. plantarum) in chicken meat storing; 3) to compare the efficiency between commercial packages and novel active packages.

8.3 | MATERIALS AND METHODS

8.3.1 | 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 1). All the packages were fabricated and characterized 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 hours under Biohazard hood (FASTER, Milan, Italy). 0.5 grams of *L. plantarum* IMC 509 lyophilized powders (10^{11} CFU g^{-1}) (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-PLA0.5% (whole paper and PLA with composite), H0.5%-PLA0.5% (paper and PLA with graphene), H-PLA-LP (whole paper and PLA, added with probiotic), H-PLA0.5%-LP (whole paper and PLA with composite, further added with probiotic), H0.5%-PLA0.5%-LP (paper and PLA with composite, with addition of probiotic).

In a second phase, paper-PLA package - H-PLA0.5% - with recovered composite was also produced and treated with probiotic suspension (H-PLA0.5%-LP).

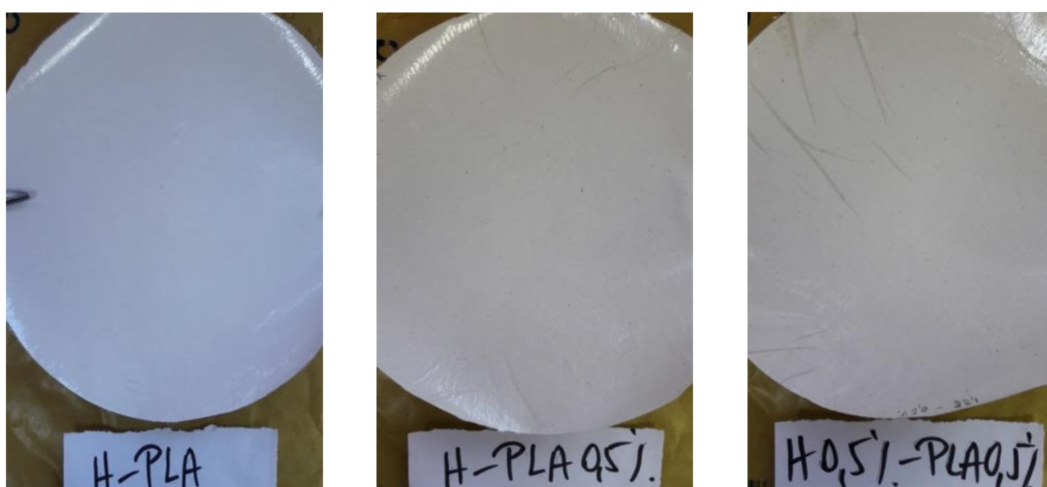


Figure 1. H-PLA: paper and polylactic acid sandwich; H-PLA 0.5%: paper and polylactic acid with 0.5% Ag-GN-TiO_2; H 0.5%-PLA 0.5%: paper with 0.5% Ag-GN-TiO_2 and polylactic acid with 0.5% Ag-GN-TiO_2

8.3.2 | Meat and packaging preparation

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

8.3.3 | Microbial counts, pH measurement and sensorial analysis

The methods used in the present study were the same as described in Chapter 6.

8.3.4 | Chemical analysis

BAs were extracted from chicken samples and analyzed by HPLC-DAD according to the method of Sagratini *et al*. [5] and as reported by Huang *et al*. [6].

8.3.5 | Statistical analysis

All experiments were performed in triplicate. The results were subjected to ANOVA test and using the Student's *t* test, for the significant difference $p<0.05$ was considered (IBM SPSS Statistics- Version 23).

8.4 | RESULTS

8.4.1 | 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 being analyzed by our partners UTCN and CEPROHART (Romania).

The thickness, grammage and smoothness were ranked in the following order: H0.5%-PLA0.5% > H-PLA0.5% > H-PLA. The addition of 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 elongation characteristic 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*.

8.4.2 | Microbiological analysis of active paper-PLA packages

Figure 2A 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-PLA0.5% 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 the threshold 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. In fact the assay of probiotic viability on this packaging surface showed around 8 log CFU/cm^2 (Chapter 9), which was 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 2C) 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-PLA0.5%-LP was able to maintain the level of presumptive *Pseudomonas* spp. counts (Figure 1D) 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-PLA0.5%-LP did not exceed the threshold during the whole storage time.

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

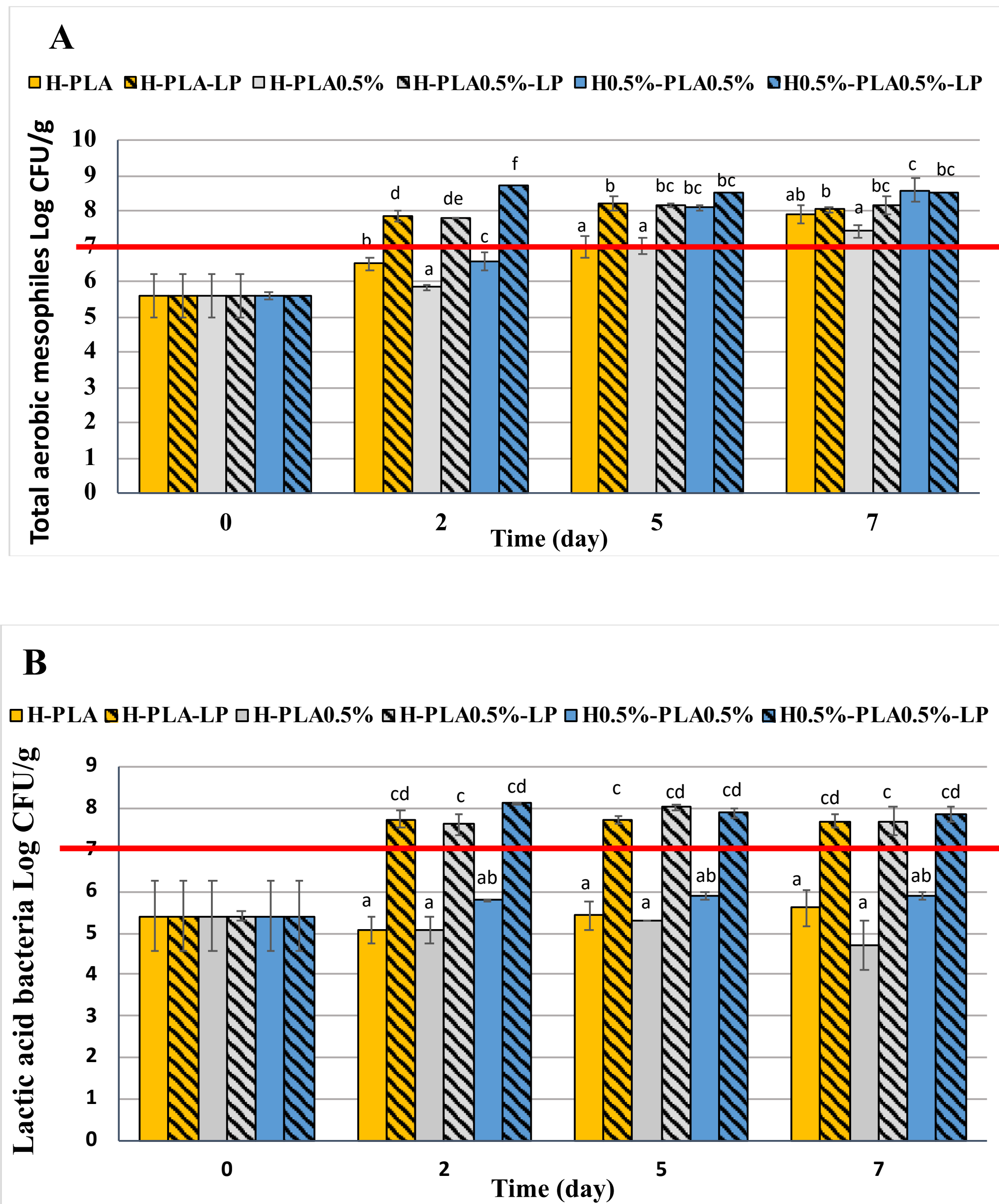
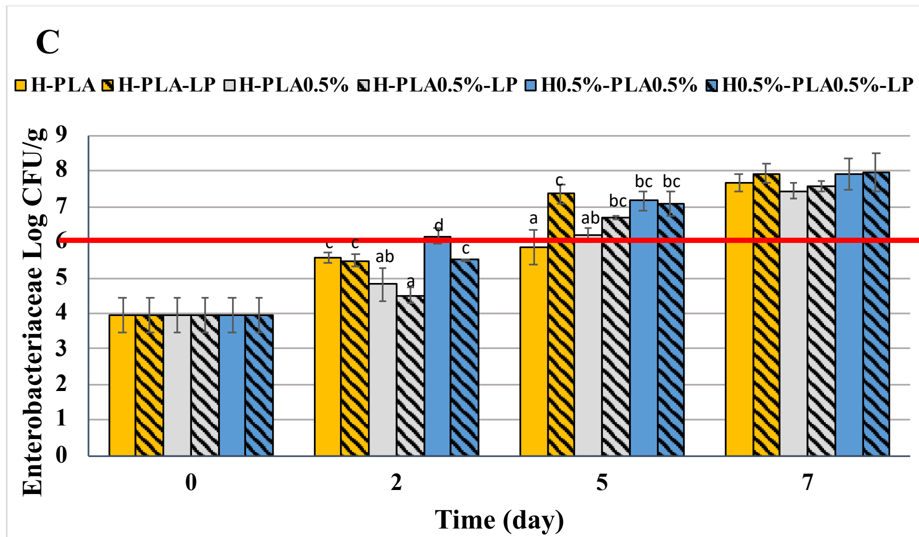


Figure 2. Bacterial counts (Total mesophiles – A; Lactic acid bacteria – B) in chicken breast meat samples during 7 days of storage at 4°C. [a, b, c, d, e, f] statistically significant different by ANOVA test.



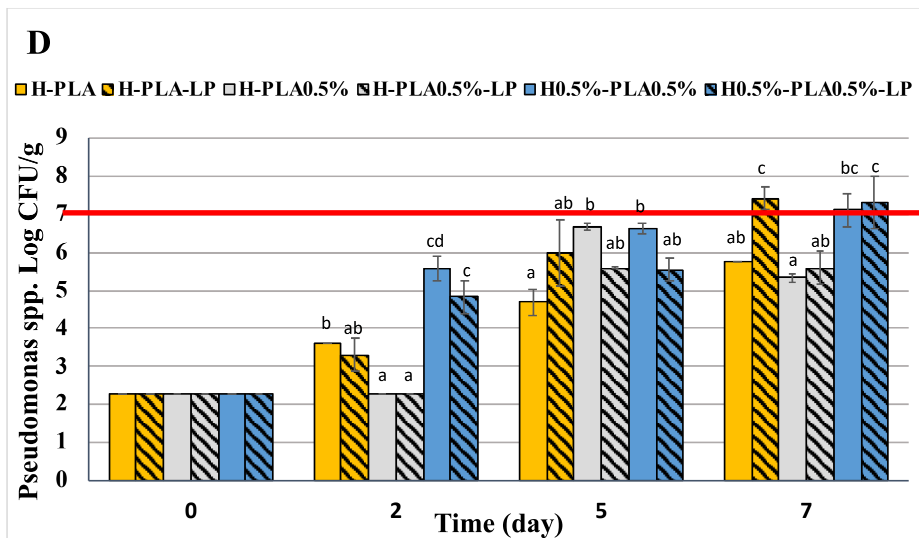


Figure 2a. Bacterial counts (Enterobacteriaceae – C; presumptive *Pseudomonas* spp. – D) in chicken breast meat samples during 7 days of storage at 4°C. $^{a, b, c, d, e, f}$ statistically significant different by ANOVA test.

8.4.3 | Microbiological analysis of sample in active paper-PLA packages made of recovered graphene composite

The microbial groups detected on chicken meat stored in recovered composites are shown in Figures 3 A-F. Sulfite-reducing anaerobes and *Cl. perfringens* were under detection limit during study. The counts of microbes generally were low during the first 5 days of storage, and increased afterwards, apart from total aerobic mesophiles (Figure 3A) and lactic acid bacteria (Figure 3B). Moreover, in these two figures, H-PLA0.5%-LP sample revealed similar amount, suggesting the main total aerobic mesophiles of H-PLA0.5%-LP sample were lactic acid bacteria-*L. plantarum*. On the contrary, H-PLA0.5% meat sample had low number of total mesophiles and lactic acid bacteria, which was below the threshold during storage. Similar to the results obtained from active paper-PLA packages (mentioned above), the count of Enterobacteriaceae (figure 3D) in LP-modified pack were higher than non-LP-modified pack at t5 ($p<0.05$) and t7. The antagonistic effect of *L. plantarum* was hampered by the complex ingredients of food matrixed. Although the amount of *Pseudomonas* spp. was higher in H-PLA0.5%-LP than H-PLA0.5% at the end of storage, the samples stored in LP-packs showed lower amount of β-glucuronidase positive *E. coli* and coagulase-positive staphylococci, suggesting the capacity of *L. plantarum* in controlling the growth of these two bacterial groups.

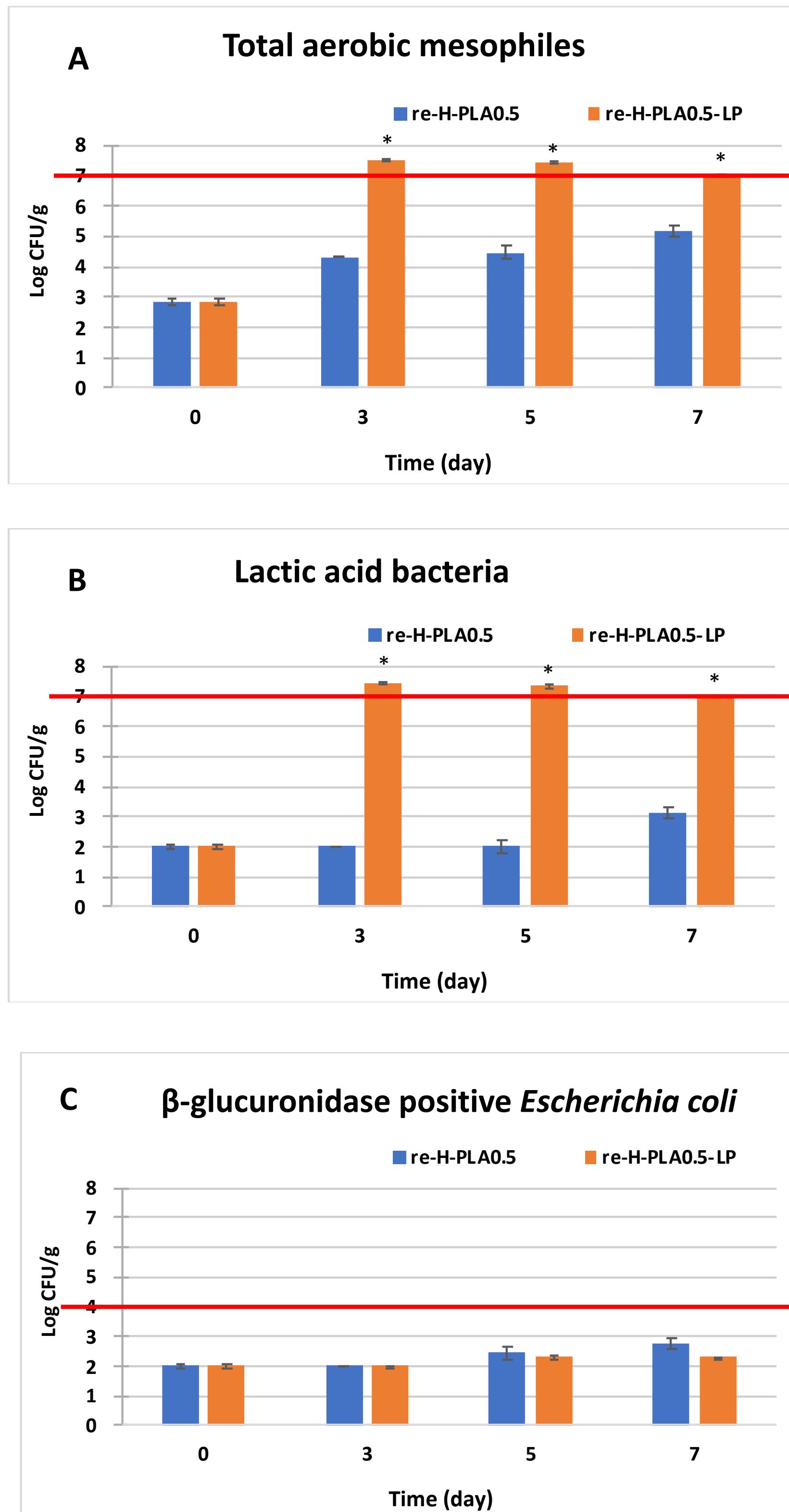
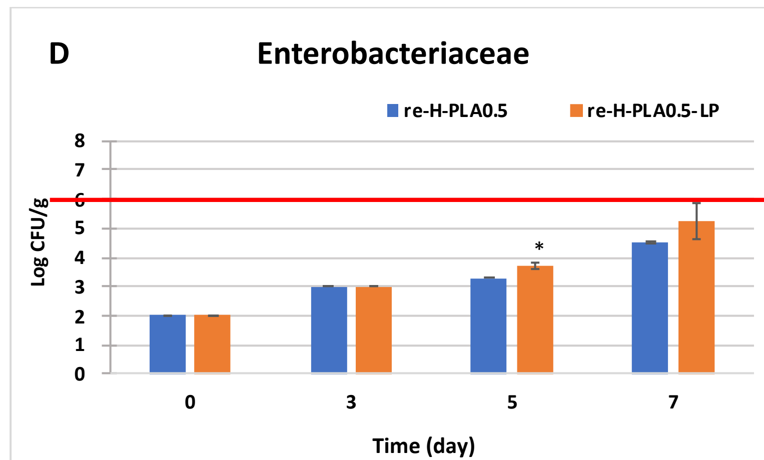
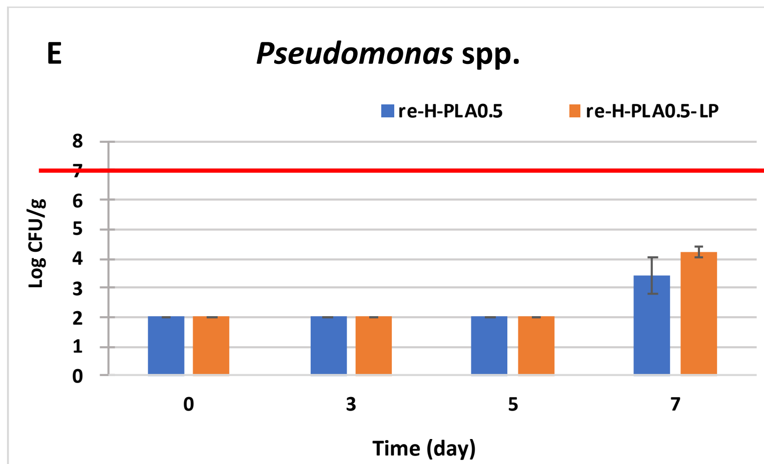


Figure 3. Bacterial counts (Total aerobic mesophiles bacteria –A; lactic acid bacteria –B; β-glucuronidase positive *E. coli* - C) in chicken breast meat samples stored in active packages with recovered composites during 7 days of storage at 4°C. *statistically significant different by Student's *t*-test ($p < 0.05$).





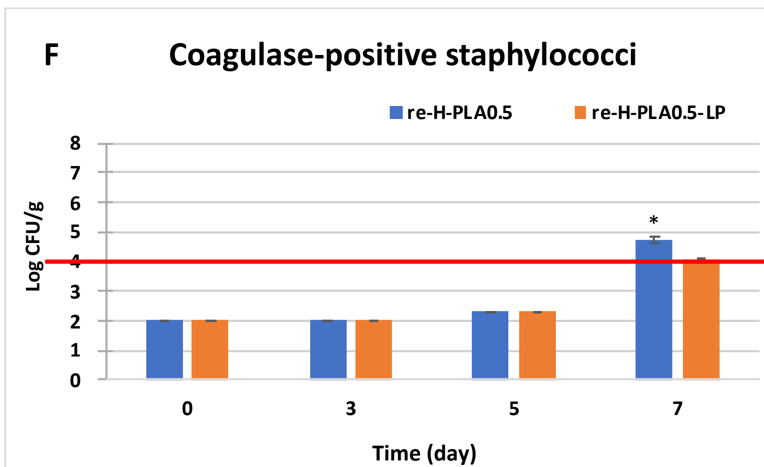


Figure 3a. Bacterial counts (Enterobacteriaceae – D; *Pseudomonas* spp. – E; Coagulase-positive staphylococci – F) in chicken breast meat samples stored in active packages with recycled composites during 7 days of storage at 4°C. *statistically significant different by Student's *t*-test ($p < 0.05$).

8.4.4 | pH values

The pH values presented similar variation in all meat samples either stored in active packages with newly manufactured composites or with recovered composites, no significant difference was found between samples (data not shown).

8.4.5 | 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 4A-D). 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 for odor and elasticity, during the first 5 days, H-PLA0.5%-LP (brown line) sample was higher in values than other samples. After another two days storage, meat stored in H-PLA0.5% (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).

Similar to the abovementioned result, meat stored in recycled packages with modification of probiotic suspension showed better aspect and odor of the meat, whereas the color and elasticity of the meat among two packages were similar (data not shown).

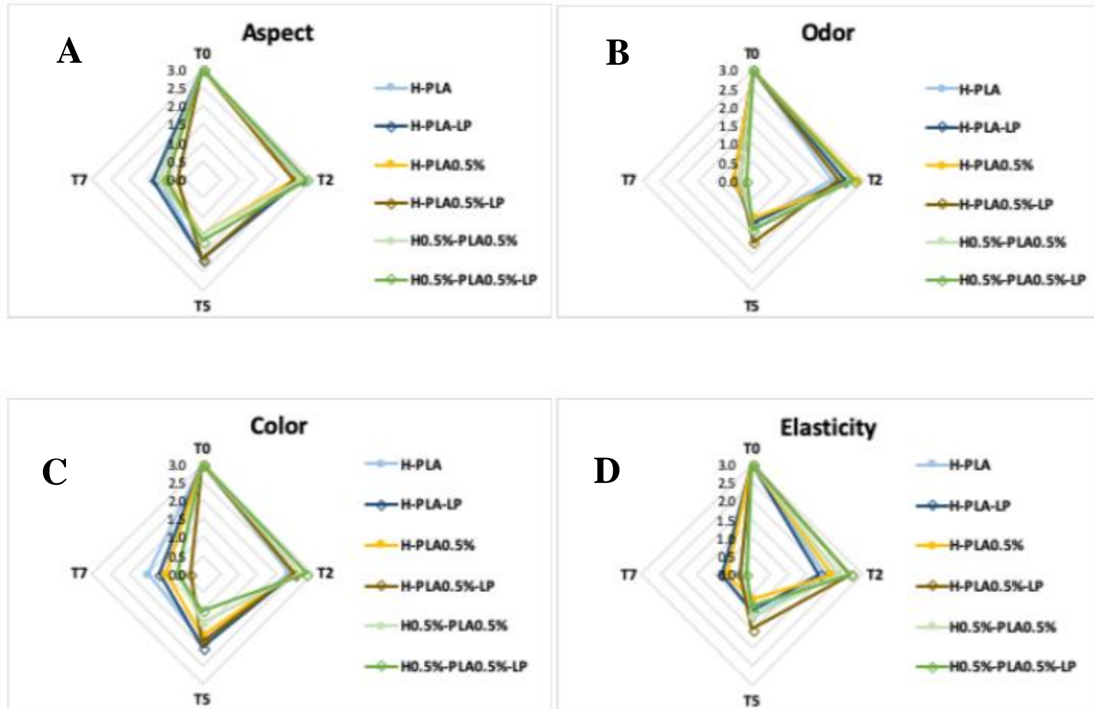


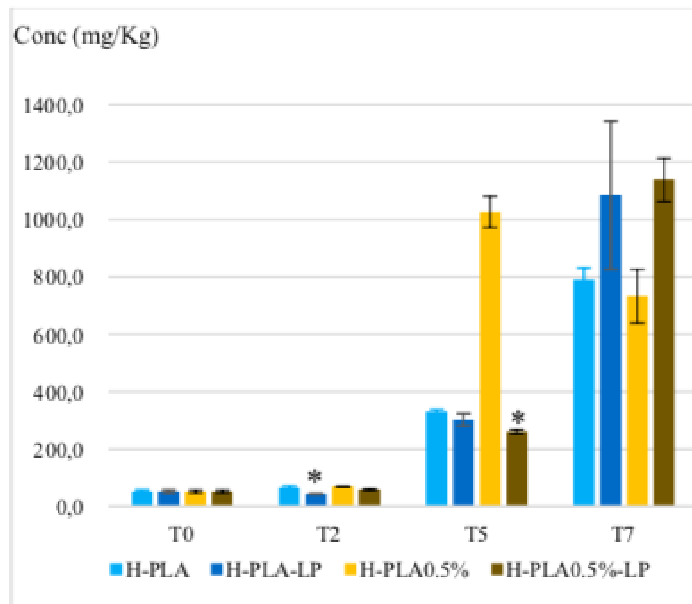
Figure 4. Sensorial analysis of meat stored in the different sandwich paper-PLA based packaging for 7 days. A-Aspect, B-Odor, C-Color, D-Elasticity. Hedonic scale: 0-3.

8.4.6 | Chemical analysis of sample in active paper-PLA packages

To evaluate the biogenic amines production during storage, three indexes were taken into consideration such as BAI (Biogenic amines index): PUT + CAD + HIS + TYR. Each index was calculated and compared between samples.

Due to the arrangement of the experiment, the chemical analysis was subdivided into two separate analyses.

In the first experiment, four samples: H-PLA, H-PLA-LP, H-PLA 0.5% and H-PLA0.5%-LP were studied. The second analysis included H-PLA, H0.5%-PLA0.5%, H0.5%-PLA0.5%-LP. Figure 5 (A-B) show that the increased level of BA during storage was observed in all tested samples. At T2, the BAI in chicken sample stored into H-PLA-LP had the lowest amount compared to all other samples, followed by H-PLA0.5%-LP. Their levels were statistically significant lower ($p < 0.05$) than either H-PLA or H-PLA0.5%. At T5, chicken samples into H-PLA0.5%-LP showed the lowest BAI compared to the other samples. At T7, H-PLA0.5% presented significantly lower BAI compared to H-PLA0.5%-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 (Figure 5B). At T7, chicken samples stored in H0.5%-PLA0.5%-LP showed the lowest BAI ($p < 0.05$) compared to all other samples (H0.5%- PLA0.5%-LP > H0.5%-PLA 0.5% = H-PLA).



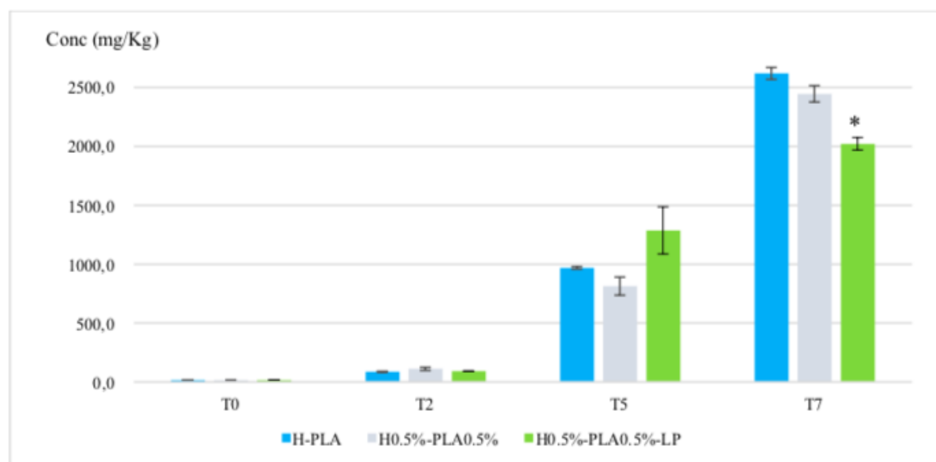


Figure 5. Biogenic amines index (BAI) of chicken samples stored in (A) H-PLA, H-PLA-LP, H-PLA0.5%, H-PLA0.5%-LP and (B) H-PLA, H0.5%-PLA 0.5% and H0.5%-PLA0.5%-LP.

8.4.7 | Chemical analysis of samples in active paper-PLA packages made of recovered graphene composite

B.A.I. index values (Figure 6A) showed two statistically significant differences at day 5 and day 7 of storage. In these cases, chicken breast in H-PLA0.5%-LP packaging exhibits higher values at T5 and T7, with 8.97 mg kg^{-1} and 14.9 mg kg^{-1} at T5 and T7, respectively.

From a more in-depth study of individual BAs levels, it appears that these statistically significant differences are due to the differences in CAD (cadaverine) concentration (Figure 6B), which showing similar trend as observed in Figure 6A.

In summary, comparing BAs concentrations of chicken breast samples stored in the two packages, statistically significant differences were reported for B.A.I. index values and they were due to different levels of CAD. In general, all monitored BAs levels indicated no spoilage signs in either of the two studied packaging.

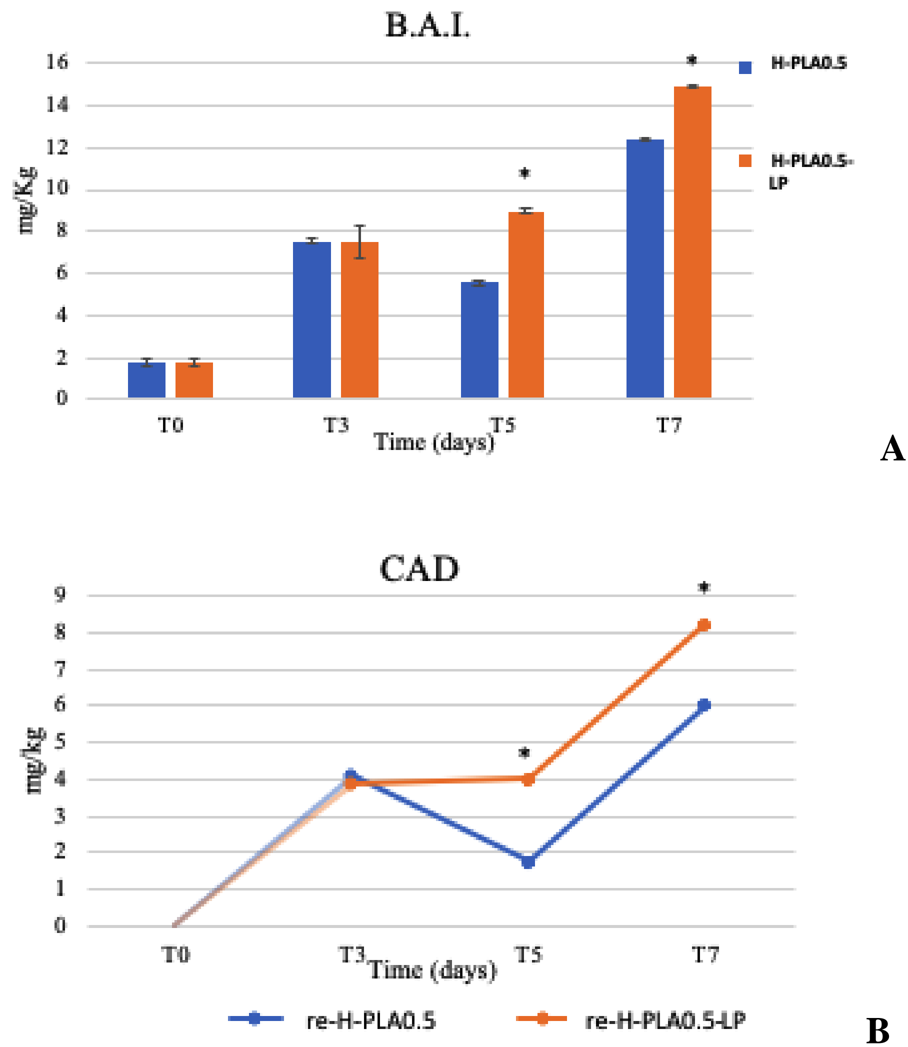


Figure 6. Biogenic amines index (BAI, A) and cadaverine (CAD, B) level of chicken samples stored in H-PLA0.5% and H-PLA0.5%-LP with recovered composite.

8.5 | Comparison of commercial meat packages, active paper-PLA packages and active paper-PLA packages made of recovered graphene-based composite

8.5.1 | Microbial maximum growth rate

Here, we tried to compare the maximum growth rate of each bacterial group detected from each sample, and the results are summarized in Table 1. In general, the growth rate of total mesophiles was lower in active packages without probiotic modification, taking into consideration that probiotic treatments contributed part of the total mesophile count. The lowest rate was detected in H-PLA0.5% meat sample. For the growth of lactic acid bacteria (LAB), the minimum value was observed in H-PLA 0.5%, in which LAB count was reduced during time. However, in LP-modified packs, LAB proliferated in a relatively fast way, demonstrating their capacity in growing on meat surface. Enterobacteriaceae grew rapidly on samples that stored in commercial packs and in H-PLA, H-PLA-LP, and H0.5%-PLA0.5% packages. Although there were variations among different packs, the lowest growth rate of *Pseudomonas* spp. was detected on re-paper-PLA 0.5% package (with recovered graphene) and followed by re-paper-PLA 0.5%-LP package. To be noticed that commercial packages (SP and RO) and active packages (H-PLA, H-PLA-LP, H-PLA0.5%, H-PLA0.5%-LP) demonstrated better ability in controlling the proliferation of staphylococci.

In summary, taking into consideration that probiotic treatment increased the total mesophile and LAB counts and subsequently increased proliferation rates. Generally, the samples in the active packages (except H0.5%-PLA0.5%) limited the proliferation of Enterobacteriaceae compared to commercial used packages. In our study, the preservation efficiency of active package was not compromised using recovered composite in packaging, especially in delaying the growth of Enterobacteriaceae and *Pseudomonas* spp. However, it is difficult to draw a conclusion due to the variances occurred during the whole study period.

Table 1. Maximum growth rate of each bacterial group in different packages: IT to SLO-commercial meat packages, H-PLA to H0.5%-PLA0.5%-LP-active paper-PLA packages, re-H-PLA0.5% and re-H-PLA0.5%-LP were paper-PLA packages made of recovered composites.

Package	Maximum growth rate				
	Total mesophile	LAB	Enterobacteriaceae	*Pseudomonas*	*Staphylococcus*
IT	1.717	0.354	1.024	0.846	0.837
SP	0.872	0.438	1.196	0.959	0.296
RO	0.582	0.255	0.621	0.53	0.363
SLO	0.694	0.351	0.911	0.968	0.912
H-PLA	0.305	0.107	0.775	0.476	0.342
H-PLA-LP	1.176	0.274	0.705	0.84	0.389
H-PLA0.5%	0.287	-0.175	0.53	0.652	0.298
H-PLA0.5%-LP	1.123	1.163	0.561	0.69	0.338
H0.5%-PLA0.5%	0.509	0.187	1.111	1.659	0.576
H0.5%-PLA0.5%-LP	0.342	1.407	0.63	1.261	0.562
re-H-PLA0.5%	0.344	0.136	0.391	0.173	1.291
re-H-PLA0.5%-LP	0.595	0.708	0.57	0.267	0.905

8.5.2 | Chemical analysis-Biogenic amine index (B.A.I)

Moreover, we tried to compare the B.A.I. among different samples by subtracting the amount of B.A.I. at T0 from each sampling time point (T2, T5, T7). The Table 2 shows at each sampling time, the increased level of biogenic amine index of samples. It is interesting to notice that B.A.I. increased rapidly at the first two days of storage in commercial packages, whereas in active packages, the increased amount was low. However, this effect was not maintained at day 5 and continued to increase for 7 days. For longer storage up to 7 days at 4 °C, the B.A.I. was highly increased in active packages. The packages made of recovered composite revealed very low B.A.I increment during study; this effect was probably caused by the relatively low initial microbial count of raw meat. Moreover, as the biogenic amines production ~~is~~ highly correlates with microbial numbers and metabolic activities, and thus the initial microbial load and packaging properties as well as storage conditions all can affect the microbial, chemical quality of raw meat.

Table 2. Increasing value of biogenic amine index (BAI).

B.A.I. (mg/kg)	Day			
Package	**0**	**2**	**5**	**7**
IT	0	30	550	550
SP	0	60	400	540
RO	0	50	225	400
SLO	0	53	380	497
H-PLA	0	15	298	723
H-PLA-LP	0	-9	247	1036
H-PLA0.5%	0	17	967	670
H-PLA0.5%-LP	0	5	237	1092
H0.5%-PLA0.5%	0	19	694	2325
H0.5%-PLA0.5%-LP	0	20	1240	1930
re-H-PLA0.5%	0	7.5	5.7	4.8
re-H-PLA0.5%-LP	0	7.5	9	7.5

8.6 | DISCUSSION AND 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-PLA0.5% and H-PLA0.5%-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-PLA0.5%-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.

Moreover, the impact of packages made of recovered composite (H-PLA0.5% and H-PLA0.5%-LP) on meat microbial, chemical, pH and sensorial parameters were also evaluated. The microbial counts results showed that LP modification caused significantly increase of total mesophile and LAB. Moreover, the higher amount of Enterobacteriaceae and *Pseudomonas* spp. in LP-packed samples suggested the compromised anti-spoilage activity of *L. plantarum* on food surface. The majority of investigated microbial groups were under the defined threshold during storage. However, the counts of *β*-glucuronidase positive *E. coli* and coagulase-positive staphylococci in H-PLA0.5%-LP were lower than H-PLA0.5%. Moreover, the packages with recovered composite had little impact on meat pH values and meat sensorial properties. Although the concentration of biogenic amine index was higher in LP-packaging samples, their B.A.I. was far below the acceptance limit.

From the microbial and chemical comparison of commercial meat packages with active packages (containing recovered composite), 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 activity, reducing the amount of biogenic amines produced during extended storage. Moreover, the recovered composite-packages were efficient in controlling meat quality under refrigerated 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 efficiency 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. Further complementary tests are highly needed to conclude the evaluation of the packaging.

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CHAPTER IX

ASSESSMENT OF PROBIOTICS VIABILITY ON PACKAGES SURFACE

9.1 | ABSTRACT

In food industry, probiotics are used to maintain food quality and safety throughout storage as bio-preservative or as active component in packaging system. The most investigated form is the probiotics-incorporated edible coating/film. In this case, the immobilized probiotics are not only acting as food preservative, but also bringing additional health benefits. The applications of probiotics and their active metabolites in food packaging can also be conducted on non-edible polymer film, such as polyethylene, polylactic acid. To exert their protective roles for food storage, it is essential to maintain and monitor the viability of probiotics on packaging matrix under storage condition, which is normally unfavorable for the growth of probiotic bacteria. Moreover, using live bacteria as bio-preservative, their proliferation may lead to unexpected result. Therefore, our study examined the viability and counts of probiotic *Lactobacillus* strains (SYNBIO® and *Lactobacillus plantarum* IMC 509) on polyethylene-laminated paper film, polylactic acid film, polylactic acid-laminated paper film with the presence of graphene-based composite or not, through plate count method. Our results showed that due to the homogeneity of the spraying process, packaging surface properties, and high tolerance of probiotic bacteria in tested environment, there were little variations in cell counts during storage at 4°C even for extended period. Compared to polyethylene film, probiotic suspension was homogenously distributed over PLA film. Moreover, the presence of graphene-based composite on packaging material had no effect on the viability of probiotic strains. In summary, *L. plantarum* IMC 509 showed better adaptability than SYNBIO® and it can maintain viable on different packaging materials at 4°C for extended period. *L. plantarum* IMC 509 is of great potential in active food packaging applications.

9.2 | INTRODUCTION

The incorporation of probiotics into packaging system has become popular among researchers as its health-related benefits, its antimicrobial activities, abundance in foods, adaptability to different environment [1]. Various probiotics-containing packages in the form

of edible coating (bioactive packaging) were developed and investigated to against the growth of food spoilage or pathogenic bacteria by agar method or on real food models [2]. Apart from the concept of bioactive packaging, which also intends to bring additional health benefits to consumers. Probiotics can also be incorporated in inorganic materials as part of active packaging techniques that mainly function as bio-preservative for preserving foods quality and safety during storage. Under this condition, the viability of probiotics needs to be improved or kept fulfilling their active functions during food processing and storing environment. Therefore, our current aims were to evaluate the viability of two probiotic products; SYNBIO® which is composed by 1:1 ratio of *L. rhamnosus* IMC 501® and *L. paracasei* IMC 502®, and *L. plantarum* IMC 509 on packaging films made by different materials during long storage period at 4°C.

9.3 | MATERIALS AND METHODS

9.3.1 | Probiotic-packaging preparation

All tested probiotic strains were provided from SYNBIOTEC® (Camerino, Italy), SYNBIO® and *L. plantarum* IMC 509 were supplied as vacuum packed white powder form. The packaging films included: polyethylene laminated paper packaging film (PE) (commonly used as packaging by Sì con te, Camerino); PLA (polylactic acid) film; H (paper)-PLA packaging with composite or without were produced by Andaltec, UTCN, CEPROHART. All packaging films with the inner surface being sterilized under UV light for 3 hours. To prepare the spraying solution, 0.5g of each probiotic lyophilized powder was dissolved in 10 ml of sterile phosphate-buffered saline (PBS) solution. The dissolved probiotic solution was homogenously sprayed on the inner surface of previously sterilized films. The sprayed films were drying in lab oven (Heraeus, Hanau, Germany) at 45°C until the weight of the film reached a constant value. Furthermore, all films were stored in fridge at 4 °C for the further analysis.

9.3.2 | Viability assay

The viability of probiotic strains on packaging films was measured by viable plate count methods. A defined area of the probiotic-film (3x3 cm^2) was brushed with a cotton swab wetted with sterile saline solution and then the cotton head was transferred into tube with 5 ml saline solution and vortexed for 2 minutes. Then, 10-fold serial dilutions were performed, and the corresponding dilution was plated on MRS agar media (de Man, Rogosa, Sharpe, by VWR). All the plates were incubated at 30°C for 72h ± 3 h (ISO 15214:1998(E)). Each procedure was repeated for three times.

Table 1. Type of packaging and the sprayed probiotic

Probiotic film	PE	PLA	PLA-GN	H-PLA	H-PLA-GN
SYNBIO®	+	+	-	-	-
***L. plantarum* IMC 509**	+	+	+	+	+

Note: (+): the viability on the film was assessed; (-): the viability test on the film was not performed. PE: paper packaging film; PLA: polylactic acid film; PLA-GN: polylactic acid film with graphene (GN); H-PLA: paper coupled with polylactic film; H-PLA-GN: paper coupled with polylactic film with graphene composites.

9.4 | RESULTS

After incubation, colonies were firstly identified morphologically through the Gram staining, then the results were calculated and expressed as mean value ± standard deviation.

Table 2 and Figure 1 show that on PE, the count of SYNBIO® was around 2 log CFU/9cm^2 at the starting point, however, after 2 days storage, the number increased and reached around 5.5 log CFU/9cm^2 at day 7. The variation in bacterial counts was probably due to the probiotic suspension was not homogenously sprayed over the packaging surface. The level of SYNBIO® was maintained up to 90 days in fridge. Therefore, it can be presumed that SYNBIO® strains can tolerate the current storage conditions, while the lower amount at starting time point was probably due to the inhomogeneity of sprayed SYNBIO® suspension. On PLA packaging film, the counts and viability of SYNBIO® was maintained for 50 days (Figure 2)

Regarding the viability of *L. plantarum* IMC 509 (Figure 2), the probiotic bacteria was stable and able to survive at 4°C until 60 days on PE film, then the counts decreased to 2 log CFU/9cm^2 after 90 days in fridge. On PLA film, the counts of *L. plantarum* IMC 509 (Figure 3) were maintained for 6 months with slightly decrement (around 1 log CFU/9cm^2) at the end. The results revealed the good adaptability of *L. plantarum* IMC 509 on the PLA film. The difference was probably due to the longer storage under refrigerated temperature. Moreover, as shown in Figure 4, the presence of 3% of Ag-GN-TiO_2 II 0.02tt composites did not affect the viability of *L. plantarum* IMC 509 on PLA film for 30 days (Figure 4). Similar effects were also observed on paper-PLA package and paper-PLA-graphene 3(%) (Figures 5 and 6).

Table 2. Probiotics cell counts (expressed as log CFU/9cm^2) of SYNBIO® and *Lactobacillus plantarum* IMC 509 on polyethylene laminated paper film for 90 days storage at 4°C.

Strain \ Day	1	2	5	7	30	60	90
SYNBIO®	2.0±0.01	2.0±0.06	3.0±0.01	5.5±0.01	5.6±0.06	5.4±0.14	5.6±0.06
***L. plantarum* IMC 509**	6.5±0.01	6.5±0.06	6.2±0.01	6.4±0.01	5.8±0.06	6.1±0.02	2.5±0.21

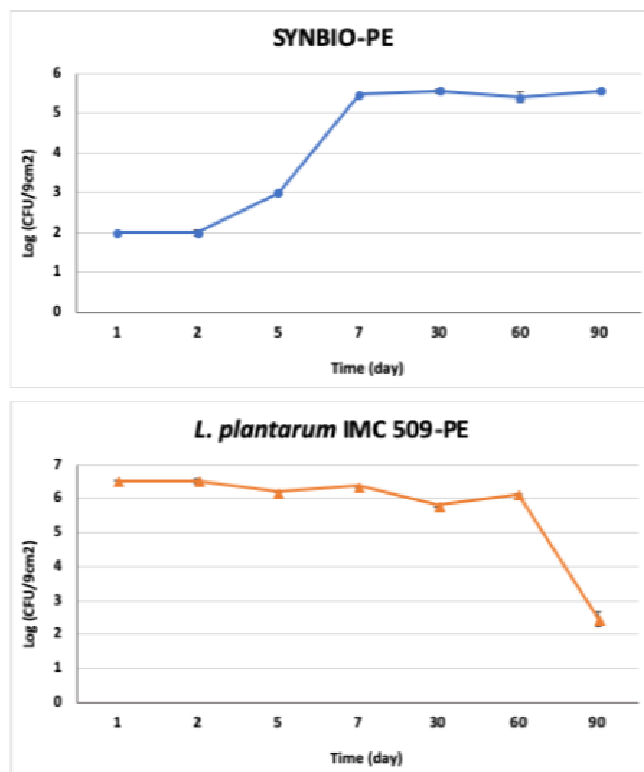

Figure 1. Bacterial cell counts (expressed as log CFU/9cm^2) of SYNBIO® (blue) and *L. plantarum* IMC 509 (orange) on polyethylene film surface for 90 days storage, at 4°C.

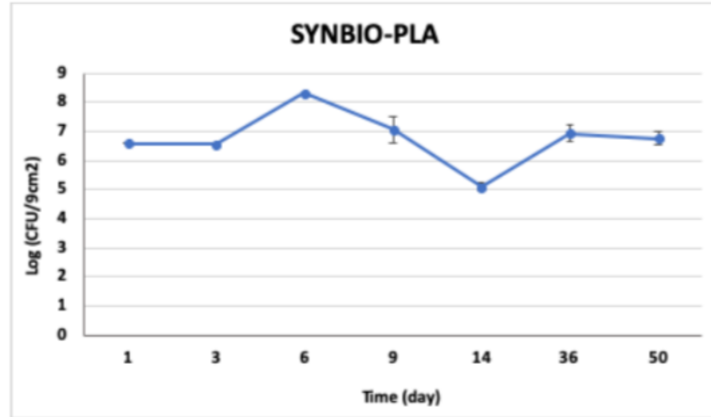

Figure 2. Bacterial cell counts (expressed as log CFU/9cm^2) of SYNBIO® on polylactic acid. (PLA) film surface during 50 days at 4°C.

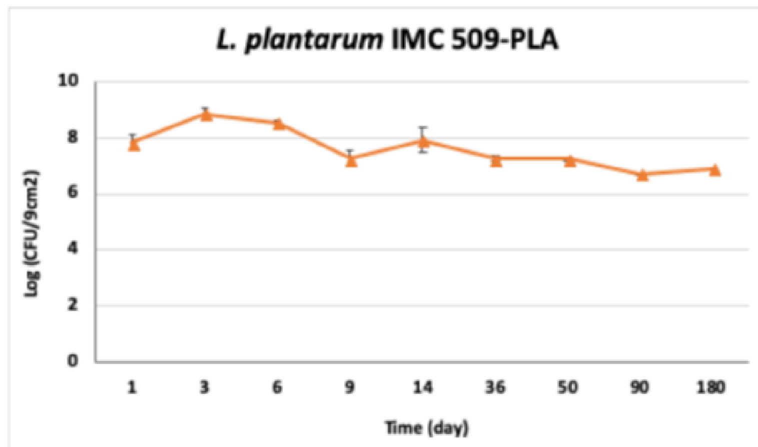

Figure 3. Bacterial cell counts of *L. plantarum* IMC 509 on polylactic acid (PLA) film. surface for 180 days storage at 4°C

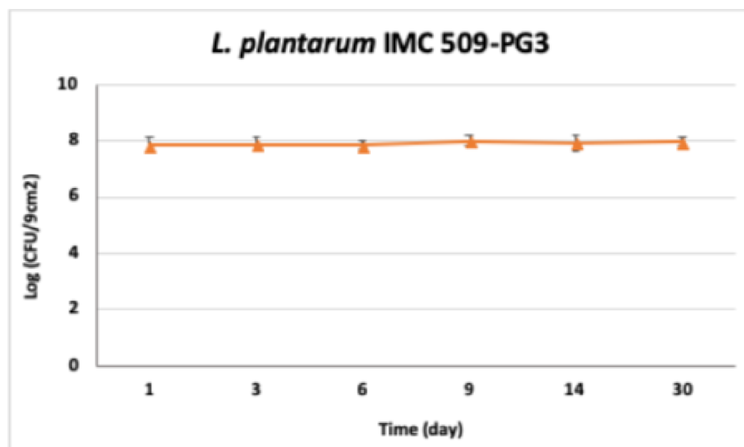

Figure 4. Bacterial cell counts of *L. plantarum* IMC 509 isolated from polylactic acid (PLA) incorporated 3% nanocomposites (Ag-GN-TiO2 II 0.02tt) film surface for 30 days storage at 4°C.

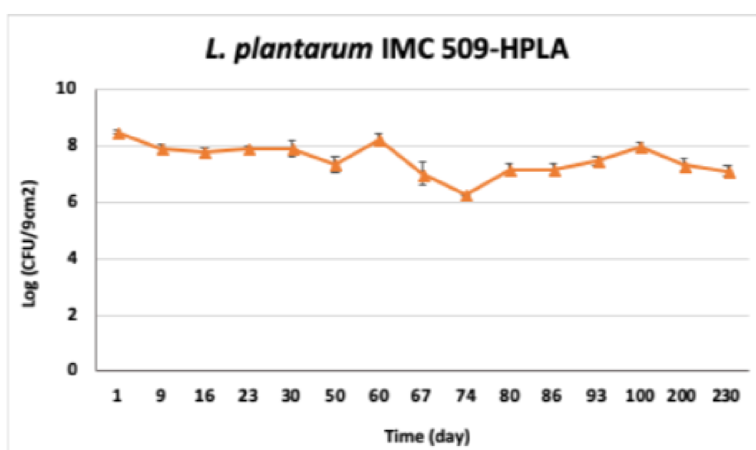

Figure 5. Bacterial cell counts of *L. plantarum* IMC 509 isolated from paper-polylactic. acid (H-PLA sandwich) disc film during 230 days at 4°C.

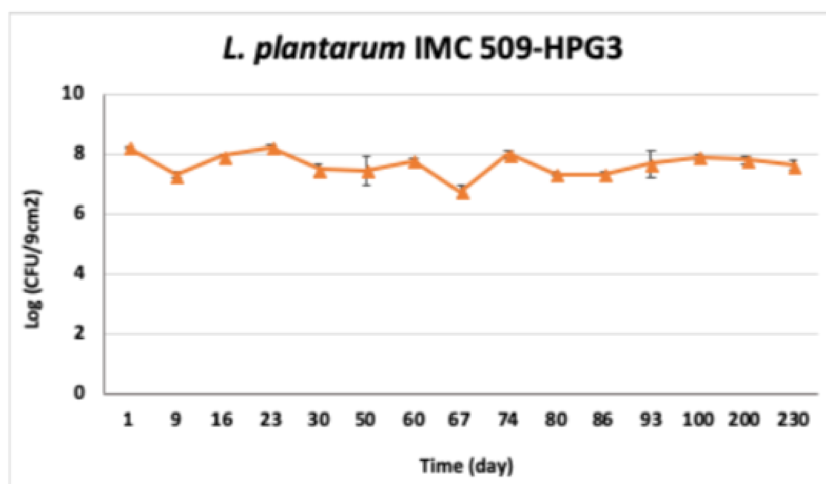

Figure 6. Bacterial cell counts of *L. plantarum* IMC 509 isolated from paper-polylactic acid (H-PLA sandwich) incorporated 3% nanocomposites (Ag- GN-TiO_2 II 0.02tt) disc during 230 days at 4°C.

9.5 | DISCUSSION AND CONCLUSIONS

The viability of probiotics either in food matrix and as part of packaging system is strongly affected by the properties of food components (acidity, preservatives), presence of oxygen, the amount and type of other bacterial groups and their metabolites [3].

Our results showed that the tested lactobacilli - *L. plantarum* IMC 509 and SYNBIO® - were able to survive on the tested packaging films at 4°C for long period. The variation of cell counts during time was due to the inhomogeneity of the spraying process as well as the mechanical properties of packaging film.

L. plantarum IMC 509 was chosen as potential probiotic bacteria used in food packaging because its good adaptability to different packaging matrixes. As the figures showed, the counts of *L. plantarum* IMC 509 presented a nearly linear line on packaging of H-PLA, H-PLA with 3% composites, PLA and PLA with 3% graphene composites for long storage period at 4°C. This effect showed that the probiotic-PBS buffer solution was homogenously sprayed over the tested film surface and the good adaptability of *L. plantarum* IMC 509 on such packaging materials. Therefore, the application of this probiotic strain for designing active packaging film under cold storage is of potential.

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CHAPTER X

MARKET SURVEY ON NOVEL COMPOSITE-PROBIOTIC PLA/ PAPER-PLA PACKAGING

10.1 | INTRODUCTION

To achieve a circular economy and to reduce economic burden and environmental pollution, the European legislation made new plastic strategy to encourage Member States to transit from single-used plastics to the biodegradable/compostable materials such as PLA. Nowadays, the most used food preservation methods include physical hurdles, chemical additives as well as intelligent and active packaging systems [1]. Active packaging indicates the packaging material modified with active compounds that enhance the packaging performances. The main functions of the active compound are regulating packaging atmosphere and moisture, preventing microbial proliferation and delaying chemical reactions [2]. Although the active packaging has been widely investigated and documented in scientific research, consumers perceptions on active packaging are poorly reported. Under such circumstances, in collaboration with the Italian Sensory Analysis Center INNOVATION (CIAS INNOVATION) for using market survey to gain consumers and food professionals feedback, understand consumers inclination and make informed business decisions on the use of this PLA/paper-PLA based active food packaging incorporated with graphene-based composites and probiotic bacteria through questionnaire analysis.

Filling in a detailed questionnaire, the perceptions and concerns of a target population on this novel designed food packages have been collected and analyzed, aiming to verify the risks of its usage and further to verify their market potentials and investment opportunities.

10.2 | MATERIALS AND METHODS

According to the requirements and background of consumers and food industrial professionals, we tailored the content of the questionnaire and distributed into 100 target consumers and 40 food industry specialists (including producers and distributors).

10.2.1 | Consumer questionnaire

The following questionnaire contained an introduction section to familiarize consumers with the background, concept and major components of the novel packages. Moreover, there are personal information collections and seven questions involved in the development of the consumers characteristics, expectations and requirements. For each question, there are five options ranging from score 1 -strongly disagree to 5 -do not know.

The consumer questionnaire is reproduced below.

Questioner for consumers

This questioner is designed to appreciate the feedback of the consumers regarding the packages based on polylactic acid (PLA) and polylactic acid - paper (PLA-H), respectively modified with graphene, Ag, TiO_2 and/or probiotics. Graphene is a carbon structure, Ag nanoparticles are already used in the market included în the washing machine systems, etc. Nanostructured TiO_2 is chemically stable and non-toxic. Graphene, as well as Ag and TiO_2 were used due to their antimicrobial activity. Probiotics are live microorganisms that when consumed in adequate amount confer a health effect on the host. In the present packaging they have antimicrobial properties.

Picture of the paper-PLA modified with graphene-Ag-TiO_2

The European legislation provides the gradually replacement of the plastic packages with low degradability with biodegradable materials such as PLA. Nowadays, the most popular method to prevent the food spoilage is the preservatives and antioxidants addition. A research team from Romania, Italy, Slovenia and Spain developed packages based on PLA and PLA-H, respectively modified with graphene, Ag, TiO_2 and/or probiotics, which extend the food availability without affecting the food safety.
Strong point: The active packages keep in safety conditions fresh cheese and chicken breast meat for a longer time than the conventional packaging.
Weak point: Price of the product increased by max 20%

Gender:		**Female**	**Male**
Marital status:		**Married**	**Single**
Age:	**18-25 years**	**25-50 years**	**> 50 years**

1. Do you think that the main attributes of the food packages are protection, easier transport, handling and storage, information, food rationalization and promotion?

1 (Strongly disagree); 2 (Somewhat disagree); 3 (Somewhat agree); 4 (Strongly agree); 5 (Don't know)

2. Do you believe that the packages can influence the quality and safety of the food stored inside?

1 (Strongly disagree); 2 (Somewhat disagree); 3 (Somewhat agree); 4 (Strongly agree); 5 (Don't know)

3. When I buy a food, I carefully read also the characteristics of the materials that are in contact with it.

1 (Strongly disagree); 2 (Somewhat disagree); 3 (Somewhat agree); 4 (Strongly agree); 5 (Don't know)

4. Would you agree to consume food stored in package based on PLA and PLA-H, respectively modified with graphene, Ag, TiO_2 and/or probiotics kept in safety conditions, knowing that the food stored in a conventional package would be more rapidly spoiled?

1 (Strongly disagree); 2 (Somewhat disagree); 3 (Somewhat agree); 4 (Strongly agree); 5 (Don't know)

5. Would you recommend to a friend to buy food stored in packages based on PLA and PLA-H, respectively modified with graphene, Ag, TiO_2 and/or probiotics?

1 (Strongly disagree); 2 (Somewhat disagree); 3 (Somewhat agree); 4 (Strongly agree); 5 (Don't know)

6. Would you feed the family with food packaged in the above mentioned active packages?

1 (Strongly disagree); 2 (Somewhat disagree); 3 (Somewhat agree); 4 (Strongly agree); 5 (Don't know)

7. Knowing the benefits, would you agree to buy ricotta cheese and/or chicken meat in the active packages even if they are more expensive by 50-100 cents?

1 (Strongly disagree); 2 (Somewhat disagree); 3 (Somewhat agree); 4 (Strongly agree); 5 (Don't know)

10.2.2 | Specialists (producer and distributor) questionnaire

To opinions of food industry specialists - producers and distributors - are fundamental for the market potentials of the investigated packages. Compared to consumers, they may take more considerations in the price and utility of packaging. Similar to consumers questionnaire, there is the section of introduction and background. In addition, questionnaire also examined questions about their opinions on price and acceptation.

The specialists questionnaire is reproduced below.

Questioner for the producers and distributors of packages for cheese and chicken meat storage

This questioner is designed to appreciate the feedback of the consumers regarding the packages based on polylactic acid (PLA) and polylactic acid - paper (PLA-H), respectively modified with graphene, Ag, TiO_2 and/or probiotics.
Graphene is a carbon structure, Ag nanoparticles are already used in the market included în the washing machine systems, etc. Nanostructured TiO_2 is chemically stable and non-toxic. Graphene, as well as Ag and TiO_2 were used due to their antimicrobial activity. Probiotics are live microorganisms that when consumed in adequate amount confer a health effect on the host. In the present packaging they have antimicrobial properties.

Picture of the paper-PLA modified with graphene-Ag-TiO_2

> ***The European legislation provides the gradually replacement of the plastic packages with low degradability with biodegradable materials such as PLA. Nowadays, the most popular method to prevent the food spoilage is the preservatives and antioxidants addition. A research team from Romania, Italy, Slovenia and Spain developed packages based on PLA and PLA-H, respectively modified with graphene, Ag, TiO_2 and/or probiotics, which extend the food availability without affecting the food safety.***
> ***Strong point: The active packages keep in safety conditions fresh cheese and chicken breast meat for a longer time than the conventional packaging.***
> ***Weak points: A more elaborated technology for packaging producing including composite production in addition to the paper and PLA production***
> ***Price of the product increased by max 20%***

Food domain for which produce/distribute the packages:

bakery; milk and dairies; meat and meat products; fruits and vegetables

Type of activity: production; distribution

Company: large; medium; small

1. Which is your opinion about the packages based on PLA and PLA-H, respectively modified with graphene, Ag, TiO_2 and/or probiotics as innovating solution for the storage of fresh cheese and chicken breast meat, respectively designed to extend the food shelf life?

I agree with the implementation of such systems only if the price of the package remains unchanged - 1
I agree with the implementation of such systems even if the price of the package is changing - 2
I`m not agree with the implementation of such systems - 3
I would like to buy/ assign a patent for the production of such innovation systems - 4
I don`t know - 5

2. Would you agree to produce/distribute the packages based on PLA and PLA-H, respectively modified with graphene, Ag, TiO_2 and/or probiotics?

Yes - 1 No - 2 I don`t know - 3

Reason of the choice

___

3. Would you agree to promote the active packages to the producers and processors of cheese and chicken meat?

Yes - 1 No - 2 I don`t know - 3

4. How much would you be willing to pay extra for the active package in comparison with the conventional one, knowing that it extends the shelf life of the product in maximum safety conditions and it is "harmless", i.e. components of the packaging materials do not migrate into food?

10% - 1; 20% - 2; 30% - 3; 50% - 4;
other, please write the maximum percentage _________________ - 5;

10.3 | RESULTS

10.3.1 | MARKETING INSIGHTS– Consumer

The following content shows the results of questionnaires collected from 100 Italian consumers, including their basic personal information, answers to each question, integrated data and observations of the survey.

10.3.1.1 | Consumers information

The selection criteria of consumers were based on their shopping frequency (at least once a week) and their roles in family as decision-makers. Figure 1 shows the basic information - sex, age, civil status of the investigated consumers. Most consumers are woman (60%), and age are older than 25 (85%), with 69% of them already married.

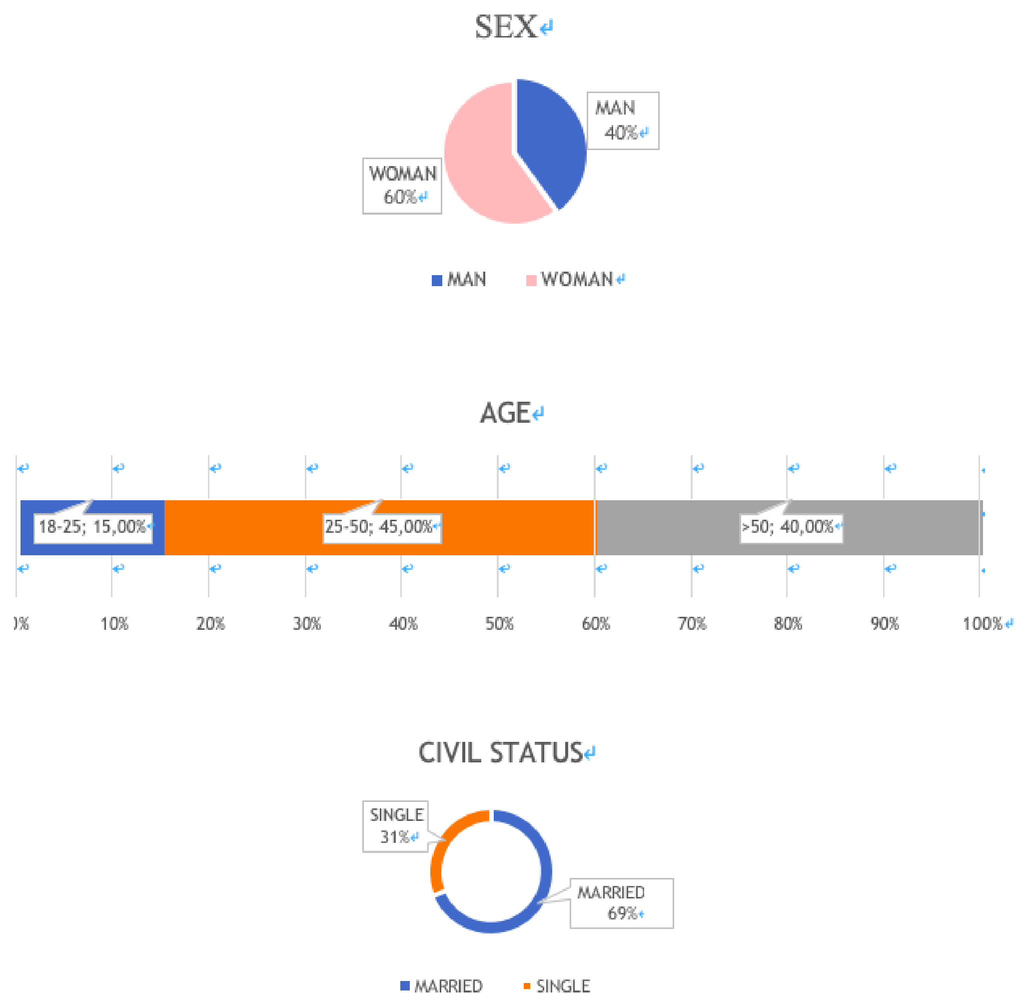

Figure 1. Gender, age and civil status of consumers.

10.3.1.2 | Integrated data of consumers perceptions

Figure 2 shows the overall opinions of consumers on active packaging functions and usage. Most of the consumers (80%) understood and agreed with the definition of food packaging that are used for protection, transportation, storage and so on. Only small portions of consumers did not know the concept, or they did not agree.

Eighty-four percent (84%) of consumers believed that it is possible that the package affect food quality and safety, indicating also consumers concerns on the function of packaging as well as substances migration from packaging to food. Despite that most people concerned about the packaging efficiency, half of them did not care or get used to read the packaging characteristics before buy a food. Lack of direct communications between food industries and consumers is probably the main contributor of this result.

The following questions are related to consumers' acceptance and willingness to use active packaging of PLA/PLA-paper modified with graphene, Ag, TiO2 with or without probiotics. More than half of them were willing to try with the family and also recommend to their friends, even when the price of the active packaging will be higher than the conventional packages. However, the reason why consumer rejected this option was that they were not familiar with the new concepts in legislative regulation, plastic transformation. Moreover, they were probably also worried about the complex terms used to describe the innovative package.

This data further demonstrated that theoretically consumers who showed more interests in novel materials and technologies were willing to try and spend a premium price. On the contrary, consumers that cared less about innovation, they were unwilling to change their perceptions and spend more to buy a product.

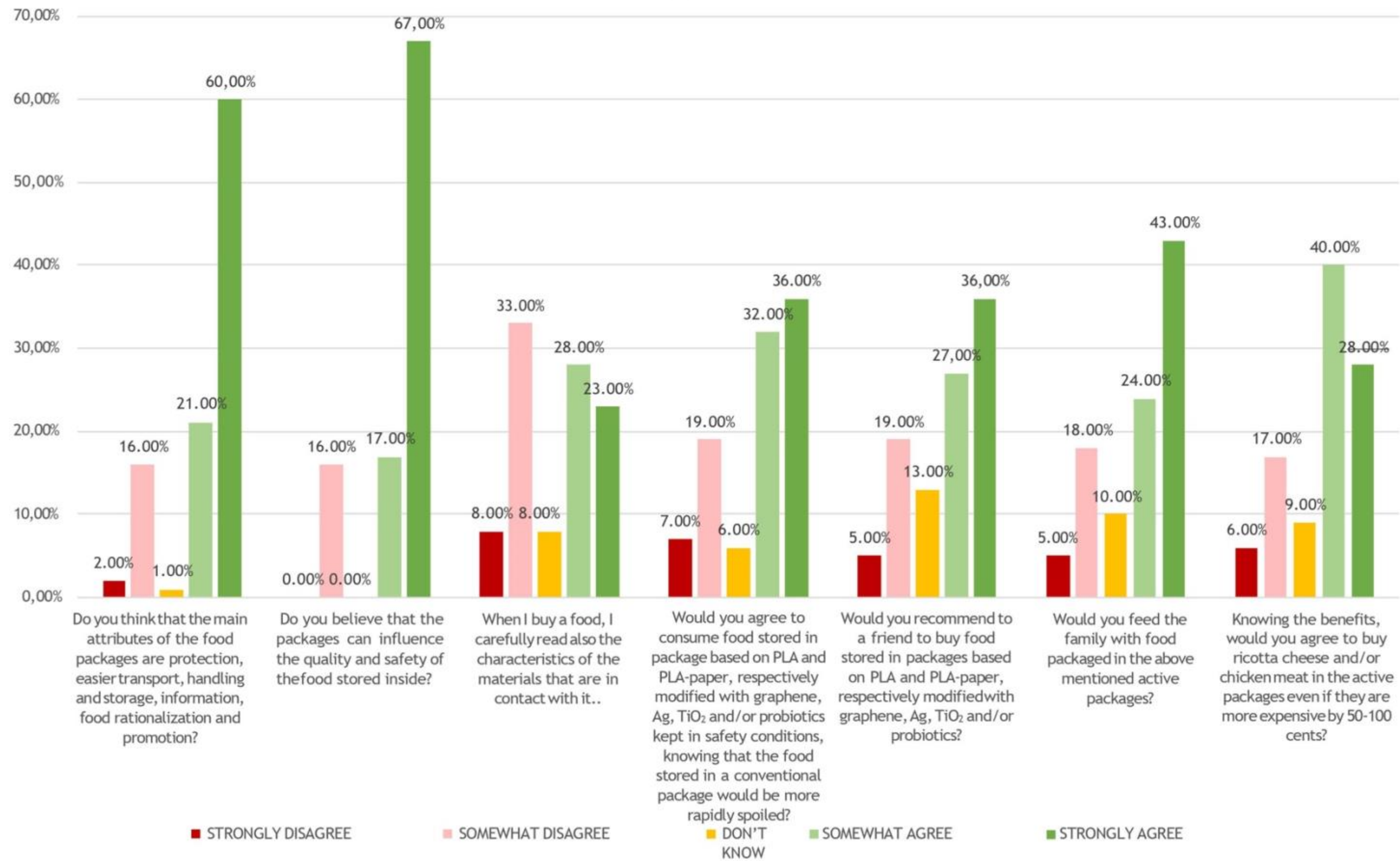

Figure 2. Integrated data of consumers

10.4 | MARKETING INSIGHTS -Food professionals

10.4.1 | Professionals information

Forty food professionals that worked in six different food sectors were involved in the market survey (Figure 3). The distribution shows among them the majority work in meat sector (37%), followed by sweet and confectionery (30%), whereas wine sector counts only 2.5% of total. Production and distribution are their main business with 40% and 30%, respectively. Other business types include R&D and consultancy. There are 40% of food specialists that work in big enterprises, while small and medium enterprises count for 30%.

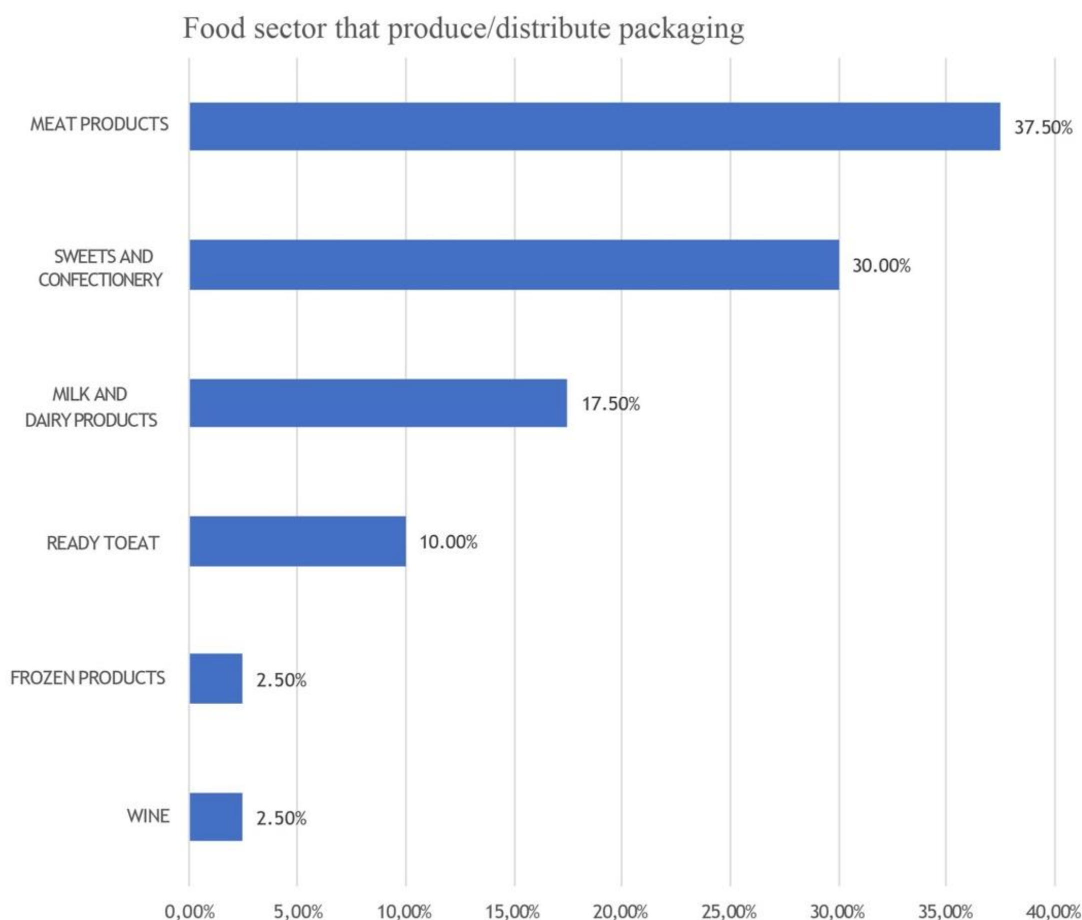

Figure 3. Professionals distribution in food sectors

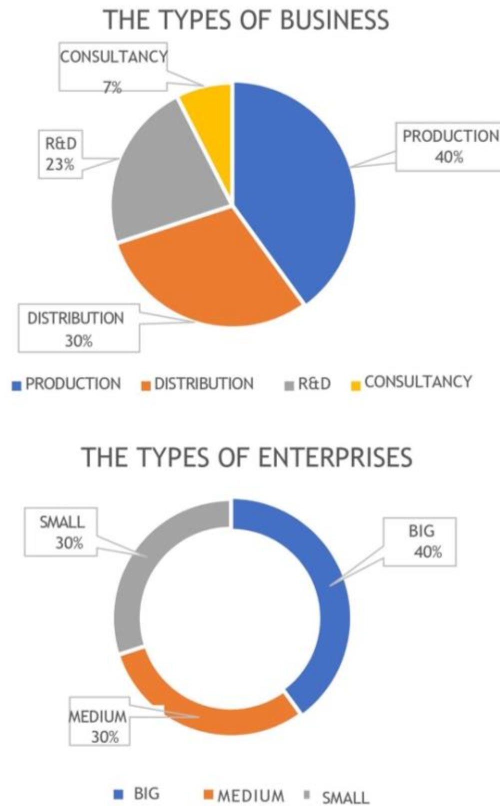

Figure 4. The types of business (upper) and enterprises (below) they involved

10.4.2 | Professionals perceptions

In summary, the majority of food professionals were willing to produce and/or distribute the innovative packaging (70%). For its application on food, the agreed percentage of the subjects slightly decreased with 60%. Furthermore, there were still high percentage - 77.5% of the tested professionals would agree with the implementation of these systems even if the price changes and the average availability to spend more is 16.75%.

In general, there was a good predisposition for innovation in the packaging sector.

The main issues observed by professionals investigation were not mainly related to the price of the product, but to the way in which the pack interact with specific food matrices and they required further information to make strategic decisions.

In summary, considering the development of packaging field, importance of food shelf-life and a better consumer service, food professionals would try and produce the novel active packaging also through the use of information claims.

In contrast, the professionals who had doubts about the production or distribution of the innovative pack were considering three reasons: the desire to study the effect of the pack on a specific food matrix, the need to have more information on the potential of the pack and which could be the unknown reaction of the consumer to this particular innovation.

10.5 | DISCUSSION AND CONCLUSIONS

Through the questionnaire survey, we collected information of consumers' and food professionals' (producer and distributor) willingness, attitudes and worries about this novel packaging to explore the packaging potential and opportunity on market.

After knowing the benefits about the active packaging, although there were concerns about possible migration of packaging materials, the majority of the consumers and professionals would try the active packaging themselves and also promoted the use of this packaging in different food sectors. The increased price did not attenuate subjects attitudes to support packaging transformation from traditional to ecological. In the meanwhile, from those subjects who showed confusion or rejection, we noticed that there was lack of communication between industry and consumers, and also widespread lack of awareness on packaging revolutions. From food professional point of view, they cared more about the impacts of packaging materials on foods, therefore, requiring detailed and specific information about the package-food interactions. Overall, there are great market potentials of the novel packaging, but efforts need to be made from decision-makers to consumers to a more sustainable development.

10.6 | REFERENCES

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CHAPTER XI

POTENTIAL OF *LACTOBACILLUS**-DERIVED CELL-FREE SUPERNATANTS AS FOOD BIO-PRESERVATIVES

***Note**: from this chapter we introduced the use of new nomenclature of the genus Lactobacillus, in accordance with the emended description of the genus Lactobacillus Beijerinck 1901 and union of Lactobacillaceae and Leuconostocaceae (Zheng et al.) [1]*

11.1 | INTRODUCTION

Food is susceptible to the biological and chemical contaminations, such as microorganisms, parasites and heavy metals that can be occurred at any stages from production to consumption. The consumption of contaminated food and beverages can affect different organs, resulting various level of symptoms from the most common diarrhea to serious tumor and even death [2]. As reported by the World Health Organization (WHO), foodborne diseases are mainly reported in developing countries, around one tenth of world population get ill and over 420,000 deaths are caused by ingestion of contaminated foods per year. Children that under 5 years old are the most at risk. Moreover, foodborne diseases also create huge global burdens on the society productivity, medical care and national social activities [3]. Under such circumstances, policymakers, research scientists, food producers have put a lot of efforts in improving food safety as well as reducing food waste.

Food spoilage is generally caused by physical, chemical and microbiological factors that may interact with each other and further affect food quality and organoleptic attributes. Environmental conditions - temperature, humidity, oxygen level, light exposure – can accelerate or delay the spoilage process by affecting the growth and metabolic activities of food microbes, enzymatic reactions in food matrices [4]. Common technologies mainly target on eliminating the potential risk factors as well as controlling the development of the spoilage process to ensure food safety and quality during whole processes. Currently, measures include physical: high temperature, high pressure, irradiation, electrical field; chemical: synthesized preservatives; microbial: bio-preservative cultures and active metabolites [5]. Furthermore, packaging systems with the incorporation of natural agents that come from have been widely investigated.

Lactic acid bacteria (LAB) exist in almost all biological niches and paly roles in human health and food safety. Fermented foods are rich in LAB that contribute to extended shelf-life, reduced antinutritional factors, enhanced organoleptic and nutritional properties [6]. Moreover, LAB in food matrix can create an environment that favor their growth while inhibiting the growth of other microorganisms by competitive exclusion and active metabolites production [7]. Bio-preservation of food using either live LAB or their metabolites have gained interest in developing natural preservatives and active packaging systems. The use of live probiotic cells offers advantages, such as natural preservatives and functional food ingredients [8]. LAB also produce active metabolites - organic acids, bacteriocins, hydrogen peroxide, enzymes, and ethanol - that are of biological activities and food potentialities and health-related benefits [9]. LAB metabolites, in the purified form or in the form of mixture (cell-free supernatant), have been widely investigated and documented for their antimicrobial, antioxidant, anti-inflammatory, anti-cancer, and immunomodulatory properties [10]. Cell-free supernatant (CFS) mainly contain metabolites of high-/low-molecular weight. Currently, numerous research studied the preservation potentials-antimicrobial, antibiofilm formation, antioxidant properties as well as the safety of CFS derived from *Lactobacillus* strains through agar media and in different food matrixes [11]. In food applications, the incorporation of CFS either in food matrix or as part of packaging system has been used to ensure the safety and quality during shelf-life. Compared to live probiotic cells, cell metabolites have several technological and health-related advantages, such as more stable and manageable during food processing, safer for the ingestion of immunocompromised patients, less impacts on food sensorial attributes [12].

Although in literature many studies have reported the method used for characterization of postbiotics, there is significant variation in the extraction time between different studies as the different metabolic profile of each producer-strain, and thus making the comparisons of bioactivities between studies is difficult. Under such conditions, the aims of the present study included: 1) comparison of the antimicrobial efficacy of individual CFS of LAB extracted after 24- and 48-hours incubation; 2) comparison of the antagonistic activity of individual CFS with CFS mixture that is prepared by co-culturing LAB strains or mixing of single CFS; 3) screening the major active compounds by different treatments and short chain fatty acids quantification.

11.2 | MATERIALS AND METHODS

11.2.1 | Lactic acid bacteria selection and culture conditions

For the preparation of CFS samples, ten lactic acid bacteria that were isolated either from human and bees were further identified by 16s RNA sequencing and MALDI-TOF analysis as shown in Table 7. The probiotic strains were composed by five strains of *Lactiplantibacillus*

plantarum, two strains of *Limosilactobacillus fermentum*, one strain of *Ligilactobacillus salivarius*, *Lactobacillus curvatus*, and *Pediococcus acidilactici*. All strains were reactivated by growing in liquid de Man, Rogosa and Sharpe (MRS) (MRS, Oxoid, Italy) broth at 37°C for 24 and 48h, aerobically.

The target bacteria were either from culture collections or food isolated (Table 1). The strains were grown in Tryptone Soya broth (TSB, Oxoid) for 24-48h, at 37°C under aerobic conditions.

Table 1. Bacterial strains used in the study, their origins and culture conditions.

LAB Strains	Origin	Growth conditions (37°C)
Lactiplantibacillus plantarum IMC 509	Human	MRS broth
Lactiplantibacillus plantarum 24H	Human	MRS broth
Lactiplantibacillus plantarum 2.1B	Human	MRS broth
Lactiplantibacillus plantarum API6	Bee	MRS broth
Lactiplantibacillus plantarum API1	Bee	MRS broth
Limosilactobacillus fermentum 27D3F	Human	MRS broth
Limosilactobacillus fermentum 22A	Human	MRS broth
Ligilactobacillus salivarius 26C	Human	MRS broth
Pediococcus acidilactici 46A	Human	MRS broth
Lactobacillus curvatus L-A1	Human	MRS broth
Target strains		
Gram negative bacteria		
Escherichia coli ATCC 13706	Culture collection	TSB broth
Pseudomonas aeruginosa DSM 1117	Culture collection	TSB broth
Salmonella enterica subsp. *enterica sv. Enteritidis* DSM 14221	Culture collection	TSB broth
*Proteus mirabilis prmi 27/77/*IMV4	Clinical isolated	TSB broth
Gram positive bacteria		TSB broth
Bacillus cereus ATCC 9634	Culture collection	TSB broth
Enterococcus faecium DSM 13590	Culture collection	TSB broth
Listeria monocytogenes 306	Food isolated	TSB broth
Staphylococcus aureus subsp. *aureus* ATCC 25923	Culture collection	TSB broth

ATCC, American Type Culture Collection; DSM, German Collection of Microorganisms and Cell Cultures; IMV, Institute of Microbiology and Virology, Ukraine; MRS, de Man Rogosa and Sharpe; TSB, tryptone soya broth

11.2.2 | Preparation of cell-free supernatant

After incubation, each strain of LAB was further isolated on MRS agar. Then, the single colony of each strain was suspended in 5 ml of sterile saline solution and the density was

adjusted to around 1x10^9 CFU ml^{-1} using spectrophotometer (Shimadzu uv-1800) at OD_{600}. 0.05ml of bacterial suspension was further transferred into 39.6 ml of MRS broth culture with final concentration of 10^7 CFU ml^{-1} (7 log ml^{-1}). All the cultures were incubated at 37±1 °C in water bath with continuous shaking (170 speed). To compare the cell metabolites produced at different incubation period, the CFS of each sample was separated from biomass after 24 and 48 hours of incubation. Before extracting the CFS, the pH value of each sample was measured and recorded three times using pH meter (Jenway™ Benchtop pH Meters) and pure MRS broth culture was used as control. The cell cultures were subjected to centrifugation at 6000 rmp for 20 minutes (4°C) and the supernatants were further sterilized using 0.22 µm pore size filters (Millex-GS, Cork). All the CFS were divided into aliquots and used immediately or stored at -20°C until use.

11.2.3 | Antimicrobial spectrum of individual CFS

The antimicrobial activity of the individual CFS was evaluated through agar-well diffusion method as described by Koohestani [13] with slight modifications. Briefly, bacterial suspension of target pathogenic strains (10^7 CFU ml^{-1}) was prepared using sterile saline. Eight mm circular wells were cut on Mueller Hinton agar with standardized thickness (around 20 ml agar/plate, MH, Merck) using a sterile cork borer and each pathogenic strain was lawn homogenously on the MH agar surface using a sterile cotton swab. 100µl of CFS was injected into the well and 100µl of sterile MRS broth was used as control. All the samples were incubated at 37°C for 24 h. After incubation, the diameter of inhibition zone was measured using a caliper, in triplicate. The inhibition efficiency was calculated by subtracting the diameter of well from the diameter of inhibition zone and expressed as mm. The CFS and the producing-bacteria that demonstrated the highest inhibitory activity against the tested pathogen were selected for the preparation of CFS-mixture.

11.2.4 | CFS mixtures preparation and antimicrobial activity assessment

The CFS mixture was firstly prepared by mixing the individual CFSs that exhibited the highest inhibitory activity, which was composed by equal volume (1ml) of the following CFS: IMC 509 (24h) + 2.1B (48h) + API1 (24h) + API1 (48h) + 46A (24h) + 46A (48h) + 26C (24h). Moreover, a concentration gradient of 100%, 50%, and 25% was obtained by two-fold dilution using sterile MRS broth. Moreover, to examine the active compounds production ability of multiple strains that grow together, three combinations of strains were proposed for co-incubation, including: 1) API1, 46A, and 26C; 2) 2.1B, 46A, and 26C; 3) API1, 2.1B, 46A, and 26C with final concentration of 10^7 CFU ml^{-1} per strain. The combination cultures were incubated at the above-described conditions, and the CFS of the combinations were extracted

after 24 and 48 hours. The inhibitory activity of the CFS mixtures was checked using agar-well diffusion test (described above) in triplicate, sterile MRS broth was used as negative control.

Table 2. Preparations of CFS mixtures by mixing individual CFSs or co-culturing of multiple strains.

CFS mixture	Combinations	Concentrations/Time
Mixing of individual CFSs	IMC 509 (24h) + 2.1B (48h) + API1 (24h) + API1 (48h) + 46A (24h) + 46A (48h) + 26C (24h)	100% 50% 25%
Coculturing	1) API1, 46A, and 26C 2) 2.1B, 46A, and 26C 3) API1, 2.1B, 46A, and 26C	24 and 48h

11.2.5 | Assessing the heat, pH, enzyme sensitivity of CFSs mixture

To preliminary characterize the major antimicrobial chemical components present in CFS mixture (CFSM, mixture of single CFSs), the latter has been treated with heat, enzymes, and pH adjustment (Table 3) and its inhibitory spectrum after treatments was assessed through agar well diffusion test as described above [14]. The function of organic acids was examined through neutralizing CFSM to pH value of 3, 7, and 9 with NaOH (1N) or HCl (1N). To verify the thermostability of the active compounds, CFSM was heated at 100°C and 121°C (autoclave) for 15 minutes. For bacteriocin-like substances and hydrogen peroxide (catalase), the neutralized CFSM (pH=7, proper for the activity of enzyme) was distributed into equal portions (2 ml) and each portion was added with the 1 mg ml^{-1} of the following enzymes: α-chymotrypsin (Sigma, USA), proteinase k (Merck, Germany), catalase (Sigma), lysozyme (Sigma). Due to the optimum pH value for pepsin is at 3, therefore, 1 mg ml^{-1} of pepsin (Sigma) was added into CFSM with pH 3. After treating with enzymes, the CFSM containing lysozyme and pepsin were incubated at 30°C for 2h, whereas the rest enzyme-treated samples were incubated at 37°C for 2h. The CFSM without any treatment was used as control.

Table 3. Different treatments on CFSM and the incubation conditions.

Treatments	Conditions
pH	3 7 9
Heat	100 °C, 15 minutes 121 °C, 15 minutes
Enzyme	α-chymotrypsin (pH 7, 37°C, 2h) catalase (pH 7, 37°C, 2h) proteinase K (pH 7, 37°C, 2h) lysozyme (pH 7, 30°C, 2h) pepsin (pH 3, 30°C, 2h)
Control	untreated CFSM

11.2.6 | Quantification of short chain fatty acids in the CFS mixture

The samples of CFSM were analised by the research team of prof. Dennis Fiorini following the methodology reported by Scortechini *et al.* [15].

11.3 | RESULTS AND DISCUSSION

11.3.1 | Antibacterial activity of individual CFS

Table 4 illustrates the antimicrobial efficiency of CFSs derived from individual *Lactobacillus* strain. For each target bacterial strain, the highest inhibition zone is highlighted in black square. In general, CFSs derived from different species demonstrated different inhibitory spectrum and the efficacy of the same CFS varies between 24 hours and 48 hours incubation. *L. plantarum*-CFSs (IMC 509, 24H, 2.1B, API6, API1) demonstrated higher inhibitory activities in limiting the growth of *E. coli, Ps. aeruginosa, S. enterica, B. cereus,* and *St. aureus*, whereas *Pc. acidilactici* 46A-CFS was more effective in inhibiting *P. mirabilis* and *L. monocytogenes*, especially after 24 hours and 48 hours of incubation, respectively. To be noticed that CFS of *L. salivarius* 26C was effective to many tested strains with exception of *St. aureus* that was resistant to it. Moreover, its inhibitory activity against *Ent. faecium* was only present after 24 hours and disappeared after extended incubation time (48 hours). Similar results were also observed in all *L. plantarum*-CFSs against *St. aureus*; *L. fermentum* 22A.2-CFS against *P. mirabilis; Pc. acidilactici* 46A against *S. enterica*. Rodrigues [16] found that the *L. fermentum* TcUESC01 metabolites were active (appear of inhibition zone) against *Streptococcus mutans* UA159 during the 14-16h of the growth (stationary phase). However, the inhibitory effect was not observed after 24 hours of growth.

Generally, the concentration of active metabolites varied according to the incubation time, and thus the antagonistic activity of the CFS derived from the same producer strain against the specific tested bacteria was different at 24 and 48 hours. For example, *E. coli, P. aeruginosa, L. monocytogenes* were more sensitive to the activity of 48h CFSs, whereas the growth of other bacterial strains was more affected by the CFSs-24h. *B. cereus* was the only strain that showed sensitivity to all tested CFSs, especially to *L. plantarum*-derived CFSs (24h). Similar to our study, *Lactobacillus plantarum* subsp. *plantarum* NC 8 supernatant exhibited stronger antagonistic activity than supernatant of *Pediococcus pentosaceus* IE 3 in limiting the growth of *B. cereus* that demonstrated the highest inhibition zone compared to other pathogens. Moreover, the authors presumed that the antagonistic activity was mainly ascribed to the organic acids, which was produced and accumulated at the end of exponential phase (around 20 hours incubation) by the *L. plantarum* subsp. *plantarum* strain [17]. On the contrary, *L. salivarius* 26C-CFS extracted after 48h showed higher efficacy in inhibiting *E. coli, S. enterica, P. mirabilis, L. monocytogenes.* Regarding the anti-listeria activity, in our study, only three CFSs showed effectiveness, especially, the CFS of *Pediococcus acidilactici* 46A of 48 hours. We hypothesized that the specific bacteriocin-pediocin was produced by the *Pc. acidilactici* and it was involved in the anti-listeria activity observed. Although pediocin had a relatively narrow antimicrobial-spectrum, its activity against food pathogen – *L. monocytogenes* has been widely used in developing active food packaging systems [18]. Another study has found that the culture pH value and growth curve (end of exponential phase) of *L. fermentum* SHY10 were stabilized at 20 hours of incubation, reaching and keeping the maximum of anti-*S. aureus* activity until 48 hours of incubation [19]. Therefore, we may conclude that due to the different metabolic profile of strains, the inhibition efficacy varies with the active metabolites concentrations and pathogen react differently to bioactive metabolites that produced at different growth stages. Thus, it is always necessary to build up the correlation between the antimicrobial activity and the different growth phases before assessing other associated bioactivities in order to maximize the desired efficiency toward target strains.

Based on the results obtained from individual CFS, several CFS mixtures, either by mixing the CFSs with highest activity or by coculturing multiple producer strains, were prepared and their inhibitory activities were compared with individual CFSs.

11.3.2 | Antibacterial activity of CFS mixture

Table 4 lists also the diameter of inhibition zones of CFS mixtures prepared by coculturing selected bacterial strains or by mixing equal portion of individual CFSs. Compared to the CFSs of coculture, CFS-mixture prepared by mixing seven individual ones showed broad inhibition spectrum that effective against all selected strains and it was highly active against the growth of Proteus mirabilis. As Table 6 shows, the efficacy of individual CFS that demonstrated the

highest values decreased after mixing them together, which may be due to the specific active metabolites were diluted in the mixture and thus the inhibitory toward the target bacteria was diminished. On the contrary, CFS mixture was still highly effective against *Proteus mirabilis*, with the biggest diameter of inhibition. Therefore, based on these findings, the CFS mixture obtained with individual CFSs was selected for future analysis.

Table 4. Inhibitory efficiency of individual CFSs and CFS mixtures detected by measuring the inhibition zone (mm) in an agar-well diffusion test, after 24 and 48 hours.

	E. coli		*P. aeruginosa*		*S. enterica*		*B. cereus*		*S. aureus*		*P. mirabilis*		*L. monocytogenes*		*E. faecium*	
CFS	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**
L. plantarum IMC 509	5.69	6.34	3.25	8.97	5.63	5.58	17.91	14.91	6.86	0.00	3.42	2.94	0.00	0.00	0.00	0.00
L. plantarum 2.1B	5.16	7.25	3.18	9.07	6.71	5.85	16.78	14.21	6.80	0.00	3.64	3.28	0.00	0.00	0.00	0.00
L. plantarum 24H	5.27	6.10	3.08	8.72	6.41	5.51	16.75	14.58	7.48	0.00	5.55	3.36	0.00	0.00	0.00	0.00
L. plantarum API6	4.93	5.10	6.87	8.24	6.28	5.16	17.68	13.20	5.81	0.00	5.27	2.79	0.00	0.00	0.00	0.00
L. plantarum API1	4.71	4.80	6.58	9.10	6.84	3.66	17.14	14.36	9.16	0.00	7.78	2.58	0.00	0.00	0.00	0.00
L. fermentum 27D3F	0.00	0.00	2.77	4.82	0.00	0.00	12.89	8.09	4.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L. fermentum 22A.2	0.00	0.00	1.51	2.33	0.00	0.00	6.71	3.75	0.00	0.00	6.46	0.00	1.74	5.20	0.00	0.00
P. acidilactici 46A	2.88	4.52	5.12	6.25	3.39	0.00	11.29	9.54	0.00	0.00	8.65	2.62	5.28	8.78	0.00	0.00
L. curvatus L-A1	0.00	0.00	0.00	0.00	0.00	0.00	4.72	4.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L. salivarius 26C	2.56	4.74	5.92	5.08	3.36	4.32	12.42	9.88	0.00	0.00	3.38	8.14	5.32	7.73	3.76	0.00
Mix coculture (A24)	3.22	2.80	5.18	5.44	3.97	5.14	11.48	11.55	3.75	0.00	9.80	10.46	4.56	6.44	0.00	0.00
Mix coculture (224)	3.33	3.07	4.08	5.76	3.10	3.62	11.69	11.33	5.36	0.00	10.26	10.07	4.79	5.68	0.00	0.00
Mix coculture (A224)	3.88	3.40	5.97	5.98	3.97	4.73	11.68	11.99	3.91	0.00	10.12	10.03	5.23	5.61	0.00	0.00
Mix (7)	**4.91**		**5.08**		**4.35**		**11.83**		**5.12**		**10.85**		**5.71**		**3.31**	

Note: Mix coculture (A24) represents the CFS mixture prepared by co-inoculating: *L. plantarum* API1, *L. salivarius* 26C, *P. acidilactici* 46A.

Mix coculture (224) represents the CFS mixture prepared by co-inoculating: *L. plantarum* 2.1B, *L. salivarius* 26C, *P. acidilactici* 46A.

Mix coculture (A224) represents the CFS mixture prepared by co-inoculating: *L. plantarum* 2.1B, *L. plantarum* API1, *L. salivarius* 26C, *P. acidilactici* 46A.

Mix (7) represents the CFS mixture prepared by mixing seven single CFS using: *L. plantarum* 2.1B (48h), L. *plantarum* API1(48h), *L. plantarum* API1(24h), *L. plantarum* IMC 509 (24h), *P. acidilactici* 46A (24h), *P. acidilactici* 46A (48h), *L. salivarius* (24h).

11.3.3 | Sensitivity of the antimicrobial compounds of CFS mixture to heat, pH, enzymes activity

In general, the heat treatments reduced the inhibitory spectrum, in which heat-treated CFSM lost its activity against *E. faecium*, suggesting the anti-*E. faecium* compounds may be heat-sensitive. Whereas the efficiency toward other strains were maintained (Table 7). Therefore, it may be presumed that most of the active compounds are thermostable, and they can resist high temperature, such as 100°C and 121°C (high pressure) for 15 minutes.

The neutralization and alkalization treatments have abolished the antimicrobial capacity of CFSM. On the contrary, the acidification slightly enhanced its efficiency, suggesting that the presence and function of organic acids.

Moreover, the results of enzyme digestion showed the active compounds were susceptible to proteolytic activity, with exception of pepsin. Thus, the antimicrobials present in CFSM are proteins that could be bacteriocin-like substances. Also, the addition of catalase negatively affected the antimicrobial capacity of CFSM, suggesting the possible presence of hydrogen peroxide. It is noteworthy that the pepsin digestion (1mg ml^{-1}, 2h) enhanced the CFSM antimicrobial efficacy, showing the highest inhibition diameter among all treatments. This result suggested that either pepsin transformed the protein substrates to peptides that possess higher activity or pepsin digestion eliminated the non-functional proteins and thus concentrating the active compounds in CFSM. Moreover, the low pH of CFSM (pH 3) also may contribute to enhanced antimicrobial activity. In summary, CFSM antimicrobials were stable under high temperature, acidic conditions, and sensitive to proteolytic enzymes, demonstrating the possible presence of bacteriocin-like substances, hydrogen peroxide and organic acids in CFSM.

Table 5. Antimicrobial efficiency of CFSM detected by measuring the inhibition zone (mm) in an agar-well diffusion test, after heat, pH and enzyme treatments.

Treatment	*E. coli*	*P. aeruginosa*	*S. enterica*	*B. cereus*	*S. aureus*	*P. mirabilis*	*L. monocytogenes*	*E. faecium*
Control	5.73	5.40	3.97	11.98	4.42	4.64	5.58	2.60
100°C	3.99	5.66	5.78	12.83	2.74	4.43	5.33	0.00
121°C	4.09	5.97	6.18	12.43	2.66	3.80	4.83	0.00
3	7.92	6.94	8.06	15.42	5.63	5.18	5.75	5.19
7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pepsin	7.20	6.43	8.50	13.70	5.89	5.61	7.21	6.00
Chymotrypsin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteinase K	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lysozyme	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Catalase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

11.3.4 | The short-chain fatty acids quantification in CFS mixture

Table 8 shows there are four short-chain fatty acids detected from CFS mixture, with high amount of acetic acid, and low amount of propionic, isobutyric and butyric acid. Organic acids have a long history as anti-spoilage agents in food applications. Under acidic conditions, undissociated form of organic acids can across cell membrane of bacteria and molds and lowering cell contents pH and interrupting cell enzymatic reactions, leading to cell death [19]. Acetic acid has been used as anti-septic compounds for contrary, it demonstrated potent and wide antimicrobial spectrum even at low concentration (0.166%). In addition, the efficiency of acetic acid was not reduced by evaporation [20]. For food bacteria, acetic acid has inhibited the growth of *Streptococcus spp., S. aureus, E. coli, P. aeruginosa, Proteus spp.* under different concentration through well diffusion method. Its antagonistic activity exerted in a concentration-dependent way [21]. The presence of high amount of organic acids contributed to lowered pH of CFS. Moreover, as we observed from treatment sections, the neutralization of CFSM abolished CFSM antimicrobial activity, suggesting that the main antimicrobial compounds of CFSM are organic acids and/or compounds that are sensitive to alkaline conditions. The quantification of short-chain fatty acids pointed out that the acetic acid is the main organic acid that present in CFS mixture and contributed to lower pH value of the CFSM. Further analysis needs to verify whether the antimicrobial activity of CFSM was ascribed to acetic acid or there are other antimicrobial compounds.

Table 6. Type and amount of short-chain fatty acids of CFS mixture.

SCFAs	Concentration (μmol/g)
acetic acid	61.24 ± 0.79
propionic acid	0.30 ± 0.03
isobutyric acid	0.09 ± 0.01
butyric acid	0.13 ± 0.01
isovaleric acid	Nonquantifiable
isocaproic acid	Nonquantifiable
caproic acid	Nonquantifiable

11.4 | CONCLUSIONS

The characterization of the major properties of CFS, such as antimicrobial, antibiofilm and antioxidant potentials were our main objectives of the study. Although the content discussed

above only was a preliminary study, there are interesting findings and potentials that worth to discover. The results of single CFS showed that the tested pathogen reacted differently to CFSs that extracted at different time. However, there was no single CFS that showed inhibitory activity against all tested strain. Therefore, we made a CFS cocktail by mixing single CFS (highest inhibitory efficiency) and also by coculturing selected LAB strains. The CFS mixture prepared by single CFS mixture demonstrated broader antimicrobial spectrum, although the efficacy was slightly decreased, with exception of *B. cereus*. Probably due to the mixing process dilute the specific active compound. Coculture CFS mixture revealed limited antimicrobial capacity, with no inhibition zone found on *E. faecium* and its anti-*S. aureus* capacity was also lost after 48-hour incubation. Therefore, we carried out the following test using CFSM of single CFSs. To identify the major active compounds, present in CFSM and gain some insights into the processability of CFSM, we firstly treated CFSM using high temperature, acids and bases, digestive enzymes to determine whether the treatments may damage its antimicrobial activity. The results revealed that heat treatments only reduced the anti-*E. faecium* ability and maintained the antagonistic activity against other bacteria. To be noticed that the acid and pepsin treated CFSM showed higher antimicrobial efficiency, suggesting that the main antimicrobials are organic acids. To confirm our presumption, the result of SCFA quantification revealed that there was significant amount organic acids that mainly acetic acid, which possess strong antimicrobial ability. Moreover, our results also showed that *Pc. acidilactici*-CFS possess strong activity against *L. monocytogenes* that is a life-threatening pathogen in risk groups. As documented by literatures, *Pc. acidilactici* can produce pediocin with anti-*Listeria* potentials. Therefore, our next analysis will proceed with the identification and quantification of active proteins – bacteriocins - in CFSM and deepen our knowledge on other potentialities of CFSM. However, it should be noted that the strong color of CFSM caused by the broth culture limited its application in light-colored food. Moreover, CFSM is more proper to be considered as a hurdle technology in food applications in combination with other techniques to guarantee the safety and quality of foods.

11.5 | REFERENCES

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CHAPTER XII

FINAL CONCLUSIONS

12.1 | INTRODUCTION

Currently, we are facing challenges from the food waste and plastic pollution. To face these challenges and achieve circular economy, sustainable food packaging was used with aims of transforming agricultural/food waste into high-quality, reusable bio-packaging materials that can replace plastic usage in market. Polylactic acid (PLA) is a recyclable and biodegradable polyester that can be synthesized by microbial fermentation of agro-food waste, improving economy and saving energy. In addition, its packaging performances and bioactivities can be tailored through adding fillers or modifying polymer matrix [1]. As the great potentials and safe for food contact, the use of PLA in food packaging is widely explored and developed [2]. However, the brittleness and inadequate barrier properties are still the main limitations for the widespread of PLA. Under such circumstances, fillers, such as composites, fibers, cellulose, agro-waste extracts, are used to reinforce the PLA-based packaging performances and confer additional bioactivities [2].

Graphene is covalently linked carbon atoms that form one-atom-thick hexagonal lattice. The large surface to volume ratio of graphene allows its applications in electronic parts, biosensors, transistors, biomedical instruments [3]. Moreover, the antimicrobial properties of graphene and its derivatives attracted researchers' attention on food packaging applications. The use of graphene-based composites in PLA-based films has improved the mechanical properties as well as brought microbial-controlling ability to the package [4].

Lactic acid bacteria (LAB) are ubiquitous in foods, their functions involve fermentation, flavor development and preservation. Recently, the preservative roles of LAB and their active metabolites have been extensively studied and reviewed. As bio-preservatives, their food applications possess advantages like less impacts to food texture, safe and health-associated benefits to consumers [5].

The aim of our study was to use active graphene-based composites and probiotic bacteria as active substances for novel packaging perspective.

The study was carried out to explore the concept of "active graphene-based food packaging for a modern society" aiming 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. To achieve this objective, we firstly characterized the efficiency of commercial (current used) plastic/paper-based packaging on selected foods - meat and cheese. Secondly, through different investigations, we selected the proper graphene-based composite and probiotic bacteria to be used for designing the novel active packaging. Thirdly, the efficiency of novel active packaging on meat and cheese had been established and compared with commercial counterparts. In addition, the study also though to evaluate the efficiency of packaging with recovered composites, to understand the market potentials and to explore other possible probiotic-derived bio-preservatives.

12.2 | EMPIRICAL FINDINGS

The main empirical findings are chapter-specific and, the empirical evidence obtained to fulfill the requirements of each specific objective, are synthesized in this section.

1) **Characterization of commercial polyethylene packages (for fresh cheese) and modified-paper packages (for raw meat) on food**.

   The packaging efficiency was mainly determined and summarized by their impacts on cheese microbial, pH, chemical and organoleptic parameters during storage. Conventional reference packages for fresh cheese and meat storage were collected from four European countries.

   a. Although the pH value, and sensorial characteristics of ricotta cheese were maintained during the first 9 days storage, polyethylene-based packages had little impact on the growth of cheese microbiota under cold storage and aerobic storage, showing reduced storage time than suggested (7 days).
   b. To prevent moisture loss, polyethylene/oxidized starch/wax laminated paper were used for raw meat storage. Regarding the microbial quality, Romanian package was slightly better in limiting the proliferation of meat microbes. Under cold and aerobic conditions, the shelf-life parameters revealed that the wax paper caused a great water loss on meat, and highest biogenic amines production due to the increased microbial metabolic activities.

   Despite the variations among samples, differences in packaging properties, we tried to evaluate and compare their behaviors through monitoring the parameters of food stored inside during common storage conditions. By doing this, we gained insights into commercial used packaging on food – ricotta cheese and raw chicken meat – main

parameters during time and also, we used these data to compare with that obtained from active packaging.

2) **Selection of graphene-based composite and probiotic bacteria**

a. Graphene composites with only graphene and graphene-titanium dioxide exhibited no inhibitory activity neither to pathogens nor to probiotics, whereas composites containing silver nanoparticles inhibited microbial growth, especially to pathogens. Regarding the growth behavior of probiotics with the presence of composites in liquid broth, *L. plantarum* IMC 509 was considered better as its highly stable growth and high tolerance to composites Ag-GN-TiO_2 II 0.02 tt in broth. The Ag-GN-TiO_2 II 0.02 tt composite was considered as an active filler for designing active packaging.

b. The application of *L. plantarum* IMC 509 on polyethylene (PE) packaging lowered the biogenic amines produced during storage and had no impact on meat pH value and positively affected meat sensorial qualities. Moreover, its stability on PE packaging during storage further proved its potentials in food packaging.

Through the tests, we decided to incorporate different concentrations of Ag-GN-TiO_2 II 0.02 tt in PLA film and paper as active packaging for food preservation.

3) **Assessment of PLA-based packaging with modification of composite and *L. plantarum* (LP) IMC 509 and comparison with commercial package in preserving ricotta cheese and chicken breast meat**

a. Although probiotic bacteria and composite addition (at higher concentrations) increased cheese microbial counts, this result was caused by addition of probiotic bacteria, weakened barrier properties of the packaging. But probiotic-containing film reduced the amount of *B. cereus* and also slowed the rate of the bacterial proliferation during storage.

b. Graphene composite incorporation improved the mechanical strength of PLA-paper packaging, but the barrier properties were still weaker than polyethylene film. Although LP incorporation increased meat microbiota, but it positively preserved meat sensorial and chemical qualities. Moreover, the packaging contained with recovered composites still possessed good preservation efficiency.

c. By using food models, we examined the efficiency of active packaging with different formulation of active compounds. Contrary to our expectations, the active packaging did not inhibit the growth of food microorganisms, this was probably due to the LP contributed to part of food microbial count and the active film could not prevent moisture and gas exchange that favored the growth of cheese microbes. Moreover, LP-modified package delayed the microbial proliferation rate.

4) Probiotics strains – *L. plantarum* IMC 509 - showed good adaptability to different packaging materials for extended period under cold storage. Marketing investigation revealed that the active packaging was popular for professional workers and consumers who are aware and open-minded. In general, research, policymakers, packaging and food industrials, and consumers are all equally important to progress the transformation from plastic to biodegradable materials. Moreover, as above, some microbial groups of food were not limited by the probiotic modified packaging.
   We thought about the active metabolites of probiotic bacteria and other LAB strains as alternatives. Through the testes performed, the cell metabolites showed broad inhibitory spectrum and resistant to heat treatments and pepsin digestion, demonstrating a potential role in food processing and preservation.

12.3 | LIMITATIONS AND FUTURE PERSPECTIVES

For specific food storage, fresh cheese and raw chicken meat, we characterized and tailored the active package to meet the specific composition and requirements of the food while maintaining the bioactivity and biodegradability of the package. Through using different food matrixes and mimicking the home-storage conditions, the current studies revealed the direct impacts of packaging on three important food shelf-life parameters, including microbial composition and their growth trend during storage that are associated to food safety and quality during time; chemical indexes reflected the chemical reactions caused by multiple storage factors, such as moisture, gas, light exposure as well as microbial metabolic activities. Moreover, by monitoring and judging the sensorial attributes of food, we tried to perceive food from the view of consumers. The market surveys on consumers and food professionals gave us valuable opinions on the potentiality of active packaging for large production. The exploration of food preservation potentials of LAB and their metabolites allowed us to understand their interactions with food components and packaging material, to evaluate their impacts on food qualities, to broaden their applications in food sector.

However, the studies encountered several limitations, which need to be considered. Regarding sample properties, although in each test we tried to standardize samples by using the same piece of meat or cheese, the heterogeneity of food (blood vessels distribution in meat and fat in cheese) and different production batches made the results difficult to analyze and interpret. In addition, the intrinsic properties, analyzing methods and instrument requirements limited the sample size being analyzed at the same time point, creating difficulties for comparison. For future work, there is necessary for adopting fast screening methods to analyze large number of

samples in order to minimize the potential variances. Although sensorial evaluation by trained panelists is the "fundamental standard" to judge from consumers' view, but to scientific research, devices such as colorimeter, texture analyzer could provide additional information to result interpretation. The currently investigated food packaging was just a prototype for lab-scale studies, to realize large-scale production, the packaging performances and the incorporation methods for probiotic bacteria/active metabolites are parameters need to be improved. Moreover, evaluations of food chemistry parameters – headspace gas composition, moisture variations, macromolecule variation (lipid oxidation) – and the impacts on food under different storage conditions (temperature variations, light exposure) can also provide valuable insights and expand knowledge in package characterization. Due to the lack of shelf-life guidance that cover major food aspects, we can only draw conclusions based on specific criteria, future works are needed to standardize methodologies and to link associations between different aspects of food and packaging studied. Recently, as the well-documented bioactivities and health-benefits associated with probiotic metabolites, their protective roles and technical advantages are also attracting researchers attention. In future, the use of these natural preservatives can be validated and tailored according to food matrix properties. Another important role of graphene composites is their anti-viral activity through physical damage. Study shown that SARS-CoV-2 can remain active on meat and fish at cold (4°C) and freezing temperature (-10°C to -80°C) up to 21 days, which leading to possible spread of disease through contaminated foods [8]. It could be interesting to examine the antiviral activity of decontamination treatment using graphene-based packaging on food.

12.4 | CONCLUSION

Despite the long history of plastic as food packaging, the serious problems caused by plastic waste and environmental pollution urges the usage and development of bio-based, biodegradable and compostable materials. Our project aimed to provide a sustainable active food packaging based on PLA/paper-PLA (biodegradable and compostable) with active graphene-based composites (reinforced packaging properties and antimicrobial ability) and probiotic strain (natural and beneficial preservative) to preserve food quality, to maintain food safety, and further to reduce food waste.

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List of publications

1. Peter A., Mihaly Cozmuta L., Nicula C., Mihaly Cozmuta A., Apjok R., Talasman C.M., Drazic G., Peñas A., Calahorro A.J., Kamgang Nzekoue F., **Huang X**., Sagratini G., Silvi S. (2021). Morpho-structural and chemical characterization of paper based materials with functionalized surface. *Mater. Chem. Phys.* 267, 124693.

2. Peter A., Mihaly Cozmuta L., Nicula C., Mihaly Cozmuta A., Apjok R., Talasman CM., Drazic G., Peñas A., Calahorro AJ., Kamgang Nzekoue F., **Huang X.**, Sagratini, G., Silvi S. (2021) Paper materials with functionalized surface. Part 2. Barrier properties, migration into the food simulants and antimicrobial activity, Cellulose Chemistry and Technology – *under submission*

3. Peter A., Mihaly Cozmuta L., Nicula C., Mihaly Cozmuta A., Apjok R., Talasman CM., Drazic G., Peñas A., Calahorro AJ., Kamgang Nzekoue F., **Huang X**., Sagratini, G., Silvi S. (2021). Paper materials with functionalized surface. Part 3. Analysis of the environmental impact using the life cycle assessment (LCA), Cellulose Chemistry and Technology – *under submission*

4. Sagratini G., 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. (2021). A shelf-life study for the evaluation of a new biopackaging to preserve the quality of organic chicken meat. *Food Chem. – under submission*

5. **Huang X**., Sagratini G., Silvi S. (2021). Natural antimicrobial bioactive compounds as novel solutions for counteracting spoilage and pathogen microbes in cheese – *In preparation*

6. **Huang X**., Kamgang Nzekoue F., Coman M. M., Peter A., Talasman C. M., Drazic G., Peñas A., Verdenelli M. C., Sagratini G., Silvi S. (2020). *Lactobacillus* Strains Treatment on Commercial Packaging Paper as Preliminary Study for Extending the Shelf-Life of Chicken Meat. *J. Biotech Res Biochem.* 3; 007.

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Patents

- Patent Application OSIM (reg. A/00328 of 12.06.2020): Peter A., Mihaly Cozmuta A, Mihaly Cozmuta L, Nicula C, Talasman C, Caprita F, Constantin C, Dumitrascu I, Drazic G, Bele M, Chernyshova E., Silvi S, Sagratini G, Huang X, Kamgang F, Verdenelli C, Orpianesi C, Coman M, Penas A, Calahorro A, Cano Galey M, Ramirez Rodriguez M. PROCEDEU DE OBȚINERE A UNOR AMBALAJE ACTIVE PE BAZĂ DE ACID POLILACTIC MODIFICAT CU NANOCOMPOZIT.

- Patent Application EPO (EP20020299.2 of 25/06/2020): Peter A., Mihaly Cozmuta A, Mihaly Cozmuta L, Nicula C, Talasman C, Caprita F, Constantin C, Dumitrascu I, Drazic G, Bele M, Chernyshova E., Silvi S, Sagratini G, Huang X, Kamgang F, Verdenelli C, Orpianesi C, Coman M, Penas A, Calahorro A, Cano Galey M, Ramirez Rodriguez M. PROCESS FOR OBTAINING ACTIVE PACKAGES BASED ON POLYLACTIC ACID MODIFIED WITHNANO-COMPOSITE.

Contribution to conferences and workshops

Posters

1. Salvesi C., **Huang, X**., Fanizzi, M., Scipioni, T., Silvi, S. Potential Effects of Commercial Kefir Milk Consumption on Gut Microbiota in Cancer Patients. Poster at IPC 2020-14th International scientific conference. PROBIOTICS, PREBIOTICS GUT MICROBIOTA AND HEALTH, Virtual conference. 11/11/2020 – 11/11/2020.

2. **Huang X.,** Kamgang Nzekoue F., Coman MM., Peter A., Talasman CM., Drazic G., Peñas A., Verdenelli MC., Sagratini G., Silvi S. Antimicrobial activity of cell-free supernatant of Lactobacillus plantarum IMC 509 against common food spoilage microbes. Poster at 10th PROBIOTICS, PREBIOTICS & NEW FOODS, NUTRACEUTICALS AND BOTANICALS FOR NUTRITION & HUMAN AND MICROBIOTA HEALTH, Università urbaniana, Roma, Italy. 08/09/2019 – 10/09/2019.

3. **Huang X**., Kamgang Nzekoue F., Coman MM., Peter A., Talasman CM., Drazic G., Peñas A., Verdenelli MC., Sagratini G., Silvi S. Characterization of probiotics as active

agent in PLA packaging for counteracting spoilage in ricotta cheese. Poster at CIBO E NUTRACEUTICI: PAROLA CHIAVE “CARATTERIZZAZIONE”. 4° Convegno a cura delle Piattaforme Tematiche di Ateneo, Camerino, Italy. 09/07/2019 – 09/07/2019.

4. **Huang X.,** Kamgang Nzekoue F., Coman MM., Peter A., Talasman CM., Drazic G., Peñas A., Verdenelli MC., Sagratini G., Silvi S. Probiotics as active agents in paper based wrapping system for increasing the shelf-life of raw meat. Poster at IPC 2019 - International scientific conference. PROBIOTICS, PREBIOTICS GUT MICROBIOTA AND HEALTH, Prague, Czech Republic. 17/06/2019 – 20/06/2019.

5. Kamgang Nzekoue F., Caprioli G**.,** **Huang X.**, Silvi S., Sagratini G. Activegraphene based food packaging systems for a modern society (GRAFOOD). Poster at CHIMALI - XII ITALIAN FOOD CHEMISTRY CONGRESS. Camerino, Italy. 24/09/2018 – 27/09/2018

6. **Huang X**., Sagratini G., Silvi S. Sensory and microbiological assessment of foods stored in packaging from different European countries. Poster at CIBO E NUTRACEUTICI: DIREZIONE SALUTE. 3° Convegno a cura delle Piattaforme Tematiche di Ateneo. Camerino, Italy. 10/07/2018 – 10/07/2018.

Courses, conferences and workshops attendances

1. Cibo e nutraceutici: direzione salute. 3° Convegno a cura delle Piattaforme Tematiche di Ateneo (Poster presentation) – Camerino
   10/07/2018 – 10/07/2018

2. Management and training meeting of GRAFOOD Project, Active GRAphene based FOOD packaging systems for a modern society (Oral presentation) – Camerino, Italy
   23/10/2018 – 24/10/2018

3. Management and training meeting of GRAFOOD Project, Active GRAphene based FOOD packaging systems for a modern society (Oral presentation) – Jaén, Spain
   06/05/2019 – 10/05/2019

4. IPC 2019 -International scientific conference. PROBIOTICS, PREBIOTICS GUT MICROBIOTA AND HEALTH (Poster presentation) – Prague, Czech Republic
   17/06/2019 – 20/06/2019

5. Cibo e nutraceutici: parola chiave “CARATTERIZZAZIONE”. 4° Convegno a cura delle Piattaforme Tematiche di Ateneo (Poster presentation) – Camerino, Italy
   09/07/2019 – 09/07/2019

6. 10TH PROBIOTICS, PREBIOTICS & NEW FOODS, NUTRACEUTICALS AND BOTANICALS FOR NUTRITION & HUMAN AND MICROBIOTA HEALTH (Poster presentation) – UNIVERSITÀ URBANIANA, Roma, Italy
   08/09/2019 – 10/09/2019

7. IPC 2020-14th International scientific conference. PROBIOTICS, PREBIOTICS GUT MICROBIOTA AND HEALTH– Virtual conference
   11/11/2020 – 11/11/2020

COURSE

Food Shelf Life: Challenges, Pitfalls and Packaging Innovation, Università degli Studi di Udine, Udine, Italy.
23/05/2019 – 24/05/2019

Academic Activities

Placement Tutor of Final Reports of Bachelor's degree students

1. Microbiological and sensory analysis of Italian "ricotta" cheese stored in packaging from different European countries. Final Report of Bachelor's degree in BIOSCIENCES and BIOTECHNOLOGY, Curriculum: Biotechnology. Candidate: Siyao Lu. University tutor: Prof. S. Silvi; Placement tutor: Dr. X. Huang. Academic Year: 2017-2018.

2. Study of graphene oxide-based composites and probiotic bacterial strains interaction. Final Report of Laboratory Experience within the BIOSCIENCES and BIOTECHNOLOGY Course, Curriculum: Biotechnology. Candidate: Xiayu Shen. Tutors: Prof. S. Silvi and Dr. X. Huang. Academic Year: 2018- 2019.

3. Probiotic strains as active component for PLA packaging. Final Report of Laboratory Experience within the BIOSCIENCES and BIOTECHNOLOGY Course, Curriculum: Biotechnology. Candidate: Xiang Li. Tutors: Prof. S. Silvi and Dr. X. Huang. Academic Year: 2018-2019.

4. Assessment of probiotic bacteria viability in the presence of graphene-based composites. Final Report of bachelor's degree in BIOSCIENCES and

BIOTECHNOLOGY Course, Curriculum: Biotechnology. Candidate: Siyao Xiao. Tutors: Prof. S. Silvi and Dr. X. Huang. Academic Year: 2018-2019.

5. Microbiological assessment of ham stored in several paper packaging systems. Final report of bachelor's degree in BIOSCIENCES and BIOTECHNOLOGY Course, Curriculum: Biotechnology. Candidate: Polina Makarycheva. Tutors: Prof. S. Silvi and Dr. X. Huang. Academic Year: 2018-2019.

6. Probiotic strains as active component for novel packaging system. Eramus Student Final Report - Alba García Ruiz (University of Zaragoza, Spain), Tutors: S. Silvi & X. Huang AY 2018-2019.

7. Evaluation of bacterial activity of a hygiene product. Final report of Bachelor's degree in BIOSCIENCES and BIOTECHNOLOGY Course, Curriculum: Biotechnology. Candidate: Umani S. Galbada Liyanage. Tutors: Prof. S. Silvi and Dr. X. Huang. Academic Year: 2020-2021.

8. Activity assessment of cell-free supernatant of *Lactobacillus* strains against foodborne pathogens. Final report of Bachelor's degree in BIOSCIENCES and BIOTECHNOLOGY Course, Curriculum: Biotechnology. Candidate: Chenxin Zhang. Tutors: Prof. S. Silvi and Dr. X. Huang. Academic Year: 2020-2021.

Other activities

1. Member of exam Commission as Subject Expert on "General and Industrial Microbiology" (Bachelor's degree – Biosciences and Biotechnologies) and on "Functional Food" (Master's degree – Biological Sciences).

2. Teaching activities (within curriculum of Ph.D.) – International School Of Advanced Studies (University Of Camerino) at "General and Industrial Microbiology" and "Laboratory II" (Microbiology module) within the Biosciences and Biotechnology Course.

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