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Development of Silver Based Antimicrobial Films for Coating and Food Packaging Applications

DOCTORAL THESIS

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CERTIFICAN

Que la presente memoria "***Development of Silver Based Antimicrobial Films for Coating and Food Packaging Applications***" constituye la tesis doctoral de Antonio Martínez Abad. Asimismo, certifican haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

Y para que conste a los efectos oportunos, firmamos la presente en Valencia a 27 de Febrero de 2014

Fdo. María José Ocio Fdo. José María Jagarón

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ABSTRACT

Although silver is used as key component to control microbial proliferation in countless applications, available silver based technologies are scarce. This relies in the difficulties in assessing silver efficacy due to stability and speciation issues. In the present dissertation, silver ions were directly incorporated into biopolymer matrices as to obtain materials with prolonged antimicrobial performance based on the sustained release of silver ions. A profound insight into the chemical interactions between the active silver species and both bacteria and the environment of action was carried out as to correctly assess silver efficacy in the subsequent design of the antimicrobial materials. Silver was found to be effective at the nanomolar level under optimum conditions. However, time dependent chemical interactions with several ligands drastically affected silver efficacy and the assessment of viability by traditional enumeration methods. The incorporation of silver ions into EVOH films produced by casting and electrospinning did not alter the physicochemical properties of the materials and showed a rapid release of the whole silver content upon contact with moisture. This was reflected in inactivation of bacteria at very low silver loadings (0.0001wt.%) under optimum conditions. When incorporated in PLA by casting or melt compounding, the release of silver ions and antimicrobial performance was prolonged from days to months depending on the silver content or the method for incorporation. Release was also found to be highly dependent on moisture content and pH. An initial burst release stage was attenuated by the application of a beeswax layer, which allowed the release profiles to be tailored to suit a specific release profile and comply with current legislation. The films demonstrated a high antibacterial and antiviral effect against the most common food-borne pathogens in synthetic media, real liquid and solid food samples as well as on the surface of the films. This study represents a step forward in the understanding of silver antimicrobial efficacy and puts forth its possible suitability for food packaging, food contact or other applications.

RESUMEN

Aunque la plata se usa como componente clave en el control microbiano en incontables aplicaciones, las tecnologías basadas en plata disponibles son escasas. Esto radica en la dificultad para evaluar su eficacia debido a problemas de estabilidad y de especiación. En la presente tesis, iones de plata se incorporaron en matrices biopoliméricas para obtener materiales de prolongada capacidad antimicrobiana basados en su liberación sostenida. Se realizó un estudio profundo de las interacciones químicas entre las especies activas de plata, las bacterias, y posibles ligandos presentes en el medio de acción. En condiciones óptimas, la plata demostró ser eficaz en el rango de los nanomoles. Sin embargo, interacciones químicas con varios ligandos afectaron drásticamente tanto su eficacia como la evaluación de la viabilidad bacteriana. La incorporación de iones de plata en películas de EVOH no alteró las propiedades físico-químicas de los materiales que mostraron una rápida liberación del contenido de plata al entrar en contacto con la humedad. Esto se reflejó en la inactivación de las bacterias a concentraciones muy bajas (0.0001wt.%) en condiciones óptimas. Cuando se incorporaron iones de plata en PLA por casting o mezclado-fundido, la liberación y el rendimiento antimicrobiano se prolongaron de días a meses, dependiendo del contenido, el método de incorporación, la humedad o el pH del medio de liberación. Una etapa inicial de liberación mayor pudo ser atenuada gracias a la aplicación de una capa de cera de abejas, lo que permitió adaptar los perfiles de liberación a demanda y cumplir con la legislación vigente en diversas condiciones de liberación. Las películas demostraron un alto efecto antibacteriano y antiviral contra los patógenos transmitidos por los alimentos más comunes en medios sintéticos, en superficie y en alimentos líquidos y sólidos. Este estudio representa un avance en la comprensión de la eficacia antimicrobiana de la plata y destaca su posible idoneidad para la fabricación de materiales de envasado de alimentos, de contacto con alimentos u otras aplicaciones.

RESUM

Encara que la plata s'usa com component clau en el control microbià en inenarrables aplicacions, les tecnologies basades en plata disponibles són escasses. Açò radica en la dificultat per a avaluar la seua eficàcia degut a problemes d'estabilitat i d'especiació. En la present tesi, ions de plata van ser incorporats en matrius biopolimériques per a obtindre materials de prolongada capacitat antimicrobiana basats en el seu alliberament sostingut. Es va realitzar un estudi profund de les interaccions químiques entre les espècies actives de plata, els bacteris, i possibles lligants presents en el mig d'acció. En condicions òptimes, la plata va demostrar ser eficaç en el rang dels nanomols. No obstant això, interaccions químiques amb diversos lligants van afectar dràsticament tant la seua eficàcia com l'avaluació de la viabilitat bacteriana. La incorporació d'ions de plata en pel·lícules d'EVOH no va alterar les propietats fisicoquímiques dels materials que van mostrar un ràpid alliberament del contingut de plata a l'entrar en contacte amb la humitat. Açò es va reflectir en la inactivació dels bacteris a concentracions molt baixes (0.0001wt.%) en condicions òptimes. Quan es van incorporar ions de plata en PLA per càsting o mesclat-fos, l'alliberament i el rendiment antimicrobià es van prolongar de dies a mesos, depenent del contingut, el mètode d'incorporació, la humitat o el pH del medi d'alliberament. Una etapa inicial d'alliberament major va poder ser atenuada gràcies a l'aplicació d'una capa de cera d'abelles, la qual cosa va permetre adaptar els perfils d'alliberament a demanda i complir amb la legislació vigent en diverses condicions d'alliberament. Les pel·lícules van demostrar un alt efecte antibacterià i antiviral contra els patògens transmesos pels aliments més comuns en mitjans sintètics, en superfície i en aliments líquids i sòlids. Este estudi representa un avanç en la comprensió de l'eficàcia antimicrobiana de la plata i destaca la seua possible idoneïtat per a la fabricació de materials d'envasament d'aliments, de contacte amb aliments o altres aplicacions.

List of Abbreviations

ASTM	American Society for Testing and Materials
ASV	Anodic Stripping Voltammetry
BPW	Buffered Peptone Water
CECT	Spanish Type Culture Collection
CFSAN	Center for Food Saftey and Applied Nutrition
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
Cys	Cysteine
ΔC_p	Specific Heat Capacity
DMF	Dimethylformamide
DSC	Differential Scanning Calorimetry
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy Dispersive X-ray microanalysis
EFSA	European Food Safety Agency
EO	Essential Oil
EU	European Union
EVA	Ethylene vinyl acetate
EVOH	Ethylene vinyl alcohol
FC	Flow Cytometry analysis
FCV	Feline Calicivirus
FDA	Food aand Drug Administration
FSI	Free silver ions
FT-IR	Fourier Transformed Infrared Spectroscopy
GRAS	Generally Recognized As Safe
HIV	Human Inmunodeficiency Virus
H_m	Melting Enthalpy
JIS	Japanese Industrial Standard
LB	Luria broth or Luria Bertani Broth
LDPE	Low Density Polyethylene
LPS	Lipopolysaccharide

MBC	Minimum Bactericidal Concentration
Met	Methionine
MHB	Müller Hinton Broth
MIC	Minimum Inhibitory Concentration
MICINN	Ministry of Science and Innovation
MINECO	Ministry of Economy and Competitiveness
MMT	Montmorillonite
NOM	Natural Organic Matter
PBS	Phosphate Buffered Saline
PE	Polyethylene
PEG	Polyethyleneglycol
PLA	Poly (lactic acid) or Polylactide
ppb	Parts per Billion (ng/mL)
ppm	Parts per Million (μg/mL)
PTFE	Polytetrafluoroethylene
PU	Polyurethane
PVOH	Poly (vinyl alcohol)
RCF	Relative Centrifugal Force
RH	Relative humidity
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
$TCID_{50}$	Tissue Culture Infectious Dose (50%)
T_g	Glass Transition Temperature
THF	Tetrahydrofuran
T_m	Melting temperature
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WHO	World Health Organization

Introduction

1. Food Packaging

Packaging materials are essential components in the daily life of any developed society. Their global use and the development of modern technologies allow foods of all kind to be consumed anywhere, easily overcoming seasonal or distance barriers and at reasonable costs. Packaging technologies have greatly evolved in response to social factors such as population growth, globalization of food trade, central processing, the incorporation of women into labour or the pursuit for healthier or environmentally friendlier products. Today, a wide variety of packaging strategies can be found to meet each very specific requirement within the diversity of products that come into the markets of developed societies. Effective packaging, ensuring high standards of quality and safety, but also suitable, appealing and affordable, is nowadays crucial for commercialization of most food products from fresh produce to ready-to-eat foods.

From these general considerations, it is clear that the introduction of polymers as materials for food packaging has meant a revolution over the past decades. The advantages of plastic compared to traditional paper, glass or tin packaging are many. One of the most important features is the diversity of available materials and compositions that allow fit the specific needs of each product. Always within the limitations inherent in plastics, polymer technologies allow the manufacture of very flexible (pouches, bags, envelopes), semi-rigid (trays, tubs) or stiff materials (bottles, tanks, caps, etc) in any imaginable size or shape. Another very important feature is their ability to be formed, filled and sealed within an in-line process, which can be highly advantageous from an economic point of view. Optical properties (transparency, colour) can also be adapted to suit specific product requirements.

1.1. Biodegradable and bio-based polymers

The fossil fuel derived origin of most of the plastic packaging production and the difficulty in recycling these kind of polymer based materials are probably the most important drawbacks for their use in food packaging and has arisen much public and governmental concern. As a means to alleviate the former drawback, the so-called drop-inn biopolymers are foreseen by many as the family of biopolymers which will most rapidly increase market volume within this decade. The so-called drop-inns, such as bio-PET, bio-PE or bio-PP, are chemically identical to their petroleum-based counterparts, but are synthesized from biomass, mostly from bioethanol. However, this kind of polymers does not solve the problem of public waste management, as they are non-biodegradable. 31.75 million tons of plastic waste and 14.9 million tons of plastic waste from packaging alone were produced in the U.S. and the European Union (EU), respectively, in 2011 [1-2]. Therefore, developed societies are doing many efforts to improve recycling systems and develop sustainable, biodegradable or bio-based polymeric materials.

Biodegradable polymers are defined as materials which are able to decompose into carbon dioxide, methane, inorganic compounds or biomass as a result of being exposed to the enzymatic action of microorganisms (ASTM, 2010). Biopolymers, on the other hand, are either extracted from renewable resources (starch, cellulose, zein, chitosan, polyhydroxyalkanoates, etc) or polymerized from renewable monomers, such as poly(lactic) acid (PLA), and are also biodegradable. Biopolymers usually have worse mechanical or barrier properties as compared to their petroleum based counterparts, which until recent has much limited its applicability in food packaging. However, public concern about plastic management have prompted legislative bodies to promote research attention in this sense, and new technologies have been developed which allow the manufacture of biopolyners and biodegradable plastics with similar characteristics as other oil based benchmark materials [3]. As a result of globally increasing legal limitations to plastic waste, these new technologies are being

rapidly developed and gradually introduced in developed societies. A good example is the European Union, whose members have been constantly improving recycling rates in the last decade. The 2001- and 2008-targets of plastic recycling rates of 15% and 22.5%, respectively, were fulfilled by nearly all EU-members before time [2]. Some countries, such as Germany, the Czech Republic or Slovenia already reached plastic packaging recycling rates of ≥ 50% by 2010. These figures are inspiring and evince the huge and versatile potential of polymeric materials, which will most probably continue to be key components in food packaging as well as in many other applications.

1.2 Active packaging

Traditionally, packaging materials were defined as passive barriers which should protect the food from the outer environmental conditions and thus slow deterioration. Arising around the turn of the century, the concept of functional packaging, including active/bioactive or intelligent/smart packaging, provided the package an active role in the preservation, health promoting capacity or provision of information.

Active packaging is an innovative concept, which seeks to improve food preservation extending the shelf-life and/or improving the sensory or nutritional properties while maintaining product quality. These new packaging concepts have been developed in response to consumer demands for minimally processed foods of better quality and freshness, as well as to corporate sales changes with a clear increase in exports, centralized processing and greater distribution distances. Among the different active packaging strategies aiming to extend the shelf-life of food products, we find oxygen, moisture or ethylene scavenging systems, as well as strategies to release ethanol, flavours, functional ingredients or antimicrobial substances.

1.3 Antimicrobial packaging

Health risks associated with microbial contamination continues to be one of the main public and governmental concerns as to food consumption and food packaging. Despite the evident progress in health risk assessment throughout manufacture, transport and commercialization of food products, the incidence of foodborne illnesses in developed countries has not waned in the last decades [4]. In the United States, Salmonella spp., Campylobacter spp. and Listeria monocytogenes appear as the main causes of food poisoning with around 76 million cases estimated annually [5]. In the European Union, 5000-6000 foodborne outbreaks are recorded each year, of which *Salmonella* spp and Campylobacter jejuni represent about 50 %, each causing more than 100,000 annual toxiinfections [6]. In the case of listeriosis, Europe registers a relatively small number of cases (about 1400/year), but the clinical prognosis may result in severe life-threatening complications (meningitis, abortions, pneumonia etc.) with a mortatily rate of 12.7%. Although data concerning the incidence of enteric viruses is still fragmented, epidemiological evidence indicates that human norovirus may be the one of the major cause of foodborne outbreaks of gastroenteritis [6] In addition, human norovirus and *Salmonella* have been listed in the top 5 highest-ranking pathogens with respect to the total cost of foodborne illness in the United States [7].

One explanation for the high incidence of food-borne illness in developed countries may be the current growing consumer demands for minimally processed ‘fresh’ food products, which may allow proliferation and persistence of pathogenic organisms. Therefore, alternative technologies such as lower thermal or high hydrostatic pressure, or other treatments combined with or without the assistance of milder thermal treatments are being considered. The reduction of conventional aggressive thermal treatments could, however, result in inefficient elimination of pathogens [8]. Moreover, even if foodborne pathogens are totally eliminated by efficient thermal treatments, microbial recontamination of the food surface could take place during post-processing

steps. As a result of the above, a reduction of food shelf-life and the risk of foodborne illnesses is consequently increased. In this context, combination of new hurdle technologies with antimicrobial packaging can result in shelf-life extension and foods with improved quality and safety characteristics.

In antimicrobial packaging, a substance with biocide properties is included in the packaging system to extend shelf-life and reduce the risk of contamination by pathogens [9]. This task is approached by different strategies including:

1. Addition of sachets or pads containing volatile antimicrobial agents into packages.

2. Incorporation of volatile and non-volatile antimicrobial agents directly into polymers.

3. Coating or adsorbing antimicrobials onto polymer surfaces.

4. Immobilization of antimicrobials to polymers by ion or covalent linkages.

5. Use of polymers that are inherently antimicrobial.

The most successful commercial application of active packaging has been sachets enclosed loose or attached to the interior of a package mostly containing moisture absorbers, oxygen scavengers or ethanol vapour generators [10]. But for antimicrobial packaging, this approach is only feasible with volatile compounds, which extremely limits the range of available antimicrobials. Volatile compounds may not be suitable for plastic processing due their poor heat resistance. Additionally, the incorporation of volatile compounds may release off-flavours within the packaged product. The direct application of antibacterial substances onto foods has limited benefits because the active substances are neutralized by product constituents on contact or diffuse rapidly from the surface into the food mass. As bacterial contamination occurs primarily on the surface, the incorporation of the biocide in a film or as a coating has many advantages due to the high exposure areas for the biocide and a reduction

of the loss of the antimicrobial compound into the bulk of the food [11-12]. The incorporation of antimicrobials into polymers constitutes a solution, as it allows the biocide substance to be released from the package during an extended period, prolonging its effect into the transport and storage phase of food distribution [9].

Antimicrobial substances reduce the viability of microbial populations, extending their lag phase, reducing their growth rate and/or their maximum growth, so as to extend the shelf life and maintain product quality and safety [13]. Ideally, packaging materials incorporating these substances should encompass a broad spectrum of antimicrobial activity at low concentrations, exert no adverse sensory effects on the product and comply with current food legislation. Low production costs are also a must considering the very low mark-ups in the food sector. Among the different antimicrobials which have been incorporated in polymers for food packaging applications we find organic acids, enzymes, bacteriocins, essential oils, fungicides, other common preservatives, and, of course, silver based compounds [10, 14].

1.4. Antimicrobial substances incorporated in food packaging

The most traditional and commonly used antimicrobials are the family of organic acids, such as acetic, benzoic, lactic, citric or propionic acid. Most of them have a long history as generally recognized as safe (GRAS) food preservatives and are usually used in different combinations with other antimicrobial substances, like nisin, or incorporated in chitosan [15-18]. Chlorine and sulphur dioxide gases are also very effective in preventing mould growth. However, accumulation of these gases can lead to inacceptable colour alterations and toxicological problems in the treated food [19]. However, there is a growing trend to prevent artificial chemicals from being incorporated to food products or food contact materials. Therefore, many efforts are being done to replace artificial chemicals for natural substances, like enzymatic extracts, bacteriocins, essential oils, etc.

Among enzymes, lysozyme, most commonly extracted from egg white, is capable of breaking the glucosidic bonds in the peptidoglycan of Gram positive bacteria and has been successfully incorporated in zein or cellulose acetate films [20-21]. Lactoferrin, on the other hand, covalently binds iron, producing a biocide effect in bacteria with medium or high iron requirements [22].

Bacteriocins are antibacterial peptides produced by a broad range of bacteria, although the great majority of these compounds are ascribed to the genus *Lactococcus*. Although many bacteriocins (such as pediocines, lacticin and plantaricin) have potential application in food products, the antibiotic nisin is currently the only bacteriocin approved as a GRAS (generally recognized as safe) food additive by both the Food and Drug Administration (FDA) and the World Health Organization (WHO)[23]. The main current industrial use of nisin aims the prevention of contamination of cheese surfaces by *Clostridium species* and *L. monocytogenes*. However, there is abundant literature as to the incorporation of bacteriocins in polymer matrices, such as polyethylene [24], ethylene vinyl acetate (EVA) [25] or polyvinyl alcohol (PVOH) [26]. Moreover, Iseppi et al. (2008) were able to entrap bacteriocin producer *Enterococcus casseliflavus* in the PVOH matrix, which demonstrated higher effectiveness than the bacteriocin alone [26].However, lysozyme and bacteriocins are only active against Gram positive bacteria. The further addition of the chelating agent EDTA, endowing the polymer with combined synergistic effects, has been proposed as an approach to extend the spectrum of action [27].

Chitosan is an aminopolysaccharide obtained by deacetylation of chitin, a structural component present in the shell of some crustaceans. Being a biodegradable, inherently antimicrobial polymer, it presents a promising potential for the development of new antimicrobial packaging concepts and there is extensive literature as to its antimicrobial efficiency, either alone or in blends with other polymers [28-30].

Another set of volatile antimicrobial compounds of great importance in research are natural essential oils (EOs), extracted from integrated plant material

(flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits or roots)[31] As they are naturally occurring substances, they pose an alternative to other more toxic or polluting antimicrobials and comply with a current trend in many sectors of the society for more natural and environmentally friendly products. There is abundant scientific evidence in relation to the effectiveness of EOs fractions of many spices and herbs and their components as antimicrobial, antifungal, and antiviral compounds as well as other positive effects (antioxidant, anti-inflammatory, etc). One of the main drawbacks of incorporating EOs into polymers is their high chemical and thermal instability as well as a very high volatility. This problem limits the possibility of producing the films by melt compounding and compression moulding as is typically done with conventional polymers. Additionally, the antimicrobial effectiveness of EOs is usually lower than other traditional antimicrobials, which implies the need for higher filler contents. These issues pose difficult challenges as to the development of cost-effective polymer technologies releasing EOs.

Last, but not least, there is the use of naturally occurring metals or metal complexes with antimicrobial activity. Metals and metal nanoparticles are of great interest for food packaging applications, due their broad spectrum of action (including Gram positive, Gram negative bacteria, yeasts and viruses), higher chemical and thermal stability as compared to organic antimicrobials, and ease of processing and cost-effective production.

Zinc is a ubiquitous trace metal and essential for a large number of metalloenzymes in living organisms. At superphysiological levels, zinc inhibits the growth of bacteria, which has prompted its worldwide use as antiseptic in dental care, skin therapy, etc for more than half a century [32-33]. More recently, interest has arisen about the possibility of incorporating zinc oxide nanoparticles into packaging polymers to endow them with antimicrobial properties. ZnO nanoparticles have been incorporated in different materials including glass, low density polyethylene (LDPE), polypropylene (PP), polyurethane (PU), paper and chitosan using different methods [34].

Copper is an essential element and is present in most food in the form of ions or salts at levels, in most cases, below 2 mg/kg (meat, fish, pecans, green vegetables, etc.), but up to 39 mg/kg in cocoa or liver. Although copper is typically used for surface treatment of medical devices, direct application in the food arena has also been reported for copper salts. Copper cast alloys were evaluated in food processing work surfaces and diminished the risks associated to *E. coli* O157:H7, although the presence of beef residues was a limiting factor for the achieved growth inhibition [35]. The growth of *Salmonella*, *E. coli* O157:H7 and *Cronobacter* spp. could be impaired by sublethal concentrations of copper (II) ions (50 mg/kg) combined with other antimicrobials, such as lactic acid, in infant formula [36] and carrot juice [37].

The antimicrobial properties, based on its photocatalytic effect, of titanium dioxide (TiO_2) are also well established. Maximum performance is reached when UV light excites the anatase form of nano TiO_2 [38]. TiO_2 photocatalysis induces the production of reactive oxygen species (ROS), glutathione depletion and cell membrane disruption [39]. In food processing, the most promising application of nano-sized titanium dioxide particles as antimicrobial is to diminish the risks associated to biofilms in food contact surfaces [40], although it has also been tested with typical packaging materials [41]. Though less effective, a reduction of bacterial viability has been demonstrated when TiO_2 nanoparticles incorporated in quartz glass or polypropylene (PP) films were put in contact with real food matrices such as lettuces [42-43].

Among metallic cations, ionic silver is known to have the greatest antimicrobial capacity against bacteria, yeast or viruses; it has long-term biocide properties and low volatility while at the same time being much less toxic to eukaryotic cells and non-toxic to humans. As it is the key antimicrobial compound used throughout this doctoral thesis, the next section will extensively cover the mechanisms of action, chemical interactions, current applications, safety concerns, release issues and other matters concerning its incorporation in polymer matrices.

2. Antimicrobial silver

2.1. Historical use of silver as an antimicrobial agent and renewed interest

Silver has been attributed antimicrobial properties since ancient times. Even before the Neolithic era, it was known that cooking or storing water or wine in silver pots would keep it safe [44-45]. Alexander the Great, for example, used to drink only from silver vessels. Silver nitrate, known in antiquity as "lapis infernalis", was first recorded to be used for therapeutic purposes in the 8th century. In 980, Avicenna reports the use of silver as a blood purifier, for bad breath and for heart palpitations. In the 17th and 18th century, the use of silver nitrate was quickly generalized in the treatment of venereal diseases, fistulae and abscesses. By the 19th century, physicians realized that silver not only prevented wounds from being infected, but it also showed to allow epithelisation and promoted crust formation [46-47]. Consequently, pencils charged with a 0.5% silver nitrate solution were introduced in the basic surgical equipment, and silver nitrate became the most useful tool in the treatment of burns or wounds. However, in 1928, penicillin was discovered, and with the advent of antibiotics after World War II, a new system was developed that could fight infections systemically as well as superficially. As a result, the use of silver against microbes was pushed to the background, being limited to the occasional treatment of burns and ulcers until recent time.

A combination of multiple factors has promoted the rediscovery of silver as antimicrobial agent, and its rapid rise to the forefront in the development of antimicrobial systems. First, the emergence of antibiotic resistant microbes manifest ever more rapidly every time a new version is introduced in the healthcare system. Therefore, the faith so long deposited on antibiotics is waning and the healthcare sector is in the need of finding new antimicrobial systems to fight the increasing number of nosocomial infections [48] and assure the proper functioning of the public health system. Second, our society is increasingly more aware of the ubiquitous presence of microbes in all aspects of daily life. A public opinion ever more concerned with sterility and safety is demanding

antimicrobials that can be safely and cost-effectively applied to any materials in our environment. Both the medical and consumer demands represent an immense potential market which promotes the fast development of new technologies based in antibacterial materials. While other natural substances with antimicrobial properties are either volatile, do not withstand thermal processing or are not cost-effectively synthesized or purified, the excellent thermal and chemical stability of silver and its relatively low cost make it an ideal candidate for its incorporation in a wide variety of materials. In addition to these advantages, the discovery of the antibacterial properties of silver nanoparticles has further increased the interest in silver as key component in antimicrobial materials. Of all nanomaterials, and even though regulatory issues are still unclear, nanosilver has the highest degree of commercialization [10, 45]. On the other hand, antimicrobial materials based on the delivery of silver ions are the most widely used polymer additives in food applications [9-10].

2.2. Molecular understanding of the mechanism of action

In dealing with its antimicrobial efficacy, a difference must be made between silver at the macro or microscale and silver nanoparticles. The biocide properties of the bulk material, which has no antibacterial effect in itself, rely on the sustained oxidation and release of very small quantities of silver cations (Ag^+) to an aqueous or moistured environment, according to the following reaction:

$$4Ag + 4H^+ + O_2 \rightarrow Ag^+ + 2H_2O$$

It is the presence of a tiny fraction of these oxidized cations which is responsible for the empirical appraisal of antimicrobial efficacy of silver cups, vessels, pots or cutlery, and their historical use.

Silver cations interact with atoms with a high electronic density, in particular having an extreme chemical affinity for sulphur groups, like thiol groups (-SH) in biomolecules [49-50]. The interaction of silver with L-Cysteine residues causes denaturation and loss of enzymatic functions [49, 51]. This unspecific

mechanism affects bacterial viability at different levels. First, with inactivation of enzymes in the outer membrane, permeability and transmembranous energy metabolism are disrupted leading to a loss in the proton motive force [52-54].

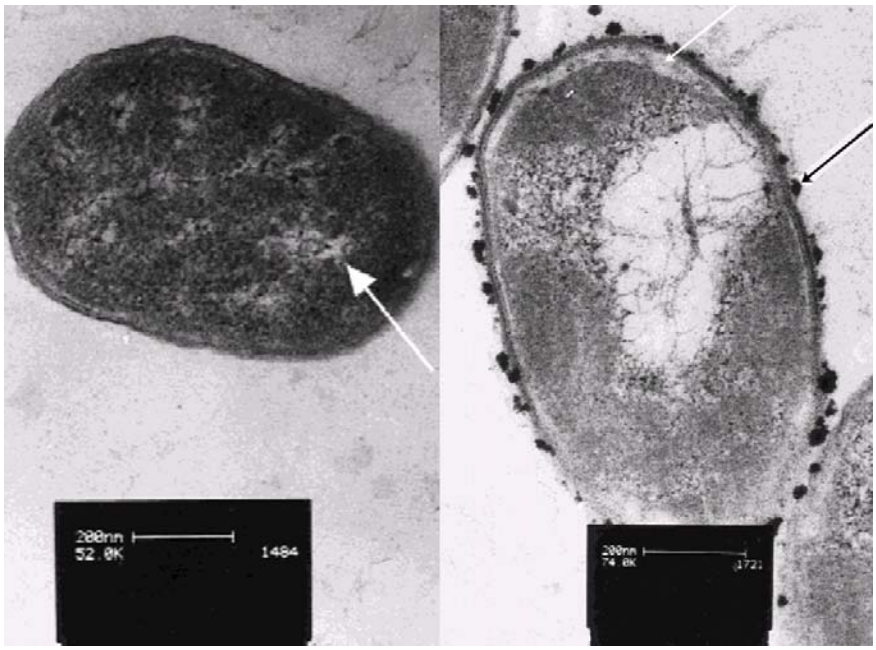

Figure 1. Internal structure of untreated *E. coli* cells (left) and treated with silver ions (right). Arrow in (left) depicts the electron-light region, DNA molecules, randomly distributed in almost all parts of the cells. Arrows in (right) signal detachment of the cytoplasm from the cell wall, and accumulation of electron dense granules around the cell wall (adapted from [52]).

This electrolyte imbalance is reflected in a massive loss of potassium out [51, 55]. Additionally, once the ions have entered the cell, they inhibit dehydrogenases of the respiratory chain, which depletes intracellular ATP levels [53-54]. Furthermore, the ions can bind DNA components, which stabilize DNA closed conformation, evidenced by a condensed region in the centre of the cell, preventing replication (Figure 1). In this respect, it remains disputed weather the silver ions are bond to the phosphate residues [50, 52] or if they intercalate between N-H bonds in purines and pyrimidines [53, 56]. When examined by

electron microscopy, a light region appears in the cytoplasm due to DNA condensation and enzyme aggregation. The membrane is noted to shrink and detach from the cell wall, and electron dense granules are accumulated at the outside [52-54, 57] (Figure 1). Additionally, it has been postulated that the depletion of protective enzymes produces an increase in reactive oxygen species (ROS), which further contributes to damage vital functions of the cell in aerobic conditions [58].

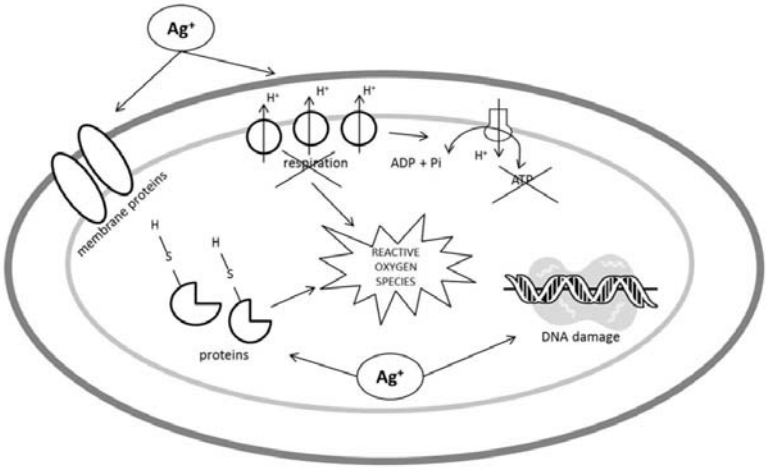

Figure 2. Antimicrobial effects of Ag^+. Interaction with membrane proteins and blocking respiration and electron transfer; inside the cell, Ag^+ ions interact with DNA, proteins and induce ROS production (from reference [59])

Bacterial defence mechanisms against the damage consist mainly on the overproduction of the targeted proteins. Lok et al., for example, detected the overexpression of envelope protein precursors in silver treated bacteria. Feng et al. describe the dense granules around the DNA as a protective proteinic envelope to prevent Ag^+ from getting to the DNA molecule. Several genes have been isolated and identified that promote a higher resistance to silver ions [60]. These genes imply the production of silver binding proteins, and efflux Ag^+/ H^+ exchange pumps. Accordingly, when silver ions are present at low concentrations, damaged bacteria can be able to re-establish homeostasis and

survive. Above a certain concentration, however, bacterial damage would be irreversible, leading to cell death.

Establishing breakpoints for the inactivation of bacteria with silver is a particularly challenging task, as silver ions will not only interact with bacterial constituents but also with any chemical substance drawing silver ions, like sulphur groups or halides [61] (see section 3.4.). Moreover, bacterial susceptibility has been found to differ highly among different strains of a single species [62]. Generally speaking, it seems Gram negative bacteria are more sensitive to the presence of silver than Gram positives [52]. This has been attributed to the presence of the negatively charged lipopolysaccharide (LPS), which would attract the positively charged silver ions. Hwang et al. found concentrations below 10 ppb to show a bactericidal effect against the Gram negative *Legionella pneumophila, Pseudomonas aeruginosa* and *Escherichia coli* [63]. They further estimated only about 0.5-2.5 pg Ag h /cell were the real amount of silver needed to inactivate these bacteria. This was done by subtracting the silver content before and after sterile filtration. These findings put forth the extraordinary potential of silver as antimicrobial, and the need for understanding the complexity of its antibacterial activity.

Silver nanoparticles

Although nanoparticles are only beginning to be considered in most food legislation frames, a lot of attention has been focused on the production of silver nanoparticles and understanding its biocide activities. Thermodynamic reactivity is dependent on the surface to volume ratio or active surface of the particles. When reduced to the nanoscale (<100 nm) and most remarkably below 25 nm, silver nanoparticles are a meta-stable high energy form of elemental silver, which leads to similar effects on bacteria as compared to silver ions [64]. These imply, as mentioned before, interaction with bacterial membrane constituents, disturbing permeability and forming pits; penetration inside the cell, unbalancing respiratory functions, leading to an increase of reactive oxygen species and depletion of ATP levels; and also intercalating between DNA bases, interfering

replication [65-67]. Although the explanation remains disputed, it is somewhat accepted that these effects could be related to either an increased reactivity of the particles due to the high active surface or/and the increase in released free silver cations or radicals when exposed to water [64-65]. More recently, the physicochemical conditions promoting oxidation of silver nanoparticles into silver ions have been studied. It has been postulated that the oxidation process on the surface of silver nanoparticles involves the concerted effects of dissolved oxygen and acidic pH [68-69]. The enhanced antibacterial effect of nanosilver as compared to silver ions, in the nano- and micromolar levels, respectively, could be due to higher chemical stability of the nanoparticles, which would enable them to penetrate more efficiently to the inside of the cells.

2.3. Advantages and limitations of the use of silver

An advanced spectrum: bacteria, yeast, fungi and viruses

The nature of the mechanism of action, combining many unspecific action levels, makes silver active not only against bacteria, but also against other harmful microorganisms, like fungi or viruses. Although not many studies have focused on the inactivation of fungi, it has been found the antimicrobial activity of silver was similar to that of itraconazole, exerting a biocide effect with concentrations of 6.6 -13.2 μM [70].

More interesting is, however, the inhibitory effect that silver ions and silver nanoparticles exert on viruses. Among the various studies, it has been found that silver inhibits respiratory syncticial virus [71], murine norovirus and bacteriophages like MS-2 [72] and UZ-1 [73]. Several studies have focused on the inactivation of HIV virus. In this respect, silver nanoparticles exclusively in the range of 1-10 nm were found to complex gp120 glycoprotein knobs, preventing CD4 dependent virion binding and post-entry stages [74-75]. This could pose further opening perspectives for the use of nanosilver in alternative therapies against AIDS and the HIV virus or application for transmission-proof devices.

Effects on human health: benefits and toxicity

When studying the potential toxicity of silver, it is compelling to differentiate between silver, silver ions or silver nanoparticles. Silver and the ions that naturally leach from it in minute concentrations are not a cause of concern to humans, as the ions are rapidly inactivated by biomolecules. Historical evidence throughout centuries in contact with it has proven this form of silver to be innocuous. However, if big quantities of silver ions are ingested, like in form of silver nitrate, deposits of silver sulphides can result in a brownish discoloration of the skin called argyria. This discoloration resolves with cessation of the therapy, remaining a cosmetic concern [51].

On the other hand, the possible side-effects of the recently discovered silver nanoparticles on human health are mostly unknown and constitute a topic of dispute and concern [45]. Although systemic toxicity of ingested nanoparticles is not to be expected, in form of aerosol they may easily reach alveoli and increase the oxidative stress of lung epithelial cells, accumulating in the liver [76]. In the skin, results on nanosilver toxicity remain disputed [45]. These hints on possible toxicity do not threaten the use of nanosilver in the medical field at any rate, where a highly positive benefit-risk balance has enabled its approval and widespread use, but rather questions if its use on other applications, such as in textiles, cosmetics or food related applications are sufficiently justified.

The same remarkable mechanism of action to which some authors attribute toxicity may allow promising benefits to human health to be gathered from the study of silver. As mentioned in section 1.1, since the 19th century it has been empirically observed that silver application on wounds promotes fast epithelisation and scarring. Recently, it has been discovered that silver also exhibits excellent anti-inflammatory properties by selectively promoting apoptosis of damaged cells, by interfering the electron transport chain and the intrinsic signalling pathway of down-stream pro-caspases,[44, 65] which reduces erythema, oedema and promotes scarring and epithelisation [77]. The overall

effects were found to be comparable and more rapid than that of steroids [46, 65].

Apart from anti-inflammatory properties, the use of silver nanoparticles poses great expectations in other fields of interest, like in cancer therapy, as they predominantly target cancer cells rather than normal functioning cells [44].

Effects on the environment

The wide range of action of silver can be seen as a threat to the environment if we consider non-pathogenic bacteria or other susceptible organisms. Environmental acute toxicity of silver is known to be derived from the free Ag^+ ion concentration in water [78-79]. These ions are shown to inhibit Na^+/K^+ ATPase and carbonic anhydrase and thus the respiratory functions of many organisms, like bacteria, algae and other small organisms [80]. Different crustacean, algae and fish species have been used as markers for environmental toxicity studies, trying to determine how silver complexation to chlorides or to natural organic matter (NOM) affect the accumulation of silver at the gill of these species or their mortality rates, either in vivo or with the help of the biotic ligand model. In general, it has been found that NOM decreases the toxicity of waterborne Ag depending on rate of uptake, most probably because of complexation [81-83]. The contribution of chlorides to toxicity is disputed, probably because bioavailability might be more influenced by the uptake mechanism and the nature of the studied organism in each case than by silver speciation itself in solution [79, 84]. The emergence of silver nanoparticles and their application for antimicrobial purposes has arisen much concern about their toxicity, as they seem to be much more toxic than the silver ions themselves [85]. This could be explained in terms of stability, considering silver ions are more reactive and could therefore be inactivated in greater extent before reaching internal environment. Extensive literature has therefore been devoted to analyze the extent of detrimental effects of nanoparticles on several organisms and the environment as a whole. [86-91]. These concerns might have been the motive

force for some legislative bodies, such as the EU, to introduce limitations for silver release to food products.

3. Antimicrobial silver in Food Packaging

3.1. Current commercial applications

As commented in section 1.2.1, silver is being marketed for countless applications. However, the innovative search for new silver-based antimicrobial composites has relied mainly on the medical and healthcare sector. The high demand for medical devices with antimicrobial properties and the high economical impact of these products promote research attention in this sense. Silver products used to treat infection can be classified in two categories depending on the presence of either silver ions (Ag^{+}) or silver nanoparticles (Ag^{0}). The incorporation of silver sulfadiazine (a silver nitrate and sulfamide combination historically used for the treatment of burns) in hydrogels, alginates or foam formulations has enabled the rise of different wound dressings (Silvercell®, Urgotul®, Silverex®, etc) or catheters (e.g Bardex Ic®) releasing silver ions. On the second category, different dressings have been embedded or coated with nanocrystalline silver, e.g Actisorb® (charcoal), Aquacel-Ag® (carboxymethyl cellulose), SilverIon®, Contreet® (polyurethane foam) or Acticoat® (Polyethylene)[46, 92-93]. The outstanding success of silver in the medical field has been the driving force for its implantation in other consumer product markets, where silver ion releasing technologies have already colonized most sectors, including the food area.

Surprisingly, one single technology of silver releasing systems predominates in these sectors: silver exchanged minerals, mostly zeolites. Zeolites are microporous, zinc sodium ammonium aluminosilicate minerals commonly used as commercial adsorbents. In silver zeolites, naturally occurring sodium ions are partially replaced by silver ions using simple ion-exchange methods [94]. The substituted zeolites are incorporated or coated into a wide range of polymers and other surfaces. In contact with moisture, silver ions are again substituted by

sodium ions present in the release environment and sustainably leach from the surface (Figure 3). This is practical, as release of silver ions will be dependent on the amount of saline moisture, which is a crucial risk factor for the development of microbes on surfaces. The combination of very low migration rates with a high melting point makes them able to withstand any kind of plastic processing or operating temperature as well and indoor and outdoor conditions, in contrast to all other antimicrobial natural or synthetic substances, including triclosan [95]. Commercial examples of silver-substituted zeolites are Zeomic®, Apacider® (Sangi Co), Croslite Ag^{+}®, Bactekiller®, RepelaCOAT®, D2P®, Novaron®, SPE®, Biomaster® (Addmaster), Irgaguard® (Ciba Specialty Chemicals), AgIon®, Biocote® or Zeargol® [9, 95] ; see "sources of further information"). The zeolites are usually manufactured as a 3-6 μm thick layer with 2-5% silver content. This layer is then coated on polymeric or stainless steel surfaces, preventing any biofilm formation on the treated surfaces. In the food sector, this technology can be applied in practically any food processing equipment: cutlery, cutting boards, counter tops, containers, or any other food-contact surface [96-97]. Additionally, the same technology is applied in tubing and filters of water purification or filtration units [98-100]. The globalization of this technology has made silver the most widely used polymer additive for food applications [9-10]. Still, the food sector only represents a small fraction of the market, as the same brands are commercialized for an extensive number of other applications and consumer products. One of the most typical and worldwide applications is the coating of inner liners in household appliances, mostly refrigerators, but also washing machines, dishwashers, microwave ovens, tiles, knobs and handles, etc. Silver zeolites can also be incorporated inside the polymer in the manufacture of, for example, antibacterial textiles. In this respect, socks and T-shirts with silver zeolites (AgIon®) technologies claim to completely eliminate body odour from sweating (www.stinkatnothing.com). Finally, we find silver zeolites in hygiene products, like in deodorants (Nivea Silver Protect), tooth brushes or tooth paste (see internet sites). Apart from silver zeolites, silver zirconium phosphates, under commercial brands like Alphasan® (Milliken Chemical) or Forfargol®, and other nanoclay based materials, such as

Bactiblock [101], among others obey the same principle of moisture induced silver-sodium exchange mechanism and are manufactured likewise and for similar applications.

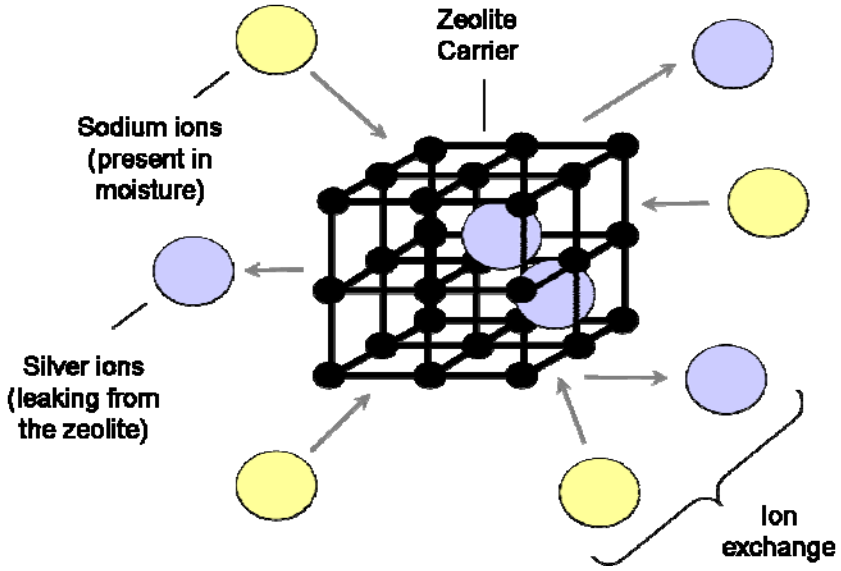

Figure 3. Schematic representation of the ion-exchange induced release mechanism in silver exchanged clays and porous minerals.

3.2. Legislation frame

When looking at regulation of silver, the fact must be taken into consideration that the rediscovery of the use of silver for antimicrobial purposes is relatively new. On the one hand, historical use of silver has proved its benefits and safety for the society throughout centuries in contact with it. On the other hand, the new applications arisen recently imply new ways of contact with the silver ion. In the case of nanosized silver, there is no previous experience or evidence of its safety or risks for the environment or for human health.

In the U.S and especially in Japan, the use of silver exchanged minerals is well established with several commercial brands incorporating silver in textiles or as coatings in different consumer products. The silver content in these materials can be up to a maximum of 3% of their formulation. In the food area, only silver nitrate is regulated with a maximum limit of 0.017mg/kg in foodstuffs and 0,1 mg/kg for drinking waters (FDA/CFSAN). As far as nanosilver is concerned, colloidal solutions are accepted in the U.S and commercialized as nutrition supplements (e.g Mesosilver®), claiming to have important benefits on human health. (www.mesosilver.com). In the medical field, different dressings containing nanocrystalline silver or silver ion releasing systems are widely spread as well as indwelling devices, like prostheses or catheters.

The confrontation between past and present circumstances is best noticed in European law. Medical devices and dressings containing nanosilver are used although not so widespread, and the council does not recommend silver for medicinal use, due to lack of sufficient information about health risk assessment. The European food safety authority (EFSA) provisionally accepts the use of silver in food-contact materials with a maximum of 5% silver in the form of silver zeolites or silver zirconium phosphate glasses. Migration to foodstuffs is restricted to max. 0.05 mg/kg food for the whole group in the EFSA provisional list of additives used in plastics 11th update: 28/10/2011. Silver hydrosols are not included in the list of food additives or supplements "because of lack of appropriate information about silver bioavailability" from them [102]. Paradoxically, silver is a colouring agent in most legislation frames, including the European. Under directive 94/36/EC, still in force, the use of silver is accepted without any restriction limits. The more recent council directive on materials and plastics intended to come in contact with foodstuffs, the food-contact directive, establishes the standard analysis protocols for determining specific migration, but does not consider any silver compounds (EC directive 10/2011).

3.3. Incorporation of silver into food packaging matrices

As commented above, although antibacterial silver based technologies have become a huge field of interest for researchers and manufacturers, the food sector covers only a small range of application. This is not surprising, as research on silver based antimicrobials is still on its early stages and new silver based technologies may be implemented in a very wide number of applications. This scenario is well reflected when browsing scientific literature. Table 1 presents the number of scientific publications dealing with silver and nanosilver for antimicrobial purposes and for food packaging applications. Publications related to antimicrobial silver in general have exponentially risen within the last years, mostly due to the substantial interest in nanosilver, whereas articles specifically devoted to the development of food packaging materials are far less numbered in comparison. Moreover, within this research field, it is the study of the preparation of silver nanoparticles which has attracted most attention, even though nanotechnology is still out of most legislation frames (Table 1).

Table 1. Publications related to silver ions or nanoparticles and in food packaging as searched on the scopus database (last accessed 25-09-2013)

	Number of publications on "antimicrobial silver"			
Publication range (years)	+ "nanoparticles"*	+ "nanoparticles" + "food packaging"	+ "ions"	+ "ions" + "food packaging"
2002-2004	28	0	96	3
2005-2007	123	5	146	1
2008-2010	640	23	209	9
2011-2013	1587	37	232	14

*The search was conducted by typing "antimicrobial or antimibacterial or biocide" and "silver" in combination with the terms referred to in this table

When looking at the articles dealing on the fabrication of silver ion releasing systems we find silver ion implantation on zirconium phosphate or other glasses [103-104], titania surfaces [105], calcium phosphate or hydroxyapatite bone cements [106-108], functionalized on polyurethane [109] , absorbed in cotton [110], and, of course, in clays or other minerals. The release of silver ions in most of these materials relies on the same mechanism if ion exchange within a porous structure when in contact with moisture. In the case of research related to food packaging materials based on silver ion technologies, again the use of clays predominates, including zeolites [111-113] and montmorillonites (MMT) [101, 114-115]. In this sense, paper impregnated with 4% silver zaeolites was able to reduce bacterial growth rates in turkey and pork [116]. Silver-MMT incorporated into alginate or agar based films significantly prolonged the shelf-life and sensory properties of cheese without affecting the lactic acid flora [114, 117-118]. The antimicrobial performance of the silver-MMT compound was, however, much reduced when incorporated into polycaprolactone or zein. More recently, a study on the efficacy of cutting boards containing silver nanoparticles and silver zeolites further studied the influence of humidity and silver chloride content and organic matter on the antimicrobial effectiveness. They found chloride concentration and organic matter decreased the efficacy of the silver ion releasing system according to the JIS [119].Silver ions have also been absorbed onto other porous silicates [120] and cellulose [121]. The main reason why researchers and manufacturers have been choosing this technology is its excellent long-lasting antimicrobial performance together with its thermal stability and relative ease of manufacture. However, the second reason might well be the absence of possible alternatives as to silver releasing effective antimicrobial materials. This gap relies in the complexity of controlling the release, bioavailability, stability and speciation of silver from a specific material and in contact with a specific environment. These aspects pose a great challenge as to the fabrication of food packaging materials based on silver ion release and will be therefore dealt with in the next section.

3.4. Release, stability and efficacy

As explained before (section 2.2), the antimicrobial efficacy of the material relies on the leaching of silver ions to the surface and surrounding environment. Thus, it is crucial to elucidate the release kinetics of the material in question and evaluate the equilibrium between reactivity of the ions against bacteria and their stability in a specific environment.

Concerning the polymer, the release will be mainly dependent on its water-uptake capacity, as release is sorption induced. As silver ions are highly polar and water soluble, their incorporation into plastic matrices, which are typically non-polar and high barrier to water, constitutes a further challenge, as far as dispersion and release kinetics is concerned. Kumar and Münstedt published several studies with silver-polyamide nanocomposites measuring silver release by anodic stripping voltammetry during up to three months. They found release kinetics could be modified based on the crystallinity of the polymer. Furthermore, introducing hygroscopic fillers in the matrix or in a multilayer coating greatly enhanced release kinetics of the nanocomposite and its biocide efficacy [122-125] Dowling et al. approached the challenge of tuning release kinetics by incorporating platinum into a coating on polyurethane. Since platinum has a higher redox potential, silver oxidation is enhanced when the two metals are in contact, increasing release of free silver ions from the polymer [126].

The release from the material can also depend on the characteristics of the aqueous or moistured environment in contact with the material. As an example, tests on human plasma have revealed that greater amount of ions are released in these conditions than in deionized water [94]. But the role of the release environment in release kinetics is not so important compared to the enormous influence it can exert on the stability of silver.

It is inevitable to realize that if the antibacterial efficacy of silver relies on a mechanism of action so unspecific, it will be subject to many variables. Indeed,

silver ions not only bind L-Cys residues of bacterial enzymes, but any atom with relatively high electronegativity and high atomic radius. This is reflected in the solubility constants for various compounds shown in table 2. Considering the extreme affinity of silver for sulphides and halides, it is clear that antibacterial activity will be strongly influenced by the presence of these ligands in the environment of action.

Table 2. Silver ion (Ag^+) solubility constants for different selected anions (M) [50, 60]

Anion	Solubility constant (M)
NO^{3-}	51.6 (soluble)
SO_4^{2-}	$1.58x10^{-5}$
PO_4^{3-}	$2.51x10^{-18}$
Cl^-	$1.58x10^{-10}$
Br^-	$7.70x10^{-13}$
I^-	$1.50x10^{-16}$
S^{2-}	$7.94x10^{-51}$

Among the halides, attention must be paid to chloride, as it will be present in practically any substrate susceptible of bacterial contamination. According to the solubility constant, the soluble concentration of silver chloride would be of about 6.3 ppb, above which saturation is reached and the equilibrium is gradually shifted towards silver chloride complexes, a very low % of silver ions remaining active in solution [127]; ChemEQL). These quantities, though very low, are very similar to bactericidal concentrations (10 ppb) observed for some Gram negative bacteria in synthetic water [63]. Hence, in the presence of chloride, a very small fraction of free silver ions will still remain soluble which would be enough to exert an antibacterial effect. This could explain the reported antimicrobial activity of silver chloride [85, 128]. The existence of an equilibrium between silver chloride complexes and free silver ions further

increases the complexity of the system, as new other factors like the ionic strength and presence of common or non-common ions come into play.

Attention must also be made to the extreme affinity of silver to sulphur. Liau et al. reported about the interaction of silver with different chemical groups containing it. They found silver antibacterial activity was lost when compounds with free –SH (thiol) groups were present, like in cysteine, glutathione or thioglicolate, while others like sulphates, thiosulphates, taurine or methionine with S-O or S-C bonds did not substantially alter its efficacy [49]. Any environment with natural organic matter (NOM) can be an important source of thiol-containing ligands, which can fully void or significantly reduce antibacterial efficacy. This is easily evidenced when looking at released silver bactericidal concentrations among the different publications. These go from the ppb range when water or salt buffers are used [63, 129-130] to hundreds of ppm when complex growth media, like LB, TSB or MHB come into play [57, 62, 131]. These huge differences in antibacterial response (up to four orders of magnitude) put forth the need of standardizing biocide tests if the potential of different materials throughout the literature is to be compared [61].

In addition, silver has a relative low redox potential, being easily reduced to metal particles in the presence of weak reducing agents, UV light or increased temperature. According to Kim et al., UV-reduction could take place even after the silver ions have been inactivated by -SH groups, disrupting the Ag-S bond to form nanoparticles [72]. Kasuga et al. have recently solved these problems by forming a transparent light stable, water soluble compound of silver with acetomethionine. Although methionine forms stable complexes with silver, it was found that silver methionine complexes could still exert a high antimicrobial performance, as was also demonstrated during the course of this thesis and will be discussed in the results section [132-133]. Thermal stability or possible incorporation into polymers was, however, not evaluated in this study. Thermal instability can lead to the formation of sulphides or other silver compounds which mostly lead to a strong discoloration and may have reduced or no

effectiveness at all. Moreover, the thermally or UV induced formation of metallic particles may also produce discoloration of the materials due to their plasmonic properties. Hence, the use of silver in plastics could be severely limited as melt-compounding at high temperature is the most widely manufacturing practice for plastics.

Efforts to increase the chemical stability of silver ions, either by forming stable compounds, complexes or chelates, must take into account that the mechanism of bactericidal activity relies on this reactivity against vital bacterial constituents. Therefore, it is crucial to find a compromise between stability and efficiency.

The search for new materials not only implies the incorporation of silver ions into a polymer matrix, but should also take into account all issues affecting the release and stability of silver. These include the chemical and physical characteristics of the specific material in which the silver ions are incorporated, the release kinetics from the polymer over time, the chemical environment where the material has to exert its effect and even the conditions to which the material will be exposed. All these factors point out the complexity of the aspects to face when designing a silver-based antimicrobial system.

Rationale of this thesis

In recent years, the interest in food packaging with antimicrobial properties has increased considerably, due to the fact that these systems are able to control the microbiological decay of perishable food products [134]. Many applications, including food production and storage, might benefit from the incorporation of safe and wide spectrum long-lasting biocides into polymers or working surfaces [10]. As bacterial contamination occurs primarily on the surface, the incorporation of the biocide in a film or as a coating has many advantages due to the high exposure areas for the biocide and a reduction of the loss of the antimicrobial compound into the bulk of the food [11-12]. Therefore, extensive research has been made to develop packaging strategies to retain the active agent in the polymeric network and control its release as to allow the use of less quantity while assuring the desired effect over the shelf-life of the product [134].

Among all antimicrobials used in the food sector, silver has the highest degree of commercialization. Other antimicrobials are either volatile, lack sufficient physical and chemical stability as to maintain its effectiveness over manufacture and storage, or imply expensive production costs. The use of silver as antimicrobial for food-related applications has been recognised since silver pottery and cutlery where used in antiquity [47]. A public opinion ever more concerned with sterility and safety, the increase in antibiotic resistant microbes and the absence of cost–effective alternatives among other antimicrobials has promoted the rise of silver as antimicrobial and the development of new antimicrobial materials based on the release of silver ions, either in the food or in other sectors.

However, and despite the outstanding commercial success, research on silver based antimicrobial technologies is still on its early stages. The recently revealed mechanism of action implies several unspecific pathways involving the complexation of silver to vital and non-vital bacterial constituents. This mechanism raises new questions as to how possible sublethal damage to bacteria and possible parallel inactivation of the active silver species may affect the efficacy of silver as antimicrobial. Both events may be at the origin of the huge

differences in efficacy (up to 4 orders of magnitude) found among different studies testing silver materials and put forth the need for standardizing biocide tests (see Annex A). Establishing breakpoints for silver efficacy is, however, a challenging task, as many solubility issues affecting speciation and bioavailability of silver are still unknown. Therefore, it is crucial to elucidate how exposure to the different silver species may influence bacterial growth rates, cultivability or viability, and how time-dependent chemical interactions found in the complexity of food matrices could interfere with silver speciation and its ultimate bactericidal effect.

However, these are not the only important aspects to cope with when designing silver based antimicrobial systems. Silver exchanged minerals constitute the only successful technology currently applied in the food area. This technology implies the need of silver filler contents of 1-5% and the presence of a porous mineral or clay. The addition of high contents of these additives may imply relatively high production costs and a relatively high environmental impact. This is important if we consider the application in the food packaging industry, characterized by very low mark-ups and a high impact on waste management. Moreover, the incorporation of these additives into plastic packaging may pose problems of dispersion, permeability or transparency. For the correct development and final application of silver in the food packaging industry, it is crucial to elucidate the threshold of biocide action and optimize the silver system so that tiny contents are required and the potential is fully realized.

Additionally and, considering both the physical and chemical lability of silver ions and the stringent migration limits established by some legislation frames, a sustained release of silver ions may be crucial as to assure antimicrobial efficacy over the shelf-life of the products without surpassing migration limits. Efficient tools to monitor the release of silver ions over time are therefore necessary, as well as a deep understanding in all speciation and stability issues.

Last, but not least, suitable fabrication of the polymer should be designed as to assure that silver is only in its ionic form and can sustainably leach to the outer environment. Silver ions are physically and chemically unstable, being reduced to elemental silver or silver nanoparticles by weak reducing treatments, such as many solvents, UV-light, thermal treatment, ligands, etc. This may radically alter the expected release profiles from the materials and may pose additional limitations as to the possible migration of nanoparticles. Taking all these issues into account, the physicochemical properties (mechanical strength, permeability and barrier properties, thermal and optical features) should not be neglected and kept up with traditional benchmark food packaging standards.

All these aspects together point out the need for expanding the knowledge as to antimicrobial silver, and evidence that the design of alternative technologies that may be implemented or adapted to other applications such as food packaging, is, in fact, a very challenging task.

Objectives

The overall objective of this dissertation was the following:

"*The development of antimicrobial biodegradable materials of interest in food packaging applications based on the sustained release of silver ions*".

For this purpose, several stages had to be sequentially accomplished as follows:

1. Gathering of a deep understanding of the interactions of silver ions with bacteria, the chemical environment, and how these may affect bacterial viability.

2. Incorporation of silver into polar matrices, such as EVOH, to promote the sorption induced release, and evaluation of the release of ions and the efficacy of the films under food contact conditions**.**

3. Evaluation of different methods for incorporation of silver ions into PLA as to their suitability as long lasting antimicrobial and antiviral materials for food applications.

4. Application of a functional barrier to the PLA-Ag^+ films as to tailor the release capacities of the films and achieve a prolonged antimicrobial performance without surpassing restriction limits.

5. Fabrication of PLA films incorporating silver compounds, capable of withstanding thermal plastic processing while maintaining a prolonged antimicrobial effectiveness over time.

An schematic overview of the different objectives, a brief description of each chapter and how they are related to each other, is presented in Annex B.

Results and Discussion

Chapter I

LIGANDS AFFECTING SILVER ANTIMICROBIAL EFFICACY ON *LISTERIA MONOCYTOGENES* AND *SALMONELLA ENTERICA*

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ABSTRACT

Although silver is being extensively used in food or other applications as the key component to control microbial proliferation, many factors affecting its real potential are still unknown. In the present work, the presence of specific ligands or the contents in organic matter was correlated with silver speciation and its antibacterial performance. Silver was found to be only active in form of free silver ions (FSI). The presence of chloride ions produced equilibrium of stable silver chloride complexes which were void of antimicrobial efficacy. However, even at relatively high concentrations of chlorides, a small fraction of FSI may still be present, producing a bactericidal effect with concentrations at the nanomolar level under optimum conditions. Low concentrations of thiol groups completely inactivated silver, while methylsulphur groups only affected its efficacy at very high concentrations. Antibacterial performance revealed differences of at least 3 fold between results for environments with high organic matter content and results for aqueous salt buffers. Thiol groups were nonetheless not found directly associated with the decrease in antimicrobial performance in a nutrient rich environment. These results point out the complexity of the antimicrobial systems based on silver and can have relevance in food or other applications of silver as an antimicrobial.

Keywords: Silver ions, inactivation, silver speciation, antimicrobial activity, ligands, microbial growth.

1. Introduction

In the last decade, the demand for minimally processed, easily prepared and ready-to-eat 'fresh' food products has globally increased which has encouraged the industry to the development of new technologies as an alternative to food-thermal technologies. These new alternative technologies such as lower thermal or high pressure treatments may in some instances allow pathogenic bacterial growth (Valero & Francés, 2006). However, even if foodborne pathogens are totally eliminated by efficient thermal treatments, microbial recontamination of the food surface could take place during the post-processing steps, when the risk of cross-contamination is elevated. As a result, a reduction of food shelf-life is observed and the risk of foodborne illnesses is greatly increased. Therefore, new preservation techniques, such as incorporation of antibacterial substances to the food products in order to extend its preservation is currently being investigated and applied.

The use of silver as antimicrobial for food-related applications has been recognised since silver pottery and cutlery where used in antiquity (Klasen, 2000). Although the mechanism remains disputed (Dibrov, Dzioba, Gosink & Häse, 2000; Texter, Ziemer, Rhoades & Clemans, 2007), it is generally accepted that free silver ions (FSI) bind to membrane constituents, destabilizing the membrane potential and causing proton leakage (Liau et al., 1997; Matsumura, Yoshikata, Kunisaki & Tsuchido, 2003) and it also interferes with DNA replication and ion transport across the respiratory chain (Feng et al., 2000; Semeykina & Skulachev, 1990), all of which eventually lead to cell death. Due to this combination of unspecific mechanisms, silver ions are not likely to develop any resistances and are active against a very broad spectrum of bacteria, yeasts, fungi and even viruses in tiny concentrations (Thomas & McCubbin, 2003), remaining nontoxic to human cells (Russell & Hugo, 1994; Williams et al., 1989).

Therefore, its use has become more and more popular in the past few years. Apart from the medical field, silver is nowadays incorporated as the key

component to control microbial proliferation in a wide variety of materials used in our daily life like textile clothing, coatings in home appliances and food related applications like water treatment units or a great variety of food-contact materials (see Bosetti, Massè, Tobin & Cannas, 2002; Chen & Schluesener, 2008; Gupta & Silver, 1998; Li et al., 2008; Rai, Yadav & Gade, 2009 for review). In most of these materials, the antimicrobial effect relies on the leaking of silver ions based on ion-exchange from mineral carriers, like montmorillonites (Busolo, Fernandez, Ocio & Lagaron, 2010; Malachová, Praus, Pavlíčková & Turicová, 2009), tobermorites (Coleman, 2009) and most predominantly zeolites (Cowan, Abshire, Houk & Evans, 2003; Galeano, Korff & Nicholson, 2003; Nakane et al., 2006). The versatility and cost-effectiveness of these materials have made silver the most widely used polymer additive for food applications (Appendini & Hotchkiss, 2002; Quintavalla & Vicini, 2002).

However, despite its widespread use, there is still much to be learnt about the chemical interactions taking place between the active silver species, the different bacteria and the matrix with which they interact. Most studies focus on the characterization of silver particles and the release rates from different materials, neglecting the crucial effect that the chemical environment of action may have on their antimicrobial performance. Looking at the final concentrations achieved in solution that have been reported to exhibit antibacterial properties, these values go from the ppb range (Bjarnsholt et al.2007; Hwang, Katayama & Ohgaki, 2007; Kim et al., 1998) to hundreds of μg/mL (Hamilton-Miller & Shah, 1996; Nomiya et al, 2004; Ruparelia, Chatterjee, Duttagupta & Mukherji, 2008; Sondi & Salopek-Sondi, 2004; Thomas, Yallapu, Sreedhar & Bajpai, 2007) (4 orders of magnitude difference). Highlighting that, standardization of silver ion biocidal tests is difficult, as many solubility issues affecting speciation and bioavailability of silver are still unknown (Chopra, 2007).

In this respect, some studies in the branch of environmental toxicology have been dealing with the effect of ligands on the toxicity of silver to fish and algae. Computational modelling has also been used to predict silver chloride complexes available at different salinities (Ward & Kramer, 2002) and the influence of organic matter or food in the bioavailability and toxicity of silver to

these organisms has been investigated (Glover, Sharma & Wood, 2005; Kolts, Boese & Meyer, 2006; Nichols et al., 2006; VanGenderen, Ryan, Tomasso & Klaine, 2003).

However, the bioavailability of silver in these cases is more influenced by the uptake mechanism and the nature of the studied organisms than by silver speciation itself (Bielmyer, Brix & Grosell, 2008; Lee, Fortin & Campbell, 2005). Accordingly, controversy arises when deciding how much natural organic matter and which silver chloride complex are responsible for toxicity or protection against silver and to the best of our knowledge, no literature has yet been published about these effects on foodborne pathogenic bacteria.

As the antibacterial mechanism of silver seems to imply different unspecific pathways, and thus probable sublethal damage (Junghanns & Müller, 2008), it is crucial to elucidate how exposure to ligands present in complex matrices like food may alter the speciation of silver and how this speciation is correlated with the bactericidal effect.

In the present work, antimicrobial assays in different growth media were performed against two of the most relevant foodborne pathogenic bacteria, i.e. *Salmonella* and *Listeria monocytogenes*. The results were correlated to the FSI concentrations, as measured by anodic stripping voltammetry (ASV) (Joyce-Wöhrmann & Münstedt, 1999; Ward & Kramer, 2002). The impact of various ligands on silver speciation was also examined.

2. Materials and methods

2.1. Bacterial strains and preparation of inoculum

L. monocytogenes CECT 5672 and *S. enterica* CECT 554 were obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain). These strains were stored in Phosphate Buffer Saline (PBS) with 10% Tryptone Soy Broth (TSB, Conda Laboratories, Madrid, Spain) and 10% glycerol at -80 ºC until needed. For experimental use, the stock cultures were maintained by monthly subculture to agar Tryptone Soy Agar (TSA) slants at 4 ºC. Previous to each study, a loopful of bacteria was transferred to 10 mL of TSB and incubated at 37 ºC overnight. A 100 µL aliquot from the overnight culture was again transferred to TSB and grown at 37 ºC to the mid-exponential phase of growth. This culture served as the inoculum for antimicrobial assays starting with approximately 5 x 10^5 CFU/mL. These CFU counts were accurately and reproducibly obtained by inoculation into 10 mL growth medium of 0.1 mL of a culture having an absorbance value of 0.20 for *S. enterica* and 0.15 for *L. monocytogenes* as determined by optical density at 600 nm by ultraviolet visible (UV) spectroscopy (SP-2000 UV, Spectrum Instruments, Shanghai, China).

2.2. Chemical preparations

Experimental tests were performed using different aqueous silver nitrate solutions (from now on referred to as silver) prepared daily from serial dilutions of silver nitrate powder (Sigma-Aldrich, Germany) as the antimicrobial compound. Ultrapure water (Traceselect ultra, Fluka) was used as a base for the preparation of the different chemical environments. Potassium chloride, ammonium chloride and sodium chloride (Panreac, Barcelona, Spain) were used as a source of chloride ions. L-cysteine and L-methionine (Panreac) were used as a source of thiol (-SH) and methylsulphur ($-SCH_3$) groups, respectively. For the simulation of complex environments, the bacterial growth media TSB and M9 minimal medium (Sigma-Aldrich) alone, or supplemented with 0.1 mg/mL Methionine (Panreac) (M9-Met) were selected.

2.3. Silver ions quantification

Silver ions in free ionic form were quantified by means of voltammetric analysis. Samples were prepared dissolving a silver ion solution in the sample medium to achieve a final concentration of 100 µg/mL (approx. 0.6 mM) silver, then incubated at 37 ºC and finally measured for free ions by differential pulse anodic stripping voltammetry (ASV) with an Autolab III potentiostat setup (EcoChemie, Switzerland) under conditions stated in Metrohm application bulletin nº 207/2e "Analysis of silver by stripping voltammetry". As the addition of the different components and substances in the concentrations used in the study did not affect the technique response, a calibration curve in high purity water was prepared daily for each set of measurements. The FSI working range was 0.004 – 0.4 µg/mL. All experiments were carried out in duplicate.

2.4. Antimicrobial tests

Antimicrobial capacity of silver under these various conditions was performed according to the broth macrodillution technique (M-26A) described by the Clinical and Laboratory Standards Institute (CLSI) with modifications. Briefly, a bacterial suspension in mid-exponential phase was inoculated into 10 mL of the selected environment of growth (ultrapure water supplemented with increasing concentrations of chlorides or sulphur groups, M9 medium or TSB) with silver concentrations of 0.1 µg/mL (0.59 µM) in ultrapure water or M9 and 50 or 100 µg/mL (0.59 mM) in TSB as to achieve approximately 10^5 CFU/mL and incubated at 37 ºC for 20-24 h. Cells suspensions were serially diluted in buffered peptone water (Scharlab S.L, Barcelona, Spain) and 100 µL spread on TSA. Colonies were counted after incubation at 37ºC for 24 h. Each of the experiments was performed in triplicate.

2.5. Effect of centrifugation

Samples with silver concentrations of 100 µg/mL (0.58 mM) in ultrapure water, M9-Met and TSB were incubated 24h at 37ºC with and without the presence of increasing concentrations of bacteria. The suspensions were subsequently centrifuged at 3,900 rpm and 12,200 rpm in a microcentrifuge (MiniSpin,

Eppendorff, Germany) giving a relative centrifugal force of 1,000*g* and 10,000*g*, respectively, for 1-60 min. Supernatant was decanted and measured for FSI by a voltammetric method as described above. Additionally, the antimicrobial efficacy of the supernatant of TSB was evaluated by serial dilution and subculture in TSA, as described above.

3. Results and Discussion

3.1 Influence of the growth conditions

Silver ions and silver nanoparticles are known to exhibit high antimicrobial efficacy due to chemical binding to membrane and respiratory enzymes, causing proton leakage and an increase in reactive oxygen species (ROS) to a sublethal or lethal level depending on the concentration (Feng et al., 2000; Jung, Kim, Kim & Jin, 2009). The same mechanism may be responsible for inactivation of the active silver species by different chemical components, which could be present in the environment of action. This may explain the huge differences in efficacy among the different studies found in the literature. To investigate how these different conditions affect silver speciation and antimicrobial effectiveness, TSB and M9-Met medium were selected. M9 medium is a minimal medium usually used for the propagation of *Escherichia coli* strains and is known to support growth of other Gram negative bacteria like *Salmonella*. Methionine was supplemented to enhance the growth of the tested *L. monocytogenes* strain, as reported previously (Martínez-Abad, Sanchez, Lagaron & Ocio, 2012). TSB, on the other hand, is a rich medium consisting of enzymatically digested soy and casein, which delivers an undetermined heterogeneous mixture of aminoacidic and glucosidic components. Both growth media could stand for rich or stringent conditions of contamination, respectively. In the present study, the stability of FSI was measured in ultrapure water, M9 and TSB after 1 min, 1 h and 3 days in contact with the growth media. FSI were found to be stable in ultrapure water (pH 6.5-7) recovering between 95 and 101% (<4% standard deviation) of the total silver throughout the experiment. In M9-Met, the amount of FSI was reduced drastically in the very first minute of contact, dropping to values between 0.2-0.4% of the total before 1 h. This suggests that a stable silver compound that is not reduced at -0.4V and thus not detected in the potentiometric assays is rapidly formed by the addition of M9-Met. Equilibrium of silver chloride species (i.e. Ag^+, AgCl, $AgCl_2^-$ and $AgCl_3^{2-}$) present in chloride solutions depends to a great extent on the chloride concentration.

According to mathematical modelling using the freeware software ChemEQL (Swiss Federal Institute of Aquatic Science and Technology, Switzeland), Cl^- speciation in M9-Met medium (c.a. 27.25mM Cl^-) would produce mostly AgCl and $AgCl_2^-$ species with about 0.45% of the silver in form of FSI which correlates with our experimental data.

On the other hand, in contact with the TSB medium, the FSI values were relatively stable after 3 days contact at 37ºC with 90.9±1.2% of the silver in form of FSI. Considering that TSB has a higher amount of chlorides than M9 (about 85 mM vs 27.25mM), these results signal that FSI might be reversibly bound to some constituent(s) in TSB, preventing formation of silver chloride complexes. This bond would allow silver species to be reduced in the deposition process so it would be accordingly detectable.

When the susceptibility of *L. monocytogenes* and *S. enterica* was tested with increasing concentrations of silver, huge differences were observed between M9-Met and TSB (Table 1). In TSB, a bactericidal effect (defined as a decrease in CFU/mL of 3 log units in 20-24h) was achieved with 100 μg/mL and 50 μg/mL silver against *L. monocytogenes* and *S. enterica*, respectively. Inhibition of growth during 24h incubation (defined as a bacteriostatic effect) occurred with silver concentrations of 50 μg/mL and 10 μg/mL, respectively. When M9-Met medium was selected for the assays, about 1,000 times less silver was needed to produce the same effects. Inhibition of growth was observed with 0.01 μg/mL against *S. enterica* and a bactericidal effect was achieved with only 0.1 μg/mL for both microorganisms. These huge differences in antimicrobial efficacy observed when the environment of action of the silver species is changed have previously been reported (Martínez-Abad, Sanchez, Lagaron & Ocio, 2012). Despite the great variety of silver species tested, relatively high silver concentrations of 10-500 μg/mL are needed if the assay is designed with rich, non-selective media as TSB, MHB or LB (Hamilton-Miller et al.1996; Thomas et al., 2007) (Nomiya et al., 2004; Ruparelia, Chatterjee, Duttagupta & Mukherji, 2008; Sondi & Salopek-Sondi, 2004). However, when chemically restricting environments are used for the assays, such as water or salt buffers, the

bactericidal concentrations are proven to be in the range of 0.01-1 μg/mL (Bjarnsholt et al., 2007; Hwang, Katayama & Ohgaki, 2007; Kim et al., 1998).

Table 1. Viable counts of *Listeria monocytogenes* and *Salmonella enterica* in M9-Met and TSB medium with increasing concentrations of silver.

Growth conditions	Ag^+ (μg/mL)	Log (CFU/mL)	
		L. monocytogenes	***S. enterica***
TSB	0	9.08 (0.05)[a] A[b]	8.93 (0.05) A
	10	9.14 (0.01) A	5.63 (0.34) C
	20	9.30 (0.08) A	3.13 (0.42) D
	50	3.73 (0.11) D	1.63 (0.72) E
	100	<1 E	<1 E
M9-Met	0	7.60 (0.10) B	8.95 (0.03) A
	0.001	7.75 (0.09) B	9.09 (0.06) A
	0.01	7.62 (0.12) B	5.97 (0.07) C
	0.1	1.35 (0.41) E	<1 E
	1	<1 E	<1 E

[a] Standard deviation (n=3)

[b] Different letters represent significant differences according to a one-way analysis (ANOVA) and Tukey's multiple comparison tests ($p<0.05$)

Voltammetric analysis suggests the presence of some constituent(s) in TSB which might be preventing the formation of silver chloride compounds. This phenomenon could explain the lesser efficacy of TSB compared to M9-Met. On the other hand, biocidal assays showed strong antibacterial activity in M9-Met although FSI were very much reduced (<0.4%). This could indicate that either the biocidal concentration of residual FSI is extremely low, or the silver chloride complexes formed (i.e. $AgCl_2^-$ and AgCl) retain antibacterial properties.

Moreover, our results showed higher susceptibility of *S. enterica* to silver than *L. monocytogenes*, as previously reported (Martínez-Abad, Lagaron & Ocio, 2012; Martínez-Abad, Sanchez, Lagaron & Ocio, 2012) and is suggested to be related to the attraction of silver ions for the negatively charged outer membrane of the Gram negatives (Feng et al., 2000; Lee, 2009).

3.2 Effects of sulphur groups on silver antimicrobial efficacy

It has been reported that silver activity is highly dependant on the presence of natural organic matter (NOM) because of strong complexation with chlorides, sulphides, thiosulfates, and dissolved organic carbon claiming sulphide formation may be responsible for this decrease in the bioavailability of FSI (Choi et al., 2009). To investigate if sulphur groups are responsible for the decrease in silver antimicrobial efficacy when in contact with NOM containing environments, silver aqueous solutions were put in contact with L-Cysteine and L-Methionine separately to quantify the influence of these sulphur groups on the stability of FSI by using voltammetry. The selected amount of silver (100 µg/mL) for the assays silver is equivalent to approximately 0.6 mM in the solutions, so concentrations were chosen below and above this value to study the effect with lesser ratios and with excess of these groups. Results in Table 2 showed that the presence of cysteine produces a drastic decrease in FSI. Equivalent quantities of silver and cysteine, not having been detected voltammetrically, indicated that silver binding was at least in a relation 1 to 1. This indicates that thiol groups bind silver covalently making the complex undetectable.

Table 2. Free silver ions available after incubation with increasing concentrations of the aminoacids cysteine and methionine and the salts potassium chloride, sodium chloride and ammonium chloride.

Ligand concentration (mM)	Silver ions in free ionic form (%)				
	Cysteine	**Methionine**	**KCl**	**NaCl**	**NH_4Cl**
0	98.8 (3.1) [a]A[b]	99.9 (1.2) A	95.4 (5.2) A	99.3 (5.1) A	97.8 (3.1) A
0.2	27.2 (3.4) CD	100.2 (0.9) A	76.2 (5.4) B	73.0 (9.0) B	73.5 (7.3) B
0.6[c]	<0.1 E	97.2 (3.1) A	18.6 (2.2) D	17.7 (2.4) D	17.9 (1.8) D
27.25	<0.1 E	90.1 (2.1) A	0.5 (0.2) E	0.3 (0.0) E	0.4 (0.1) E
200	<0.1 E	34.8 (3.8) C	< 0.1 E	<0.1 E	<0.1 E

[a] Standard Deviation (n=2)
[b] Different letters represent significant differences according to a one-way analysis (ANOVA) and Tukey's multiple comparison tests ($p<0.05$)
[c] 0.6 mM corresponds to 100 µg/mL of the added silver compound

When the assay was performed analogously with methionine, the amount of FSI was not significantly reduced up to 27.25 mM. Great excess of methionine (200mM) produced, however, a slight decrease in FSI, which indicates a much lower affinity of silver to -SCH_3 groups than to –SH groups.

To relate FSI concentration with antimicrobial efficacy, a bacterial suspension was inoculated in M9 medium with and without a silver concentration of 0.1 μg/mL (bactericidal) and increasing concentrations of each of the aminoacids. As the Gram positive bacterium *L. monocytogenes* did not reach the maximal growth values in the restrictive minimal medium M9 (Fig. 1) as compared to those obtained in TSB (Table 1), the starting concentration of the aminoacids was 1mg/mL (corresponding to 0.82mM cysteine and 0.67mM methionine) according to minimum requirements stated by Tsai & Hodgson (2003), followed by 10 and 50 mM. The addition of cysteine did not enhance the growth of samples without silver in any of the microorganisms tested. Furthermore, samples with silver displayed values similar to controls without silver when cysteine at any of the tested concentrations was added. This evidences that total loss of antibacterial efficacy was due to the presence of cysteine and correlates with results obtained voltammetrically.

When methionine was added to M9 medium, considerable turbidity was observed in all test tubes after 24h incubation, reaching viable counts (c.a. 10^8 CFU/mL) at least two magnitudes higher than the M9 controls (Fig. 1). A decrease in cell viability of at least 3 fold was observed in samples with silver except in test tubes with 50 mM methionine, which displayed similar values as the control without silver. This means that silver efficacy is lost when methionine is in great excess which again correlates with the voltammetric analysis. Liau et al. (1997) calculated the coordination numbers of silver nitrate with different sulphur containing compounds by cyclic voltammetry. They reported coordination numbers of 3.1 and 0.31 for cysteine and methionine, respectively. According to the present ASV analysis, we found coordination numbers of about 2 for cysteine. Considering that many food matrices contain a certain amount of proteins, including the aminoacid cysteine, these results point out the need for thoroughly investigating the final application of a silver-based

antimicrobial system, as inactivation may block its activity and potentially compromise food safety. With methionine, however, the amount of FSI does not drastically drop at a certain aminoacid concentration, but starts to gradually decrease with >10fold concentration. Differences between these assays might be associated to the use of a different technique or of a different counter-ion for silver. However, if we consider the inactivation of bacteria as a signal to calculate the coordination number, then >5000fold concentration of methionine is needed for this inactivation to be detected, which would suggest a notably lower coordination number (<0.0002). These differences might be associated with the coordination not being stechoimetric, but allowing the existence of an equilibrium which is gradually shifted towards complexed silver but where a small quantity of FSI (below the detectable threshold of the technique) are still present even at high concentrations of the aminoacid.

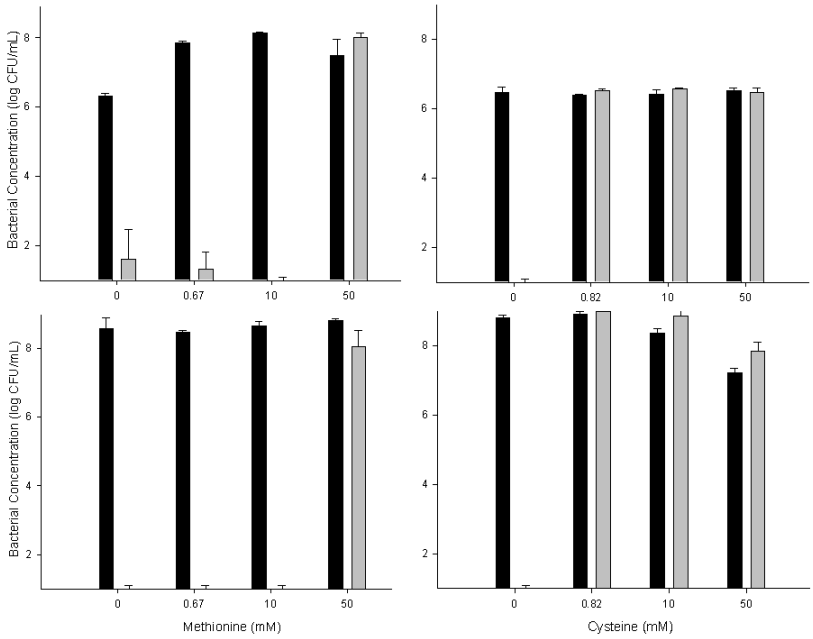

Figure 1. Viable counts numbers of *Listeria monocytogenes* (above) and *Salmonella enterica* (below) after incubation at 37ºC in M9 medium and M9 medium with 0.1 µg/mL silver , supplemented with increasing amounts of methionine (n=3).

Our results also demonstrate that M9 can be used as a satisfactory minimal medium for *L. monocytogenes* CECT 5672 if methionine is supplemented. The results, however, do not contradict findings by Tsai & Hodgson (2003) considering that a different strain was selected for the present study. Moreover, they evidenced that supplement of methionine in suitable concentrations did not negatively affect silver efficacy and could be used as model for the assessment.

3.3 Effects of chlorides on silver antimicrobial efficacy

In order to investigate the role of chloride ions in speciation of silver and its antimicrobial efficacy, voltammetric analysis was analogously performed. To assess if the counter-ion might have any relevance, three chloride salts were tested: Potassium chloride, sodium chloride and ammonium chloride. Table 2 presents the FSI concentration of 100 µg/mL (equivalent to aprox. 0.6mM) silver aqueous solutions after being incubated with increasing concentration of these salts. FSI concentration decreases with increasing chloride concentration, regardless of the counter-ion used. With approx. equivalent quantities of silver and chlorides, about 17-19% of the silver was in form of FSI. When increasing the chloride concentration to 27.25 mM (as in M9-Met), FSI concentration dropped to values between 0.2-0.5%, depending on the chloride salt. An excess of chlorides (200 mM) produced the complexation of most FSI, their concentration being too low to be detected under the experimental conditions (<0.1%). These results are in accordance with speciation as calculated with ChemEQL (0.41-0.42% for 27.25mM and 0.003% for 200 mM for the three tested salts). Furthermore, no significant differences were found among salts with the same chloride concentrations, suggesting that the cations implicated do not play an important role in silver speciation (Table 2).

Antimicrobial assays were carried out with the Gram negative *S. enterica* to correlate them with the potentiometric assays and ascertain how silver complexation with different chloride salts affects its biocidal properties. For that purpose, ultrapure water was used with chloride salt concentrations of 0.6 µM, 27.25 mM and 200 mM representing chloride amounts equal to 0.1 µg/mL silver,

the chloride concentration present in M9 medium and a relatively hypertonic environment, respectively. According to preliminary assays, incubation time was set to 30 min to assure no cell damage was attributed to growth conditions (data not shown). All samples without silver exhibited similar viable counts as the initial inoculum size except samples with 200 mM chlorides. This indicates cell death in samples with silver was only due to the presence of the antimicrobial in this range of concentrations (Table 3). Samples containing equal amount of chlorides and silver (0.6 μM each) displayed no cell viability for both bacteria in any of the three different chloride salts. The same effect was observed when 27.25 mM chlorides are added (as in M9-Met). Samples without silver and with excess of chlorides (200 mM) displayed a decrease in viability, more notable with NaCl, due the sensitivity of *S. enterica* to hypertonic environments.

Table 3. Antibacterial effect of silver against *Salmonella enterica* in ultrapure water when combined with different chloride concentrations.

	Bacterial counts of *S. enterica* (log CFU/mL)					
	Samples without silver (controls)			**Samples with 0.1 μg/mL silver**		
Cl^- (mM)	**KCl**	**NaCl**	**NH_4Cl**	**KCl**	**NaCl**	**NH_4Cl**
0	5.04 (0.03)[a] ABC[b]	5.60 (0.20) A	5.23 (0.21) AB	<1 D	<1 D	<1 D
0.0006[c]	5.25 (0.01) AB	5.61 (0.18) A	5.10 (0.35) ABC	<1 D	<1 D	<1 D
27.25	5.02 (0.09) ABC	4.76 (0.18) ABC	5.17 (0.10) ABC	<1 D	<1 D	<1 D
200	4.73 (0.06) ABC	3.92 (0.20) C	4.95 (0.16) ABC	1.95 (1.94) D	4.06 (0.06) BC	1.59 (0.83) D

[a] Standard Deviation (n=3)
[b] Different letters represent significant differences according to a one-way analysis (ANOVA) and Tukey's multiple comparison tests ($p<0.05$)
[c] 0.0006 mM corresponds to 100 μg/mL of the added silver compound

However, when silver was added in the presence of 200 mM, its antimicrobial effectiveness was notably reduced. Interestingly, the antimicrobial efficacy of silver was lost when in contact with 200 mM NaCl, while the same concentrations of the other chloride salts produced a decrease in viability of at least 2 log units as compared to the controls. According to silver speciation either measured voltammetrically or simulated with ChemEQL, these results

demonstrate that silver has no antimicrobial properties when complexed with chlorides (AgCl, $AgCl_2^-$, etc), but only in its free ionic form (Ag^+). This is in accordance with similar findings by Lansdown (2006). It also indicates real antimicrobial concentration, corresponding to the FSI fraction, is far below 0.1 μg/mL. If we consider silver speciation in the tested solutions containing chlorides, a bactericidal effect can be achieved with FSI concentrations lower than 0.4 ng/mL, corresponding to 2.35nM. These findings put forth the outstanding potential of silver ions as antimicrobial. Other authors found antimicrobial effects with concentrations as low as 0.02 μg/mL in PBS (Kim et al., 1998) and synthetic water (Hwang, Katayama & Ohgaki, 2007).

3.4 Effect of centrifugation

As explained above, silver ions readily interact with different ligands which in some cases render the silver complex undetectable by ASV. Centrifugation was used in an attempt to separate the complexed silver species from the remnant of FSI. Two different relative centrifugal forces (RCF) were selected, 1,000*g* and 10,000*g*. The first RCF is known to be able to sediment particles such as whole cells and nuclei in minutes. Therefore, silver ions attached to bacterial constituents should be part of the cell pellet and would be accordingly separated from the supernatant. The second RCF should be able to sediment smaller aggregates of the size of organelles. As sedimentation is proportional to centrifugation time, this was prolonged for 1-60 min in order to be able to centrifuge smaller aggregates.

In first place, the effect of the presence of bacteria on the active FSI concentration was studied. Different concentrations of *Salmonella enterica* and *L. monocytogenes* suspensions were mixed in ultrapure water with 100 μg/mL silver. After 24h incubation at 37ºC, samples were centrifuged at 1,000*g* for 20 min to investigate how much of the silver, in free ionic form, could remain in the supernatant after centrifugation of the cells. Satisfactory centrifugation of the cells was noted by formation of pellet and absence of turbidity in the supernatant. Results in Fig. 2 show that the amount of FSI decreases with increasing amounts

of bacteria, this decrease being more evident upon centrifugation of the cells. This demonstrates silver ions attach to bacterial constituents (i.e. membrane proteins) and become accordingly undetectable. Centrifugation of the cells contributes to better detection of the bonded silver fraction as weak bonds which may be broken in the voltammetric deposition process are removed with the cells from the supernatant. The decrease is not linear and is more drastic with *Salmonella* than with *L. monocytogenes*, probably due to attraction of the ions by the negatively charged outer membrane of the Gram negatives, which correlates with the higher susceptibility of *Salmonella* to silver ions. The high remnant of FSI when in contact with a relatively high proportion of bacteria signals a very low silver-bacterial ratio of chemical interaction, which again evinces that the amount of silver ions necessary to exert a bactericidal effect is extremely low. Hwang, Katayama & Ohgaki, 2007 reached the same conclusions by separating the bacterial bonded fraction of silver by filtration.

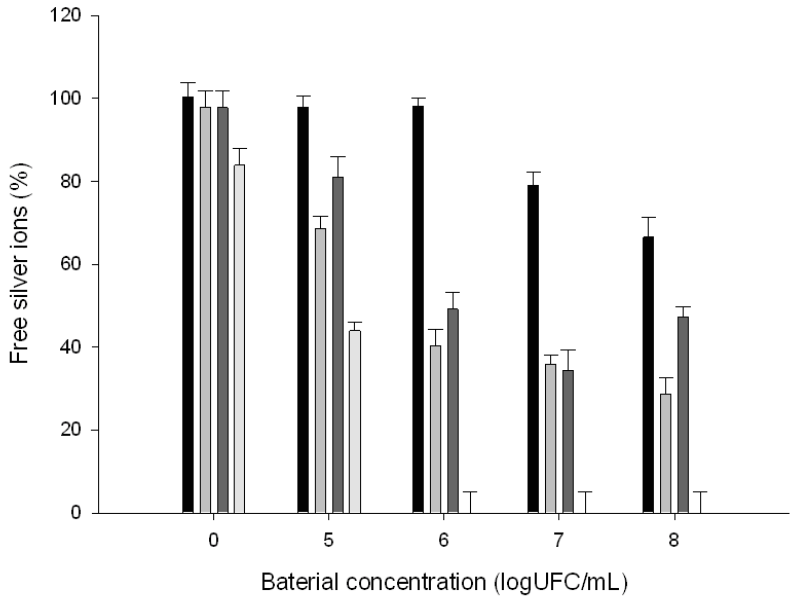

Figure 2. Free silver ions (FSI) detected after incubation of silver with aqueous bacterial suspensions of *Listeria monocytogenes* before ■ and after □ centrifugation at 1,000 g for 20 min and *Salmonella enterica* before ■ and after the same centrifugation treatment □ (n=2).

In order to investigate the nature of silver complexes formed with the different selected media, and ascertain if FSI in TSB are actually bonded as suggested above, M9 and TSB samples incubated for 24 h with silver were centrifuged for 1, 10, 30 and 60 min at 1,000*g* and 10,000*g* and the supernatant was checked for FSI. In aqueous samples measured as a control, silver was in form of FSI throughout all the centrifugation process (Fig. 3). M9-Met samples gave values (0-0.8%) close to the detection threshold under the experimental conditions and were therefore not considered. In TSB samples, the amount of FSI, which did not change with the incubation time, decreased gradually upon centrifugation (Fig. 3). At 1,000*g*, 30 min were needed to halve the concentration of FSI, while only about 1 min was needed to achieve the same effect at 10,000*g* (Fig. 3). This treatment of 10,000*g* for 60 min was enough to eliminate detectable silver from the supernatant (<0.1%). Pellets observed for samples with silver were dark-brownish whereas TSB samples without silver exhibited light-yellowish pellets.

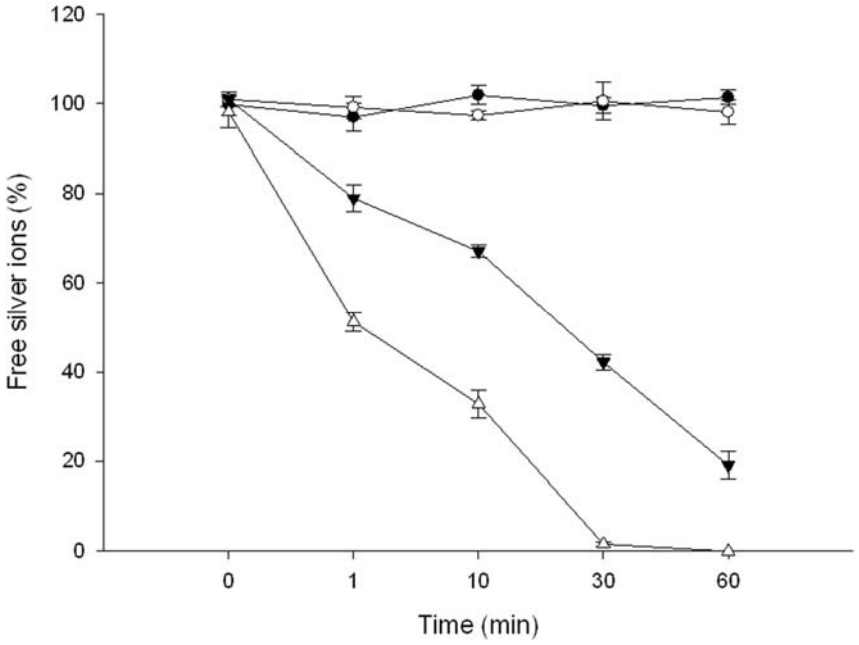

Figure 3. Free silver ions detected after centrifugation at 1,000*g* (black) and 10,000*g* (white) in ultrapure water (circles) and TSB (triangles). Experiments were performed in duplicate.

These results corroborate that most silver ions are not free in solution but bond to some constituent(s) in the TSB and evidence that the complex formed can be precipitated by centrifugation. As thiol groups were shown to drastically decrease FSI concentration even in very low quantities (Table 2), evidence suggests that silver may not be bonded to these groups when in contact with TSB. Instead, some other constituent(s) in the growth medium is/are likely to be bonded to silver. Accordingly, thiol groups may not be responsible for the decrease in availability or antimicrobial efficacy of silver in environments with natural organic matter. In agreement with these findings, Glover, Sharma & Wood, 2005 suggested in a study on the crustacean *Daphnia magna,* that compounds in the UV absorption range of about 300 nm (probably aromatic) may confer protection against silver toxicity.

The supernatant of samples centrifuged at 10,000*g* for different times was also used to correlate voltammetric results with susceptibility assays. The test tubes were incubated at 37 ºC with *S. enterica* for 24 h and bacterial viability in samples centrifuged at different times was compared to non-centrifuged controls. Centrifugation of TSB samples with 50 µg/mL silver for up to 20 min did not result in a decrease in antibacterial activity as compared to uncentrifuged controls (<10 CFU/mL). In the supernatant of samples inoculated after 60 min centrifugation, however, bacteria were able to proliferate, reaching the same number of viables as controls without silver (Table 1). These results are in agreement with voltammetric measurements. Furthermore, they suggest that the silver-TSB complexes formed, are, to some extent, also responsible for the decreased antibacterial efficacy of silver in TSB. It is therefore most likely that the signal detected voltammetrically comes from the silver-TSB complex, which may be broken during the reduction step in the deposition process, as suggested above.

A final voltammetric assay was subsequently carried out in TSB with *L. monocytogenes* to compare the affinity of FSI for the selected media or for bacterial constituents. Samples incubated with 100 µg/mL of silver and

increasing concentrations of bacteria were centrifuged at 1,000*g* for 10 min and at 10,000*g* for 30 min. In TSB, FSI concentrations for both RCF gave similar values as samples centrifuged under the same conditions but without bacteria, 75-82% and 23-25% of FSI after centrifugation at 1,000*g* and 10,000*g*, respectively. The addition of logarithmic increases of bacterial load to the solution did not produce a decrease in FSI. This proves FSI have more affinity for TSB constituents than for bacteria. Accordingly, when a high centrifugation rate was selected, TSB constituents bond to silver ions were deposited and the signal decreased, regardless of the presence or absence of bacteria as shown in Fig. 3. This suggests silver binding to these constituents may be responsible for the loss of antibacterial efficacy in TSB and the need of greater silver concentrations to achieve antimicrobial effect in complex media like LB, MHB or TSB (Hamilton-Miller & Shah, 1996; Nomiya et al., 2004; Ruparelia, Chatterjee, Duttagupta & Mukherji, 2008; Sondi & Salopek-Sondi, 2004).

4. Concluding Remarks

Although antibacterial silver has been used for centuries and is nowadays present in many aspects of our daily life, understanding the full potential of its outstanding properties is still standing. Huge differences in its antibacterial response advise the need for standardization of the biocidal tests, which is, however, difficult due to complexity of bioavailability and speciation issues. FSI seem to be the only active silver species with antimicrobial performance, exerting a bactericidal effect on *L. monocytogenes and S. enterica* even at the nM level. However, their stability is easily compromised in the presence of many ligands which may be part of any environment of action, such as sulphur groups, chloride ions or other organic substances. This work approaches the mechanisms in which these various aspects alter the antimicrobial effect of silver, stressing the importance of thoroughly investigating the environment where silver species are going to be released before designing any silver-based antimicrobial system.

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6. References

Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Science and Emerging Technologies*, *3*(2), 113-126.

Bielmyer, G. K., Brix, K. V., & Grosell, M. (2008). Is Cl- protection against silver toxicity due to chemical speciation? *Aquatic Toxicology*, *87*(2), 81-87.

Bjarnsholt, T., Kirketerp-Møller, K., Kristiansen, S., Phipps, R., Nielsen, A. K., Jensen, P. Ø., Høiby, N., & Givskov, M. (2007). Silver against Pseudomonas aeruginosa biofilms. *APMIS*, *115*(8), 921-928.

Bosetti, M., Massè, A., Tobin, E., & Cannas, M. (2002). Silver coated materials for external fixation devices: In vitro biocompatibility and genotoxicity. *Biomaterials*, *23*(3), 887-892.

Busolo, M. A., Fernandez, P., Ocio, M. J., & Lagaron, J. M. (2010). Novel silver-based nanoclay as an antimicrobial in polylactic acid food packaging coatings. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *27*(11), 1617-1626.

Coleman, N. J. (2009). Aspects of the in vitro bioactivity and antimicrobial properties of Ag^{+}-and Zn^{2+}-exchanged 11 Å tobermorites. *Journal of Materials Science: Materials in Medicine*, *20*(6), 1347-1355.

Cowan, M. M., Abshire, K. Z., Houk, S. L., & Evans, S. M. (2003). Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel. *Journal of Industrial Microbiology and Biotechnology*, *30*(2), 102-106.

Chen, X., & Schluesener, H. J. (2008). Nanosilver: A nanoproduct in medical application. *Toxicology Letters*, *176*(1), 1-12.

Choi, O., Clevenger, T. E., Deng, B., Surampalli, R. Y., Ross Jr, L., & Hu, Z. (2009). Role of sulfide and ligand strength in controlling nanosilver toxicity. *Water Research*, *43*(7), 1879-1886.

Chopra, I. (2007). The increasing use of silver-based products as antimicrobial agents: A useful development or a cause for concern? *Journal of Antimicrobial Chemotherapy*, *59*(4), 587-590.

Dibrov, P., Dzioba, J., Gosink, K. K., & Häse, C. C. (2002). Chemiosmotic mechanism of antimicrobial activity of Ag+ in Vibrio cholerae. *Antimicrobial Agents and Chemotherapy*, *46*(8), 2668-2670.

Feng, Q. L., Wu, J., Chen, G. Q., Cui, F. Z., Kim, T. N., & Kim, J. O. (2000). A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. *Journal of Biomedical Materials Research*, *52*(4), 662-668.

Galeano, B., Korff, E., & Nicholson, W. L. (2003). Inactivation of vegetative cells, but not spores, of Bacillus anthracis, B. cereus, and B. subtilis on Stainless steel surfaces

coated with an antimicrobial silver- and zinc-containing zeolite formulation. *Applied and Environmental Microbiology*, *69*(7), 4329-4331.

Glover, C. N., Sharma, S. K., & Wood, C. M. (2005). Heterogeneity in physicochemical properties explains differences in silver toxicity amelioration by natural organic matter to Daphnia magna. *Environmental Toxicology and Chemistry*, *24*(11), 2941-2947.

Gupta, A., & Silver, S. (1998). Silver as a biocide: Will resistance become a problem? *Nature Biotechnology*, *16*(10), 888.

Hamilton-Miller, J. M. T., & Shah, S. (1996). A microbiological assessment of silver fusidate, a novel topical antimicrobial agent. *International Journal of Antimicrobial Agents*, *7*(2), 97-99.

Hwang, M. G., Katayama, H., & Ohgaki, S. (2007). Inactivation of Legionella pneumophila and Pseudomonas aeruginosa: Evaluation of the bactericidal ability of silver cations. *Water Research*, *41*(18), 4097-4104.

Joyce-Wöhrmann, R. M., & Münstedt, H. (1999). Determination of the silver ion release from polyurethanes enriched with silver. *Infection*, *27*(SUPPL. 1).
Jung, R., Kim, Y., Kim, H. S., & Jin, H. J. (2009). Antimicrobial properties of hydrated cellulose membranes with silver nanoparticles. *Journal of Biomaterials Science, Polymer Edition*, *20*(3), 311-324.

Junghanns, J. U. A. H., & Müller, R. H. (2008). Nanocrystal technology, drug delivery and clinical applications. *International journal of nanomedicine*, *3*(3), 295-309.

Kim, T. N., Feng, Q. L., Kim, J. O., Wu, J., Wang, H., Chen, G. C., & Cui, F. Z. (1998). Antimicrobial effects of metal ions (Ag+, Cu2+, Zn2+) in hydroxyapatite. *Journal of Materials Science: Materials in Medicine*, *9*(3), 129-134.

Klasen, H. J. (2000). Historical review of the use of silver in the treatment of burns. I. Early uses. *Burns*, *26*(2), 117-130.

Kolts, J. M., Boese, C. J., & Meyer, J. S. (2006). Acute toxicity of copper and silver to Ceriodaphnia dubia in the presence of food. *Environmental Toxicology and Chemistry*, *25*(7), 1831-1835.

Lansdown, A. B. G. (2006). Silver in health care: Antimicrobial effects and safety in use. *Current Problems in Dermatology*, *33*, 17-34.

Lee, D. Y., Fortin, C., & Campbell, P. G. C. (2005). Contrasting effects of chloride on the toxicity of silver to two green algae, Pseudokirchneriella subcapitata and Chlamydomonas reinhardtii. *Aquatic Toxicology*, *75*(2), 127-135.

Lee, S. (2009). Multifunctionality of layered fabric systems based on electrospun polyurethane/zinc oxide nanocomposite fibers. *Journal of Applied Polymer Science*, *114*(6), 3652-3658.
Li, Q., Mahendra, S., Lyon, D. Y., Brunet, L., Liga, M. V., Li, D., & Alvarez, P. J. J. (2008). Antimicrobial nanomaterials for water disinfection and microbial control: Potential applications and implications. *Water Research*, *42*(18), 4591-4602.

Liau, S. Y., Read, D. C., Pugh, W. J., Furr, J. R., & Russell, A. D. (1997). Interaction of silver nitrate with readily identifiable groups: Relationship to the antibacterial action of silver ions. *Letters in Applied Microbiology*, *25*(4), 279-283.

Malachová, K., Praus, P., Pavlíčková, Z., & Turicová, M. (2009). Activity of antibacterial compounds immobilised on montmorillonite. *Applied Clay Science*, *43*(3-4), 364-368.

Martínez-Abad, A., Lagaron, J. M., & Ocio, M. J. (2012). Development and characterization of silver-based antimicrobial ethylene-vinyl alcohol copolymer (EVOH) films for food-packaging applications. *Journal of Agricultural and Food Chemistry*, *60*(21), 5350-5359.

Martínez-Abad, A., Sanchez-Moragas, G., Lagaron, J. M., & Ocio, M. J. On the different growth conditions affecting silver antimicrobial efficacy on Listeria monocytogenes and *Salmonella* enterica. *International Journal of Food Microbiology*(0).

Matsumura, Y., Yoshikata, K., Kunisaki, S. I., & Tsuchido, T. (2003). Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Applied and Environmental Microbiology*, *69*(7), 4278-4281.

Nakane, T., Gomyo, H., Sasaki, I., Kimoto, Y., Hanzawa, N., Teshima, Y., & Namba, T. (2006). New antiaxillary odour deodorant made with antimicrobial Ag-zeolite (silver-exchanged zeolite). *International Journal of Cosmetic Science*, *28*(4), 299-309.

Nichols, J. W., Brown, S., Wood, C. M., Walsh, P. J., & Playle, R. C. (2006). Influence of salinity and organic matter on silver accumulation in Gulf toadfish (Opsanus beta). *Aquatic Toxicology*, *78*(3), 253-261.

Nomiya, K., Yoshizawa, A., Tsukagoshi, K., Kasuga, N. C., Hirakawa, S., & Watanabe, J. (2004). Synthesis and structural characterization of silver(I), aluminium(III) and cobalt(II) complexes with 4-isopropyltropolone (hinokitiol) showing noteworthy biological activities. Action of silver(I)-oxygen bonding complexes on the antimicrobial activities. *Journal of Inorganic Biochemistry*, *98*(1), 46-60.

Quintavalla, S., & Vicini, L. (2002). Antimicrobial food packaging in meat industry. *Meat Science*, *62*(3), 373-380.

Rai, M., Yadav, A., & Gade, A. (2009). Silver nanoparticles as a new generation of antimicrobials. *Biotechnology Advances*, *27*(1), 76-83.

Ruparelia, J. P., Chatterjee, A. K., Duttagupta, S. P., & Mukherji, S. (2008). Strain specificity in antimicrobial activity of silver and copper nanoparticles. *Acta Biomaterialia*, *4*(3), 707-716.

Russell, A. D., & Hugo, W. B. (1994). Antimicrobial activity and action of silver. *Progress in medicinal chemistry*, *31*, 351-370.

Semeykina, A. L., & Skulachev, V. P. (1990). Submicromolar Ag+ increases passive Na+ permeability and inhibits the respiration-supported formation of Na+ gradient in Bacillus FTU vesicles. *FEBS Letters*, *269*(1), 69-72.

Sondi, I., & Salopek-Sondi, B. (2004). Silver nanoparticles as antimicrobial agent: A case study on E. coli as a model for Gram-negative bacteria. *Journal of Colloid and Interface Science*, *275*(1), 177-182.

Texter, J., Ziemer, P., Rhoades, S., & Clemans, D. (2007). Bactericidal silver ion delivery into hydrophobic coatings with surfactants. *Journal of Industrial Microbiology and Biotechnology*, *34*(8), 571-575.

Thomas, S., & McCubbin, P. (2003). A comparison of the antimicrobial effects of four silver-containing dressings on three organisms. *Journal of wound care*, *12*(3), 101-107.
Thomas, V., Yallapu, M. M., Sreedhar, B., & Bajpai, S. K. (2007). A versatile strategy to fabricate hydrogel-silver nanocomposites and investigation of their antimicrobial activity. *Journal of Colloid and Interface Science*, *315*(1), 389-395.

Tsai, H. N., & Hodgson, D. A. (2003). Development of a Synthetic Minimal Medium for Listeria monocytogenes. *Applied and Environmental Microbiology*, *69*(11), 6943-6945.

Valero, M., & Francés, E. (2006). Synergistic bactericidal effect of carvacrol, cinnamaldehyde or thymol and refrigeration to inhibit Bacillus cereus in carrot broth. *Food Microbiology*, *23*(1), 68-73.

VanGenderen, E. J., Ryan, A. C., Tomasso, J. R., & Klaine, S. J. (2003). Influence of dissolved organic matter source on silver toxicity to Pimephales promelas. *Environmental Toxicology and Chemistry*, *22*(11), 2746-2751.

Ward, T. J., & Kramer, J. R. (2002). Silver speciation during chronic toxicity tests with the mysid, Americamysis bahia. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *133*(1-2), 75-86.

Williams, R. L., Doherty, P. J., Vince, D. G., Grashoff, G. J., & Williams, D. F. (1989). The biocompatibility of silver. Critical Reviews in Biocompatibility, 5(3), 205+221-243.

Chapter II

ON THE DIFFERENT GROWTH CONDITIONS AFFECTING SILVER ANTIMICROBIAL EFFICACY ON *LISTERIA MONOCYTOGENES* AND *SALMONELLA ENTERICA*

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ABSTRACT

Silver is known to inhibit microorganisms and therefore it is an ideal candidate for its incorporation in a wide variety of materials for food applications. However, there is still a need for understanding how silver prolonged exposure to bacterial contamination affects the bioavailability of the active silver species. In the present study, growth curves of Listeria monocytogenes and *Salmonella* enterica were performed for 3-5 days in Tryptic Soy Broth (TSB) and M9 minimal medium (M9) in the presence of silver ions and silver solutions previously in contact with the growth media. The cultivability of the bacteria under these conditions was correlated with the viability of the bacterial populations as measured by flow cytometry analysis (FC) using a LIVE/DEAD BacLight kit. It was found that, after a period where viable counts were not detected, bacterial populations recovered and were able to proliferate in most cases. The resuscitation of the cultures was explained by both the existence of a resilient fraction of bacteria in a compromised state and the parallel inactivation of the silver species. This inactivation was found to be highly influenced by time dependant chemical reactions taking place in the environment of exposure, producing differences of at least 3 fold between results for nutrient rich environments and results for limiting environments. This study points out the need for understanding these chemical interactions and bacterial mechanisms of adaptation and may have relevance in the design of silver-based antimicrobial systems for food-related applications.

Keywords: silver antimicrobial activity, microbial growth, food-borne pathogens

1. Introduction

In the last decade, the demand for minimally processed, easily prepared and ready-to-eat 'fresh' food products has globally increased which has encouraged the industry to the development of new technologies as an alternative to food-thermal technologies. These new alternative technologies such as lower thermal or high pressure treatments may in some instances allow pathogenic bacterial growth (Valero and Francés, 2006). However, even if foodborne pathogens are totally eliminated by efficient thermal treatments, microbial recontamination of the food surface could take place during the processing steps, when the risk of cross-contamination is elevated. As a result, the risk of foodborne illnesses is increased. Therefore, new preservation techniques, such as incorporation of antibacterial substances into materials intended to come in contact with food products, is currently being investigated and applied.

The use of silver as antimicrobial for food-related applications has been recognised since silver pottery and cutlery where used in antiquity (Klasen, 2000). Although the mechanism of action still remains disputed, it is generally accepted that free silver ions (FSI), present or leaking from the materials in contact with the food matrix, are able to bind to membrane constituents, destabilizing the membrane potential and causing proton leakage (Liau et al., 1997; Matsumura et al., 2003). They also interfere with DNA replication and ion transport across the respiratory chain (Feng et al., 2000; Semeykina and Skulachev, 1990; Texter et al., 2007), all of which eventually lead to cell death. Due to this combination of unspecific mechanisms, silver ions are not likely to develop any resistances and are active against a very broad spectrum of bacteria, yeasts, fungi and even viruses in tiny concentrations, remaining nontoxic to human cells (Russell and Hugo, 1994).

Therefore, its use has become more and more popular in the past few years. Apart from the medical field, silver is nowadays incorporated as the key component to control microbial proliferation in a wide variety of materials used

in our daily life like textile clothing, coatings in home appliances and food related applications like water treatment units (Han et al., 2005) or a great variety of food-contact materials (Appendini and Hotchkiss, 2002; Bouwmeester et al., 2009; Simpson, 2003) (see Gupta and Silver, 1998; Li et al., 2008; Rai et al., 2009 for review). In most of these materials, the antimicrobial effect relies on the leaking of silver ions based on ion-exchange from mineral carriers, like montmorillonites, tobermorites and most predominantly zeolites (Busolo et al., 2010; Cowan et al., 2003; Galeano et al., 2003; Nakane et al., 2006). The versatility and cost-effectiveness of these materials have made silver the most widely used polymer additive for food applications (Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002).

However, despite its widespread use, few studies have been devoted to examine how interactions within chemically complex matrices may affect the antimicrobial efficacy of silver. Most studies focus on the release rates from different materials, using agar diffusion tests or short-time assays to test the antibacterial effect while few have performed growth curves in the long term. Additionally, it is remarkable to observe how much bactericidal concentration values differ among the various studies. Looking at the final concentrations achieved in solution that have been reported to exhibit antibacterial properties, these values go from the ppb range (Bjarnsholt et al., 2007; Hwang et al. 2007; Kim et al., 1998) to hundreds of ppm (Hamilton-Miller and Shah, 1996; Nomiya et al., 2004; Ruparelia et al., 2008; Sondi and Salopek-Sondi, 2004; Thomas et al., 2007) (4 orders of magnitude difference). This is probably due to the use of different bacterial species, incubation times and growth conditions. A standardization of silver ion biocidal tests is difficult, as many solubility issues affecting speciation and bioavailability of silver are still unknown.

The antibacterial mechanism of silver seems to imply different unspecific pathways, and thus probable sublethal damage (Junghanns and Müller, 2008). Therefore, in order to correctly assess antimicrobial efficacy, it is crucial to elucidate how prolonged exposure to silver influences bacterial growth rates, cultivability or viability, and how time-dependant chemical interactions found in

the complexity of food matrices could interfere with its bactericidal effect. In the present work, growth curves for two relevant foodborne pathogens, *Listeria monocytogenes* and *Salmonella enterica* were profiled in two different growth media during 3-5 days by traditional plate counting. These results were then correlated with the viability of the same cultures as measured by flow cytometry using the LIVE/DEAD BacLight viability kit. The purpose of this study was to investigate bacterial behaviour throughout long times of exposure to silver, as well as to demonstrate the crucial effects the different chemical environments of contamination within food applications could exert on these results.

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2. Materials and methods

2.1. Bacterial strains and preparation of inoculum

Listeria monocytogenes CECT 5672 and *Salmonella enterica* CECT 554 were obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain). These strains were stored in Phosphate Buffer Saline (PBS) with 10% Tryptone Soy Broth (TSB, Conda Laboratories, Madrid, Spain) and 10% glycerol at -80 ºC until needed. For experimental use, the stock cultures were maintained by monthly subculture to agar Tryptone Soy Agar (TSA) slants at 4 ºC. Previous to each study, a loopful of bacteria was transferred to 10 mL of TSB and incubated at 37 ºC overnight. A 100 μL aliquot from the overnight culture was again transferred to TSB and grown at 37 ºC to the mid-exponential phase of growth. This culture served as the inoculum for antimicrobial assays starting with approximately 10^4 CFU/mL for the time-course plating of growth curves and 10^6 CFU/mL for flow cytometry (FC) analysis. These CFU counts were accurately and reproducibly obtained by inoculation into 10mL growth medium of 0.1 mL of a culture having an absorbance value of 0.20 for S. enterica and 0.15 for L. monocytogenes as determined by optical density at 600 nm by ultraviolet visible (UV) spectroscopy (SP-2000 UV, Spectrum Instruments, Shanghai, China).

2.2. Silver preparations

Experimental tests were performed using different aqueous silver nitrate solutions (from now on referred to as silver) as the antimicrobial substance. The silver solutions were prepared daily from serial dilutions of silver nitrate powder (Sigma) in milli-Q grade water.

2.3. Growth conditions

L. monocytogenes and S. enterica were grown in TSB and M9 minimal medium (Sigma-Aldrich). TSB is a rich, non-selective growth medium consisting of a digest of casein and soy, which assures delivery of a heterogeneous mixture of many nutrients (including proteins and lipids) and could stand for a

hypothetically nutrient rich environment of contamination. M9 is a minimal medium made up of a mixture of salts and 20mM glucose as a sole carbon source, which would represent a more nutrient limited environment of contamination.

For the profiling of the growth curves, a bacterial suspension in mid-exponential phase was inoculated into 10 mL of each medium starting with an initial inoculum size of approximately 10^4 CFU/mL. After that, 10uL of an aqueous silver solution was subsequently added to the test tubes as to achieve the suitable silver concentration. Simultaneously, another set of tubes was incubated with the silver solutions for 72h at 37ºC prior to bacterial inoculation in order to study the influence of previous contact between the silver species and the growth media on biocidal activity. In all cases, the range of silver concentrations was set slightly above the observed threshold of antimicrobial activity for each medium, namely between 20 and 100 ppm for the TSB medium and between 0.1 and 0.3 ppm for M9 medium, as reported in previous studies (Martinez-Abad et al., 2013). The test tubes were subsequently incubated at 37ºC and samples were plated every 8 hours during 3 days except a final sample which was plated after 5 days incubation. For this purpose, cells suspensions were serially diluted in buffered peptone water (Scharlab, S.L, Barcelona, Spain) and 100 µL spread on TSA per duplicate. Colonies were counted after incubation at 37ºC for 24 h. Each of the experiments was performed in triplicate.

2.4. Viability assays using flow cytometry

Samples with and without silver were incubated in both TSB and M9 media for 24h at 37 ºC, stained with a viability kit (Baclight® viability kit, Invitrogen), incubated 15min in the dark and subsequently ran through flow cytometry (FC, FacsCantoII, BD, U.S.A.) until at least 5000 events within the population ascribed as “Live” (P1) were counted . Dead control samples were prepared by incubating live controls of both bacterial species in 70% isopropanol for 30min before staining.

3. Results

3.1. Growth curves in TSB

Growth curves of L. monocytogenes in TSB with silver concentrations of 20, 40 and 50 ppm are shown in Figure 1. Figure 1a shows results for samples where silver and bacteria were added simultaneously to the growth medium. In these cases, the lowest silver concentration (20 ppm) induces a short decrease in cultivable counts that increases up to a maximum again before 24h. The samples treated with 40 and 50 ppm of silver showed cell cultivability was below the detection limit (10 CFU/mL) up to 40 hours and 60 hours of incubation, respectively. From that time on, these samples were able to recover and reach up the maximum growth.

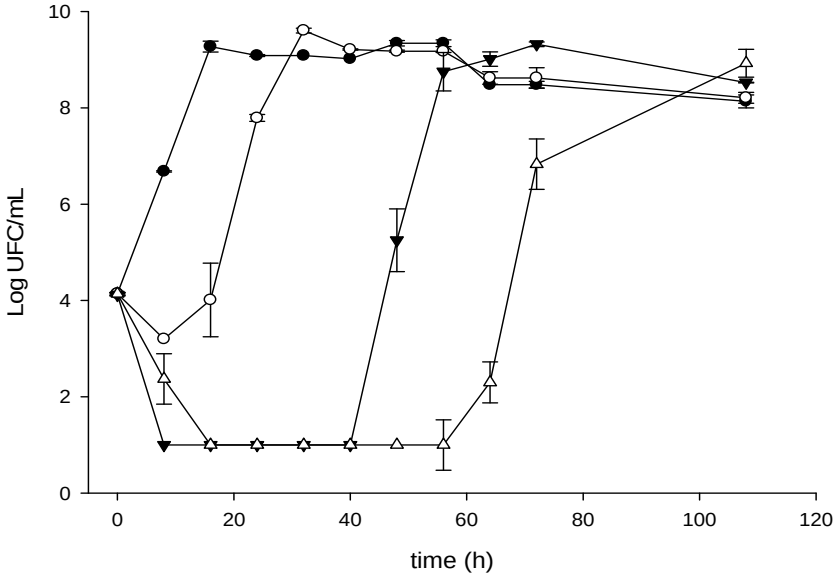

Figure 1a. Growth curves of *L. monocytogenes* at 37ºC without silver (●) and with 20 ppm (○), 40 ppm (▼) and 50ppm (Δ) silver content in TSB when the microorganisms and the silver solution are added simultaneously to the TSB (n=3).

The effect of the contact between silver and the growth medium previous to inoculation on the growth patterns of L. monocytogenes is shown in Figure 1b. When the biocide was added to the medium 72h before inoculation, all samples with silver concentrations of 20, 40 and 50 ppm grew with the same pattern as the control without silver, clearly indicating that silver antibacterial efficacy was lost.

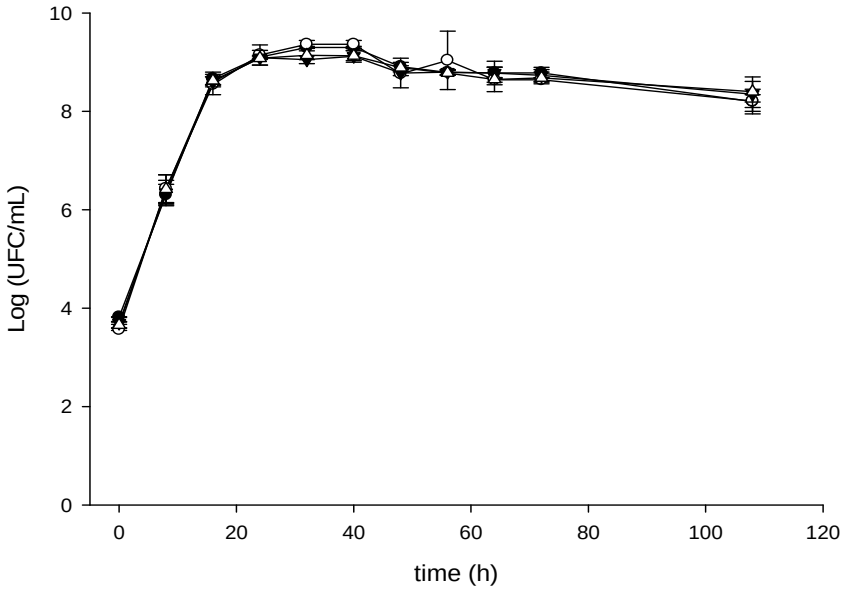

Figure 1b. Growth curves of *L. monocytogenes* at 37ºC without silver (●) and with 20 ppm (○), 40 ppm (▼) and 50ppm (Δ) silver content in TSB when the microorganisms are added after silver has been 72h in contact with the growth medium (n=3).

Growth curves with and without previous contact with TSB for S. enterica with silver concentrations of 20, 50 and 100 ppm are presented in Figure 2a and 2b. In the cases where silver and the bacteria were inoculated at the same time (Figure 2a), growth curves for 20 ppm exhibit the same phenomenon of initial absence of viables counts within 24h and subsequent recovery, reaching the same viable counts as the control (about 9 log) after 48h incubation. Curves for

samples with 50 and 100 ppm showed a similar delay in the growth as 20 ppm samples, but reached considerably lower growth rates and lower maximum values (about 5 log) after 5 days incubation.

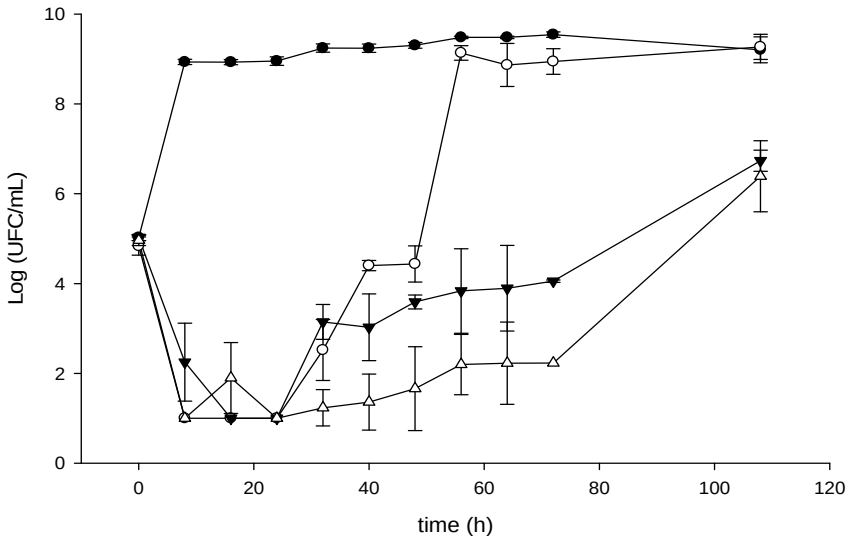

Figure 2a. Growth curves of *Salmonella enterica* at 37ºC without silver (●) and with 20 ppm (○), 50 ppm (▲) and 100ppm (Δ) silver content when the microorganisms and the silver solution are added simultaneously to the TSB (n=3).

When the silver was added to the medium 72h before inoculation (Figure 2b), 20 ppm samples showed no noticeable antibacterial properties, growing as control samples similar to the case of L. monocytogenes. The growth curves with 50 and 100 ppm silver showed similar patterns as the ones without previous contact, reaching about 10^5 CFU/mL after 5 days.

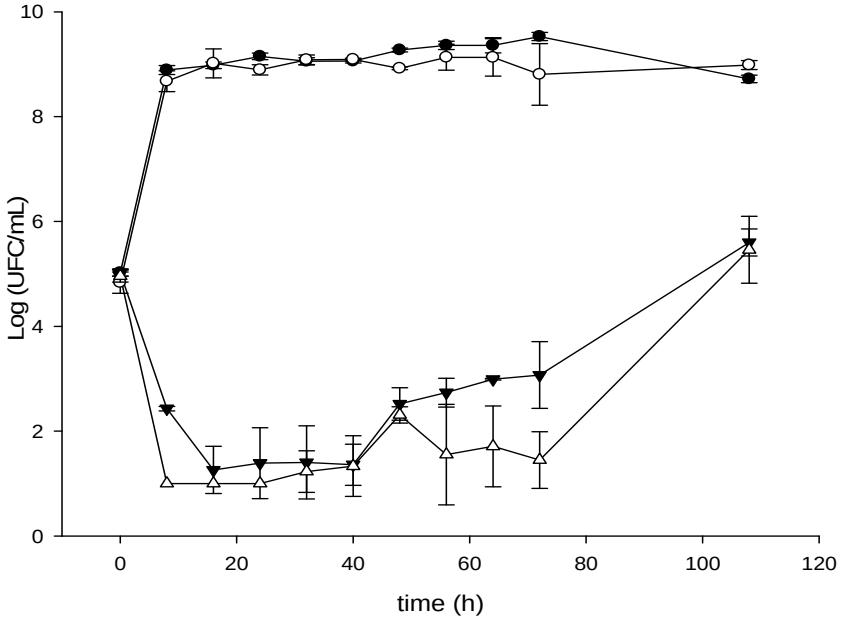

Figure 2b. Growth curves of *Salmonella enterica* at 37ºC without silver (●) and with 20 ppm (○), 50 ppm (▲) and 100ppm (Δ) silver content when the microorganisms are added after silver has been 72 h in contact with the growth medium (n=3)

3.2. Growth curves in M9 minimal medium

Strong differences in growth patterns were observed between L. monocytogenes and S. enterica when M9 minimal medium was selected as example of a hypothetical nutrient limited environment of contamination. In this medium, S. enterica grew well, reaching similar maximum growth values as when grown in TSB after 24h incubation. However L. monocytogenes, only increased their numbers up to 10^6-10^7 CFU/mL within the same time (Figure 3). Preliminary assays carried out in our lab set the threshold range of antibacterial activity in this medium between 0.01 and 0.1 ppm and bactericidal concentrations between 0.1 and 0.5 ppm (data not shown). Consequently, concentrations were set to a range where differences in efficacy might be best noticed, namely 0.1 and 0.3 ppm (Figure 3). In samples with silver, initial absence of cultivable bacteria and subsequent recovery of the cultures was noted again in both bacteria (Figure 3). Plate counts of L. monocytogenes were initially reduced to less than 10 CFU/mL

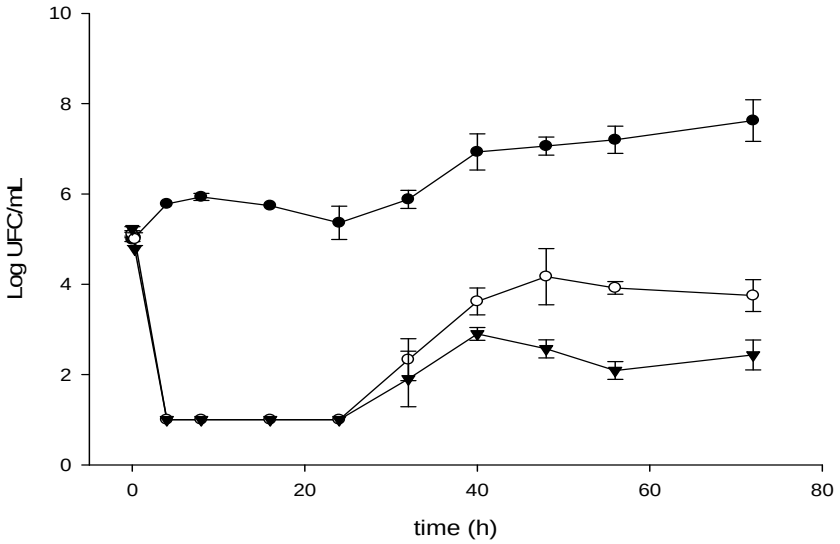

Figure 3a. Growth curves of *L. monocytogenes* at 37ºC without silver (●) and with 0.1 ppm (○) and 0.3 ppm (▼) silver content in M9 minimal medium when a) the microorganisms and the silver solution are added simultaneously to the M9 (n=3).

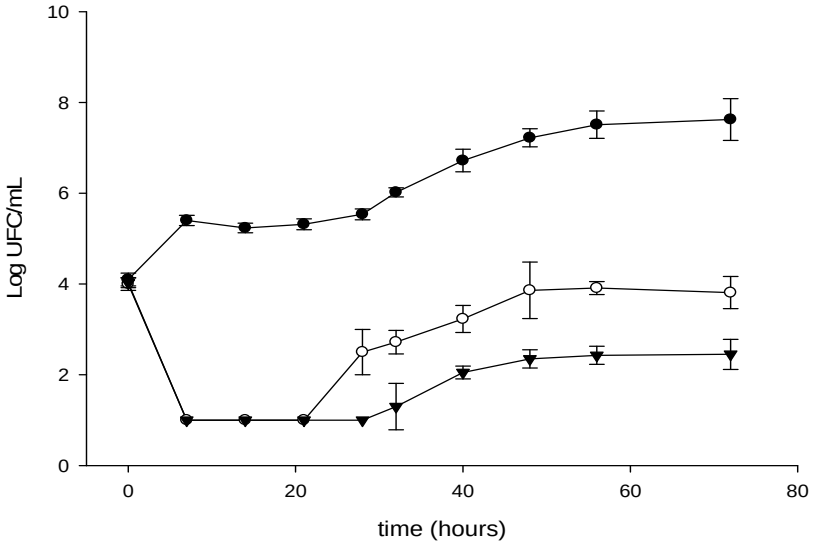

Figure 3b. Growth curves of L. monocytogenes without silver (●) and with 0.1 ppm (○) and 0.3 ppm (▼) silver content in M9 minimal medium when the microorganisms are added after silver has been 72 h in contact with the growth medium (n=3)

with 0.1 ppm silver and reach up to similar counts as the initial inoculum size after 5 days incubation (Figure 3a). Growth curves with 0.3 ppm silver developed a similar pattern with a lower maximum growth value of approximately 100 CFU/mL.

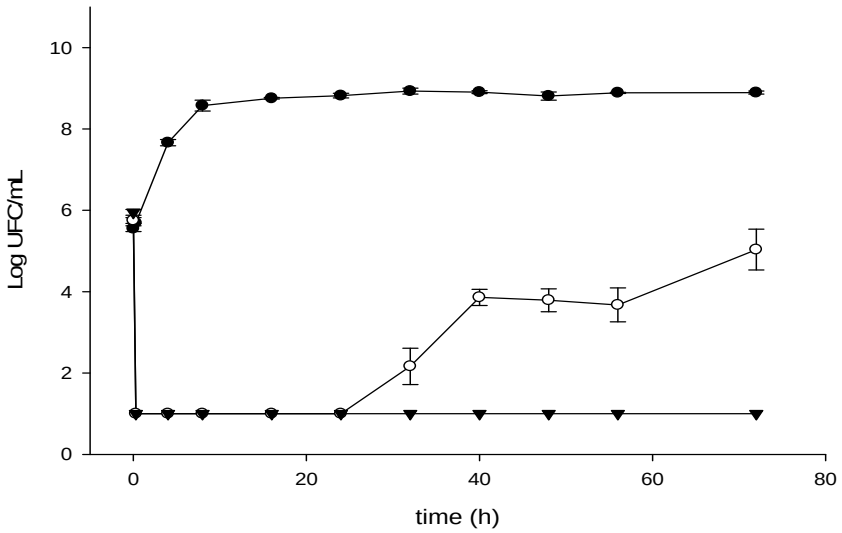

Figure 4a. Growth curves of *S. enterica* without silver at 37ºC (●) and with 0.1 ppm (○) and 0.3 ppm (▲) silver content in M9 minimal medium when the microorganisms and the silver solution are added simultaneously to the M9 (n=3).

Analogous results were reported when silver was 72h in contact with the medium previous to inoculation (Figure 3b). Cultures were initially not able to produce any CFU until at least 20h incubation. From that time on, the same tendency to recovery was noted for both samples with silver.

S. enterica manifested slightly more susceptibility to silver than L. monocytogenes showing no sign of cultivable counts with 0.3 ppm silver during the whole experiment regardless if the silver compound was in contact or not with the growth medium previous to inoculation (Figure 4). For 0.1 ppm silver, *Salmonella* cultures recovered after 24h and reach up to approximately 10^5

CFU/mL after 72h (Figure 4a). As with L. monocytogenes, previous incubation of the medium with the biocide did not result in a considerable difference in antimicrobial efficacy (Figure 4b).

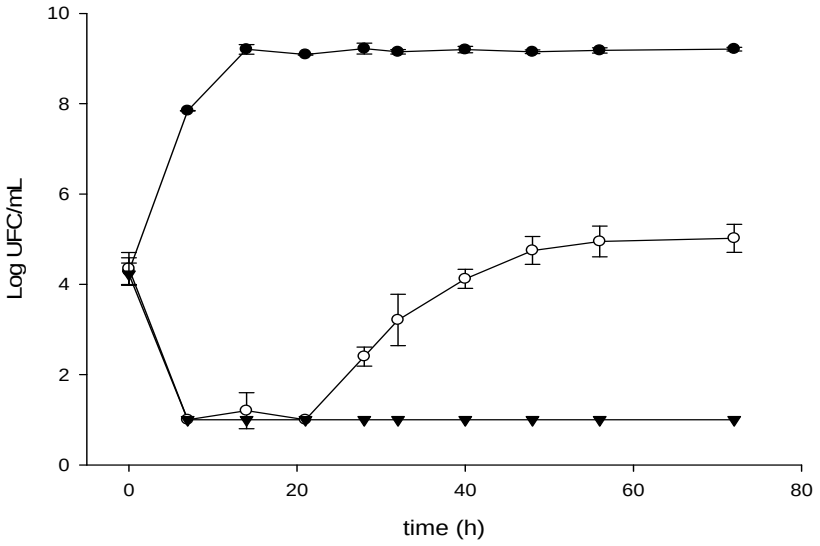

Figure 4b. Growth curves of *S. enterica* at 37ºC without silver (●) and with 0.1 ppm (○) and 0.3 ppm (▲) silver content in M9 minimal medium when the microorganisms are added after silver has been 72 h in contact with the growth medium (n=3).

3.3. Viability study using flow cytometry

To compare the antibacterial activity and as a means to evaluate and quantify the presence of compromised cells not detectable by traditional plate counts methods, bacterial viability was further determined by using flow cytometry after double staining of the nucleic acids of bacteria based on the permeability of the fluorescent dyes, Syto 9 and propidium iodide. Figure 5 shows typical dual-parameter dot plots of the fluorescence intensities of both dyes when populations of L. monocytogenes (Figure 5A) and S. enterica (Figure 5B) were exposed to 0, 20 and 50 ppm and 0, 20 and 100 ppm silver, respectively, in the medium TSB, together with the dead controls as treated with isopropanol. Based on the

differential staining characteristics with PI and Syto9 of live and dead controls, a line divides the plots into a "dead region" (red dots), and a "live region" (green dots). Following the staining patterns of the controls without silver, a "live" subpopulation (P1) was gated inside the live region. Within the dead region, another subpopulation in samples with silver identified as lysed (P3) denotes

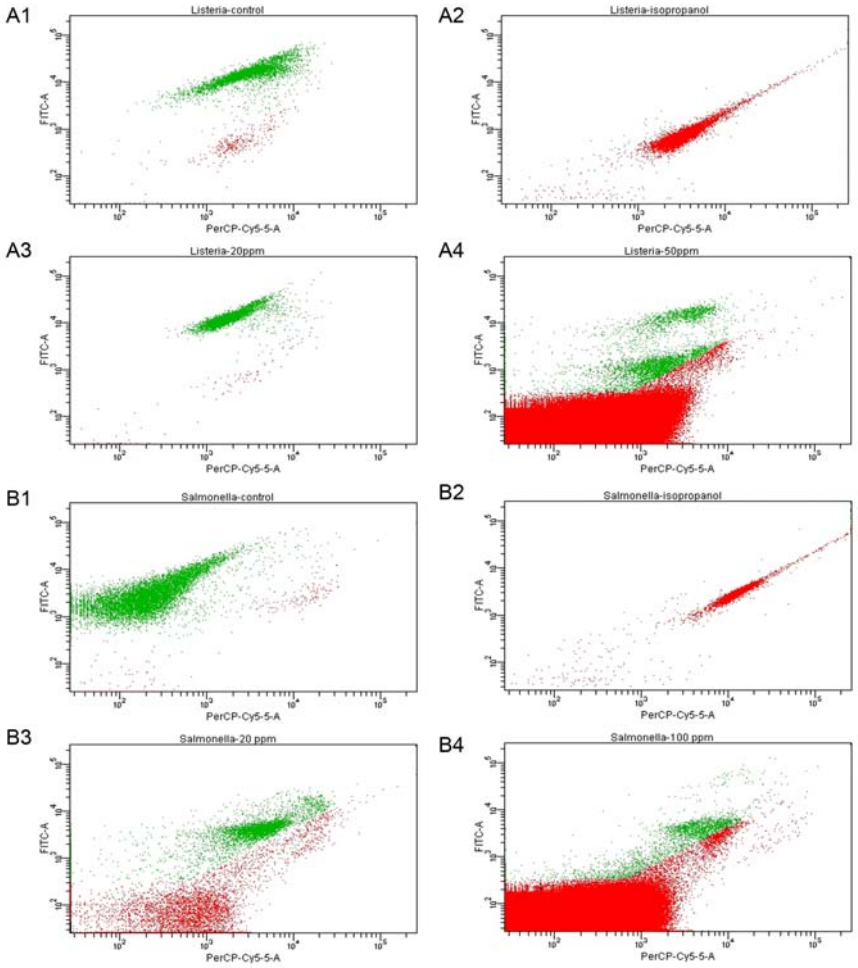

Figure 5. FC dual parameter dot plots of green and red fluorescence of bacterial populations of *L. monocytogenes* (A) and *S. enterica* (B) with 0 (A1), 20 (A3) and 50ppm (A4) and 0 (B1), 20 (B3) and 100 ppm (B4) silver, respectively, in the medium TSB, together with the dead controls as treated with isopropanol (A2, B2).

cells with similar side scatter intensities as the controls (data not shown) but with far less or completely void of fluorescence, suggesting loss of nucleic acids from lysed cells. The absence of fluorescence was confirmed by fluorescence microscopy (data not shown). In some samples treated with silver, a third population which falls between both the green and the red region was considered (P2), which would consist mainly of dead or heavily compromised bacteria. The amount of live (P1), dead/compromised (P2) and lysed (P3) cells of L. monocytogenes and S. enterica in samples incubated in TSB and M9 for 24h with different silver concentrations is listed in Table 1.

Table 1a. Viability of Listeria monocytogenes under different growth conditions as measured by flow cytometry analysis with a Baclight viability kit. P1, P2 and P3 correspond to live, dead/compromised or lysed counts as defined in the results.

Growth medium	Silver (ppm)	Viability (%)		
		P1	P2	P3
TSB	0	91.80	7.72	0.48
	20	98.25	1.10	0.65
	40	0.88	5.22	94.00
	50	0.14	6.85	93.04
M9	0	43.95	8.32	46.73
	0.1	3.64	25.45	70.95
	0.3	3.10	36.88	60.02

L. monocytogenes cultures in TSB without and with 20 ppm silver consisted mostly (91.8% and 98.3%, respectively) of bacteria ascribed as live. This is in accordance with results for cultivability at the same time-point in the growth curves. The slightly higher amount of dead bacteria in the control was probably due to longer aging of the stationary culture. Samples with 40 and 50 ppm silver consisted mostly of lysed bacteria (93-94%) with 0.88% and 0.14% in P1, respectively, which represents a reduction in viables of 2-3 log, leaving a total of 1000-10000 viables/mL. In M9, the inability of L. monocytogenes cultures to proliferate properly was reflected in a relatively low P1 value (43.95%). When

0.1-0.3 ppm silver were added to the medium, the number of live cells was reduced to 3-4%, in favour of a strong increase in both P2 and P3.

Table 1b. Viability of *Salmonella enterica* under different growth conditions as measured by flow cytometry analysis with a Baclight viability kit. P1, P2 and P3 correspond to live, dead/compromised or lysed counts as defined in the results.

Growth medium	Silver (ppm)	Viability (%)		
		P1	P2	P3
TSB	0	96.80	2.55	1.34
	20	2.54	25.26	72.20
	50	0.31	8.44	91.25
	100	0.09	5.95	93.97
M9	0	96.45	2.02	1.54
	0.1	0.92	24.95	74.13
	0.3	0.59	44.05	55.36

When S. enterica was incubated in TSB or M9 medium, controls were able to proliferate, consisting of 96-97% live cells. The addition of 50 ppm and 100 ppm silver in TSB produced the lysis of approximately 70% and 94% of the cells, leaving a live fraction of 3.54% and 0.09%, respectively. In M9, the addition of small amounts of the antimicrobial compound decreases the number of viable cells to 0.5-1%, which represents a decrease of about 2 log or 10^4 viables/mL.

4. Discussion

4.1. Viability vs cultivability

Although antibacterial activity of silver has been used for centuries and is nowadays present in many aspects of our daily life, understanding the full potential of its outstanding properties is still standing. In most studies dealing with newly emerged silver-based materials, attention is focused on ensuring the sustained release of silver ions or nanoparticles for an extended period, for example immersing the material for months or repeatedly washing it (Bedi et al., 2012; Dammet al., 2008; Guggenbichler et al., 1999; Lee et al., 2005). However, the final antimicrobial assays proving effectiveness are normally performed from few minutes to 24h incubation of the bacteria in contact with the environment containing silver (Bjarnsholt et al., 2007; Damm et al., 2008; De Muynck et al., 2010; Guggenbichler et al., 1999; Hamilton-Miller and Shan, 1996; Hwang et al., 2007; Kim et al., 1998; Lee et al., 2009; Nomiya et al., 2004; Rodríguez-Argüelles et al., 2011; Ruparelia et al., 2008; Shrivastava et al., 2007; Sondi and Salopek-Sondi, 2004; Thomas et al., 2007). These incubation times may be too short if we consider future applications in food–contact materials, where a chemically complex matrix may be constantly in contact with it. The results in the present manuscript evidence that bacteria, though very sensitive to small concentrations of silver, can actually recover and proliferate in an environment where silver is still present and even if no viable counts are detected for relatively long times. This observation puts in question previously published results based on short-term assays, stressing the importance of thoroughly investigating the conditions in which the silver species are going to be released before designing any silver-based antimicrobial system. One explanation for the initial absence of viable counts and subsequent recovery could be the survival of residual bacteria below the detection limit (<10 CFU/mL) which were able to proliferate. However, this alone cannot explain lag phases as long as 65 hours. As the antibacterial mechanism suggested generally relies primarily on unspecific binding to bacterial constituents (e.g. membrane

proteins) silver may cause in minute concentrations a sublethal damage from which bacteria would recover with time. Therefore, the presence of a certain degree of stressed or damaged bacteria should be also taken into consideration. One of the most widely accepted methods of assessing bacterial viability is through membrane integrity (Amor et al., 2002). Nucleic acid staining in FC analysis has shown to be a useful tool in identifying the presence of viable but not cultivable bacteria and differentiate compromised populations or lysed cells based on the intensities of both green and red fluorescence of viability kits (Banerjee et al., 2010; Berney et al., 2006; Muñoz et al., 2009). Based on viability results by FC, evidence is put forth of the existence of a resilient fraction of bacteria which retains similar viability as controls without silver. When examining the great differences between cultivability and viability (2-4 log depending on the sample), the presence of silver throughout the process of sampling must be taken into consideration. As silver might still be present after samples are transferred to agar plates, bacteria could still be division compromised and thus non-culturable. This fraction of live bacteria is presumed to be responsible for the resuscitation of the cultures after an increased lag phase of adaptation in the environment containing silver. Bacteria which do not sustain the damage are mostly lysed within 24h, leaving a small fraction in a dead or heavily compromised state (P2). It is feasible, however, that some fraction of this population could be able to resuscitate as well. The existence of differences between cultivability and viability with silver as antimicrobial has already been reported previously (Hwang et al., 2006; Percival et al., 2011; Woo et al., 2008).

The impact of silver on the tested bacteria is reflected on the growth patterns, mostly by an increase in lag phase, related to silver concentration, and a decrease in growth values. Few studies cover the growth patterns of food-borne pathogenic bacteria like *L. monocytogenes* or *S. enterica* when exposed to silver ions over time, these being most commonly done with silver nanoparticles and on *E. coli* or *S. aureus*. In a study with silver nanoparticles against *Salmonella typhimurium*, Irwin et al. found the increase in the lag phase with increasing silver nanoparticle concentration to be linear, all samples finally reaching

maximum growth (Irwin et al., 2010). In other reports, a delay in proliferation or increase in the lag phase can also be noted which varies from 2-4 hours (Sondi and Salopek-Sondi, 2004.) to 24h (Pal et al., 2007). This effect sometimes occurs simultaneously with a decrease in maximum growth value (Mohammed Fayaz et al., 2009; Ruparelia et al., 2008; Shrivastava et al., 2007; Sondi and Salopek-Sondi, 2004.; Zhao and Stevens, 1998). *S. enterica* manifests a higher susceptibility to silver than *L. monocytogenes* as demonstrated by a decrease in maximum growth values in TSB, and, more remarkably, by absence of growth with 0.3 ppm silver and considerably lower viability according to FC when M9 is selected. This is in accordance with previous findings suggesting Gram negative bacteria are more sensitive to silver ions or nanoparticles than Gram positive (Feng et al., 2000; Lee, 2009, Jung et al., 2009). However, the subpopulation P2 in *S. enterica* falling mostly within the live (green) region, the existence of resilient bacteria which may resuscitate should not be rejected as well.

4.2. Chemical environment and inactivation

In parallel to these phenomena, the effect the chemical environment exerts on the antibacterial efficacy of silver was evaluated. For this purpose, two different growth media, TSB and M9, were selected, as examples for rich or stringent environments of contamination.

In this sense, the antimicrobial efficacy of silver in M9 medium was found to be much greater than in TSB. These strong differences in antimicrobial efficacy observed when the environment of action of the silver species is changed have previously been reported. Despite the great variety of silver species (nano- or microparticles dispersed either in solution or incorporated in a polymer matrix), silver concentrations of 10-500 ppm are needed if the assay is designed with rich, non-selective media as TSB, MHB or LB (Hamilton-Miller and Shah, 1996; Nomiya et al., 2004; Ruparelia et al., 2008; Sondi and Salopek-Sondi, 2004; Thomas et al., 2007). If water or salt buffers are used, however, the bactericidal concentrations are proven to be in the range of 0.01-1 ppm ((Bjarnsholt et al.,

2007; Hwang et al. 2007; Kim et al., 1998). Similar results with 0.1 and 0.3 ppm in Phosphate Buffer Saline (PBS) were confirmed in our lab (data not shown). These differences suggest that silver efficacy might be lost after contact with the nutrient broth or bacterial constituents, which would further allow the delayed proliferation of the resilient viable bacteria. Therefore, the effect of prolonged exposure of silver to the different chemical environments before contamination was evaluated by incubating the silver under both conditions for 3 days before bacterial inoculation. Interestingly, antibacterial efficacy of silver was lost when previous contact has taken place in the medium TSB, except for the highest concentrations of 50 and 100 ppm with *S. enterica*, while in M9 growth patterns are similar with or without previous contact. This indicates that it is neither salts nor glucose interfering with the final antimicrobial effect, but some constituent(s) in the complex digest in TSB which produces a strong decrease in the biocidal properties (2-3 orders of magnitude) that is favoured by direct contact between the medium and the silver ions. Therefore, prolonged contact between the biocide and the growth medium allows the process of inactivation to be fulfilled before inoculation of the microorganisms. Accordingly, when bacteria are inoculated, no initial damage is produced and no delay effect in the growth patterns is observed. Over a certain concentration of the biocide, silver inactivation by growth medium constituents is not significant to produce a change in growth patterns, and these are similar with or without previous contact, as observed with the more susceptible *S. enterica* at high silver concentrations (Figure 2b). The absence of differences in growth patterns with or without previous contact in M9 could suggest that resuscitation is achieved by the resilient viables able to adapt to an environment with silver. However, as silver is known to irreversibly bind to bacterial constituents (Choi et al., 2008; Feng et al., 2000; Guggenbichler et al., 1999; Jung et al., 2009; Liau et al., 1997), inactivation of silver ions by these is also feasible.

Considering these issues, the need to clearly investigate bacterial behaviour in longer terms is required, as short-time assays could overvalue antimicrobial effect and potentially jeopardize the security of antibacterial systems. These

findings highlight the need to investigate further the activity of silver within the chemistry of the scope of application.

5. Acknowledgements

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6. References

Amor, K.B., Breeuwer, P., Verbaarschot, P., Rombouts, F.M., Akkermans, A.D.L., De Vos, W.M., Abee, T. 2002. Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead bifidobacterium cells during bile salt stress. Applied and Environmental Microbiology 68, 5209-5216.

Appendini, P., Hotchkiss, J.H. 2002. Review of antimicrobial food packaging. Innovative Food Science and Emerging Technologies 3, 113-126.

Banerjee, M., Mallick, S., Paul, A., Chattopadhyay, A., Ghosh, S.S. 2010. Heightened reactive oxygen species generation in the antimicrobial activity of a three component iodinated chitosan?silver nanoparticle composite. Langmuir 26, 5901-5908.

Baranyi, J., Roberts, T.A. 1994. A dynamic approach to predicting bacterial growth in food. International Journal of Food Microbiology 23, 277-294.

Bedi, R.S., Cai, R., O'Neill, C., Beving, D.E., Foster, S., Guthrie, S., Chen, W., Yan, Y. 2012. Hydrophilic and antimicrobial Ag-exchanged zeolite a coatings: A year-long durability study and preliminary evidence for their general microbiocidal efficacy to bacteria, fungus and yeast. Microporous and Mesoporous Materials 151, 352-357.

Berney, M., Weilenmann, H.U., Egli, T. 2006. Flow-cytometric study of vital cellular functions in Escherichia coli during solar disinfection (SODIS). Microbiology 152, 1719-1729.

Bjarnsholt, T., Kirketerp-Møller, K., Kristiansen, S., Phipps, R., Nielsen, A.K., Jensen, P.Ø., Høiby, N., Givskov, M. 2007. Silver against Pseudomonas aeruginosa biofilms. APMIS 115, 921-928.

Bouwmeester, H., Dekkers, S., Noordam, M.Y., Hagens, W.I., Bulder, A.S., de Heer, C., ten Voorde, S.E.C.G., Wijnhoven, S.W.P., Marvin, H.J.P., Sips, A.J.A.M. 2009. Review of health safety aspects of nanotechnologies in food production. Regulatory Toxicology and Pharmacology 53, 52-62.

Busolo, M.A., Fernandez, P., Ocio, M.J., Lagaron, J.M. 2010. Novel silver-based nanoclay as an antimicrobial in polylactic acid food packaging coatings. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 27, 1617-1626.

Cowan, M.M., Abshire, K.Z., Houk, S.L., Evans, S.M. 2003. Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel. Journal of Industrial Microbiology and Biotechnology 30, 102-106.

Choi, O., Deng, K.K., Kim, N.J., Ross Jr, L., Surampalli, R.Y., Hu, Z. 2008. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. Water Research 42, 3066-3074.

Damm, C., Münstedt, H., Rösch, A. 2008. The antimicrobial efficacy of polyamide 6/silver-nano- and microcomposites. Materials Chemistry and Physics 108, 61-66.

De Muynck, W., De Belie, N., Verstraete, W. 2010. Antimicrobial mortar surfaces for the improvement of hygienic conditions. Journal of Applied Microbiology 108, 62-72.

Feng, Q.L., Wu, J., Chen, G.Q., Cui, F.Z., Kim, T.N., Kim, J.O. 2000. A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. Journal of Biomedical Materials Research 52, 662-668.

Galeano, B., Korff, E., Nicholson, W.L. 2003. Inactivation of vegetative cells, but not spores, of Bacillus anthracis, B. cereus, and B. subtilis on Stainless steel surfaces coated with an antimicrobial silver- and zinc-containing zeolite formulation. Applied and Environmental Microbiology 69, 4329-4331.

Guggenbichler, J.P., Böswald, M., Lugauer, S., Krall, T. 1999. A new technology of microdispersed silver in polyurethane induces antimicrobial activity in central venous catheters. Infection 27.

Gupta, A., Silver, S. 1998. Silver as a biocide: Will resistance become a problem? Nature Biotechnology 16, 888.

Hamilton-Miller, J.M.T., Shah, S. 1996. A microbiological assessment of silver fusidate, a novel topical antimicrobial agent. International Journal of Antimicrobial Agents 7, 97-99.

Han, D.W., Lee, M.S., Lee, M.H., Uzawa, M., Park, J.C. 2005. The use of silver-coated ceramic beads for sterilization of Sphingomonas sp. in drinking mineral water. World Journal of Microbiology and Biotechnology 21, 921-924.

Hwang, M.G., Katayama, H., Ohgaki, S. 2006. Effect of intracellular resuscitation of Legionella pneumophila in Acanthamoeba polyphage cells on the antimicrobial properties of silver and copper. Environmental Science and Technology 40, 7434-7439.

Hwang, M.G., Katayama, H., Ohgaki, S. 2007. Inactivation of Legionella pneumophila and Pseudomonas aeruginosa: Evaluation of the bactericidal ability of silver cations. Water Research 41, 4097-4104.

Irwin, P., Martin, J., Nguyen, L.H., He, Y., Gehring, A., Chen, C.Y. 2010. Antimicrobial activity of spherical silver nanoparticles prepared using a biocompatible macromolecular capping agent: Evidence for induction of a greatly prolonged bacterial lag phase. Journal of Nanobiotechnology 8.

Jung, R., Kim, Y., Kim, H.S., Jin, H.J. 2009. Antimicrobial properties of hydrated cellulose membranes with silver nanoparticles. Journal of Biomaterials Science, Polymer Edition 20, 311-324.

Junghanns, J.U.A.H., Müller, R.H. 2008. Nanocrystal technology, drug delivery and clinical applications. International journal of nanomedicine 3, 295-309.

Kim, T.N., Feng, Q.L., Kim, J.O., Wu, J., Wang, H., Chen, G.C., Cui, F.Z. 1998. Antimicrobial effects of metal ions (Ag^{+}, Cu^{2+}, Zn^{2+}) in hydroxyapatite. Journal of Materials Science: Materials in Medicine 9, 129-134.

Klasen, H.J. 2000. Historical review of the use of silver in the treatment of burns. I. Early uses. Burns 26, 117-130.

Lee, D., Cohen, R.E., Rubner, M.F. 2005. Antibacterial properties of Ag nanoparticle loaded multilayers and formation of magnetically directed antibacterial microparticles. Langmuir 21, 9651-9659.

Lee, S. 2009. Multifunctionality of layered fabric systems based on electrospun polyurethane/zinc oxide nanocomposite fibers. Journal of Applied Polymer Science 114, 3652-3658.

Li, Q., Mahendra, S., Lyon, D.Y., Brunet, L., Liga, M.V., Li, D., Alvarez, P.J.J. 2008. Antimicrobial nanomaterials for water disinfection and microbial control: Potential applications and implications. Water Research 42, 4591-4602.

Liau, S.Y., Read, D.C., Pugh, W.J., Furr, J.R., Russell, A.D. 1997. Interaction of silver nitrate with readily identifiable groups: Relationship to the antibacterial action of silver ions. Letters in Applied Microbiology 25, 279-283.

Martinez-Abad, A., Sánchez, G., Lagaron, J.M., Ocio, M.J. 2013a. Ligands affecting silver antimicrobial efficacy on Listeria monocytogenes and *Salmonella enterica*. Food Chemistry 139, 281-288.

Matsumura, Y., Yoshikata, K., Kunisaki, S.I., Tsuchido, T. 2003. Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. Applied and Environmental Microbiology 69, 4278-4281.

Mohammed Fayaz, A., Balaji, K., Girilal, M., Kalaichelvan, P.T., Venkatesan, R. 2009. Mycobased synthesis of silver nanoparticles and their incorporation into sodium alginate films for vegetable and fruit preservation. Journal of Agricultural and Food Chemistry 57, 6246-6252.

Muñoz, M., Guevara, L., Palop, A., Tabera, J., Fernández, P.S. 2009. Determination of the effect of plant essential oils obtained by supercritical fluid extraction on the growth and viability of Listeria monocytogenes in broth and food systems using flow cytometry. LWT - Food Science and Technology 42, 220-227.

Nakane, T., Gomyo, H., Sasaki, I., Kimoto, Y., Hanzawa, N., Teshima, Y., Namba, T. 2006. New antiaxillary odour deodorant made with antimicrobial Ag-zeolite (silver-exchanged zeolite). International Journal of Cosmetic Science 28, 299-309.

Nomiya, K., Yoshizawa, A., Tsukagoshi, K., Kasuga, N.C., Hirakawa, S., Watanabe, J. 2004. Synthesis and structural characterization of silver(I), aluminium(III) and cobalt(II) complexes with 4-isopropyltropolone (hinokitiol) showing noteworthy biological activities. Action of silver(I)-oxygen bonding complexes on the antimicrobial activities. Journal of Inorganic Biochemistry 98, 46-60.

Pal, S., Tak, Y.K., Song, J.M. 2007. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Escherichia coli. Applied and Environmental Microbiology 73, 1712-1720.

Percival, S.L., Slone, W., Linton, S., Okel, T., Corum, L., Thomas, J.G. 2011. Use of flow cytometry to compare the antimicrobial efficacy of silver-containing wound dressings against planktonic Staphylococcus aureusand Pseudomonas aeruginosa. Wound Repair and Regeneration 19, 436-441.

Quintavalla, S., Vicini, L. 2002. Antimicrobial food packaging in meat industry. Meat Science 62, 373-380.

Rai, M., Yadav, A., Gade, A. 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances 27, 76-83.

Rodríguez-Argüelles, M.C., Sieiro, C., Cao, R., Nasi, L. 2011. Chitosan and silver nanoparticles as pudding with raisins with antimicrobial properties. Journal of Colloid and Interface Science 364, 80-84.

Ruparelia, J.P., Chatterjee, A.K., Duttagupta, S.P., Mukherji, S. 2008. Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomaterialia 4, 707-716.

Russell, A.D., Hugo, W.B. 1994. Antimicrobial activity and action of silver. Progress in medicinal chemistry 31, 351-370.

Semeykina, A.L., Skulachev, V.P. 1990. Submicromolar Ag^+ increases passive Na^+ permeability and inhibits the respiration-supported formation of Na^+ gradient in Bacillus FTU vesicles. FEBS Letters 269, 69-72.

Shrivastava, S., Bera, T., Roy, A., Singh, G., Ramachandrarao, P., Dash, D. 2007. Characterization of enhanced antibacterial effects of novel silver nanoparticles. Nanotechnology 18.

Simpson, K. 2003. Using silver to fight microbial attack. Plastics, Additives and Compounding 5, 32-35.

Sondi, I., Salopek-Sondi, B. 2004. Silver nanoparticles as antimicrobial agent: A case study on E. coli as a model for Gram-negative bacteria. Journal of Colloid and Interface Science 275, 177-182.

Texter, J., Ziemer, P., Rhoades, S., Clemans, D. 2007. Bactericidal silver ion delivery into hydrophobic coatings with surfactants. Journal of Industrial Microbiology and Biotechnology 34, 571-575.

Thomas, V., Yallapu, M.M., Sreedhar, B., Bajpai, S.K. 2007. A versatile strategy to fabricate hydrogel-silver nanocomposites and investigation of their antimicrobial activity. Journal of Colloid and Interface Science 315, 389-395.

Valero, M., Francés, E. 2006. Synergistic bactericidal effect of carvacrol, cinnamaldehyde or thymol and refrigeration to inhibit Bacillus cereus in carrot broth. Food Microbiology 23, 68-73.

Woo, K.J., Hye, C.K., Ki, W.K., Shin, S., So, H.K., Yong, H.P. 2008. Antibacterial activity and mechanism of action of the silver ion in Staphylococcus aureus and Escherichia coli. Applied and Environmental Microbiology 74, 2171-2178.

Zhao, G., Stevens Jr, S.E. 1998. Multiple parameters for the comprehensive evaluation of the susceptibility of Escherichia coli to the silver ion. BioMetals 11, 27-32.

Chapter III

DEVELOPMENT AND CHARACTERIZATION OF SILVER-BASED ANTIMICROBIAL ETHYLENE-VINYL ALCOHOL COPOLYMER (EVOH) FILMS FOR FOOD-PACKAGING APPLICATIONS

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ABSTRACT

The use of silver as an antimicrobial in the food area has raised wide interest in recent years. In the present work, 0.001-10 wt.-% silver ions were satisfactorily incorporated into an ethylene vinyl alcohol (EVOH) copolymer matrix by a solvent casting technique. The antibacterial efficacy of the composite was evaluated under laboratory conditions and in contact with some foods. The ionic compound did not affect the crystallinity or the water-induced plasticization of the materials and was homogeneously distributed across the surface and thickness of the films. When immersed in water, sorption induced release of 50-100% of silver ions took place in less than 30 minutes. In the bacterial minimal growth medium M9, minimal inhibitory concentration (MIC) of the film was in the range of 0.01-0.1 ppm. High protein content food samples displayed low susceptibility to the films (<1 log reduction in any case), while low protein content food samples exhibited no detectable bacterial counts for films with 1 wt.-% and 10 wt.-% and about 2 log reduction for films with 0.1 wt.-% silver. These results represent a step forward in the understanding of silver antimicrobial efficacy and its possible application in the food packaging industry most likely as food coatings.

Keywords: Active packaging, antimicrobial silver, Ethylene vinyl alcohol (EVOH**)**

1. Introduction

Market trends towards minimally processed, easily prepared and ready-to-eat 'fresh' food products involve the use of alternative technologies such as lower thermal, high pressure, UV irradiation or electric pulse treatments which might allow survival and proliferation of pathogenic bacteria[1-4]. Recent food-borne microbial outbreaks, globalization of food trade, and distribution from centralized processing are driving a search for innovative ways to inhibit microbial growth in the foods while maintaining quality, freshness and safety[5]. The combination of these emerging technologies with antimicrobial packaging technologies could allow extending shelf-life of foods and the prevention of recontamination with pathogens.

In antimicrobial packaging, a substance with biocide properties is included in a sachet, coated, adsorbed or immobilized on the surface, or directly incorporated in the polymer during its processing. As microbial contamination occurs primarily at the surface, the incorporation of the biocide in a film or as a coating has many advantages due to the high exposure areas for the biocide[6-7].

Various antimicrobials have been incorporated in polymers for food packaging applications such as organic acids[8-9], or triclosan[10]. Recently, natural antimicrobials such as enzymes[11], bacteriocins[12-14], essential oils[15], chitosan[16] and others have attracted much attention due to consumer demand trends (see[5, 17-18] for review). Antimicrobial silver has emerged as a new effective technology to prevent microbial proliferation on food contact surfaces in the food industry.

The antimicrobial efficacy of silver has been recognized since ancient times[19-20]. In the past few years, the use of silver or silver salts as key component to control microbial proliferation has become more and more popular. Much of the research on this compound is still focused on medical applications, like wound[21]

and burn[22] treatment, dentistry, catheters[23-25] or orthopedics[26]. However, new applications have emerged, so that silver is currently being incorporated in a wide variety of materials used in daily life, ranging from textile clothing[27-28], coatings in washing machines, refrigerators, furniture handles[29-32], ,home water treatment units, food-contact materials[5,33], deodorants[34] or tooth brush[35] (see [36-38] for review).

Due to its unspecific mechanism of action, silver ions are active against a very broad spectrum of bacteria, yeasts, fungi and viruses[39-40] and are not toxic to human cells[19-20]. In the U.S., the Center for Food Safety and Applied Nutrition in the Food and Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled waters and in the EU, silver is accepted under directive 94/36/EC as a coloring agent (E-174) with no restrictions. Additionally, the European Food Safety Authority (EFSA) has provided positive opinions for silver zeolites, silver zirconium phosphates, and silver containing glasses in its provisional list of additives for food contact materials with a general restriction of ≤0.05 mg/kg food for the whole group. Regardless of the stringent regulations, silver still remains the most widely used antimicrobial polymer additive in food applications [5,18].

The approach of the use of silver in the food industry has been mostly bounded to silver zeolites[41-44] and silver-zirconium ion-exchange resins, which are subsequently incorporated as a coating on predominantly stainless steel surfaces. These systems rely on the sustained release of silver ions via a moisture dependent ion exchange mechanism. However, the very low migration rates of the silver ions from these materials imply the need of silver filler contents of 2-5%. This high silver content could limit the application of these systems in antimicrobial packaging, due to possible permeability and dispersion problems and a negative environmental impact. For the correct development and final application of silver in the food industry, it is crucial to elucidate the threshold of biocide action and optimize the silver system so that tiny contents are required and the potential is fully realized. A feasible approach to this challenge

inside the range of food packaging polymers might be the use of ethylene vinyl alcohol copolymers (EVOH).

EVOH copolymers are a family of semicrystalline random copolymers widely used in the food-packaging sector due to their outstanding gas barrier properties, chemical resistance and high transparency. Upon contact with moisture, EVOH severely plasticizes leading to increased permeability and up to 9 wt.-% water uptake[45], which would enable the sorption-induced release of its content. This might allow the incorporation of much lower quantities of silver salts which could be activated upon direct contact with the moisture of the food and be released in its entirety during the shelf-life of the packaging. The combination of silver and plastics like EVOH would accordingly reduce the load of silver in the packaging and its environmental impact.

In this work, we have focused on the incorporation of silver nitrate to an EVOH copolymer. The materials were characterized to determine possible property and structural changes due to the incorporation of the biocide compound. Considering the instability of active silver ions in the presence of several ligands, such as sulphur groups, the antimicrobial potential of these materials was evaluated under laboratory conditions and in contact with food samples of different composition in comparison with pure silver nitrate. The aim of the study was to elucidate how the silver-based system actuates under these various conditions as a means to further gain understanding about silver antimicrobial efficacy and its possible application in active food packaging.

2. Materials and methods

2.1. Samples Preparation

Ethylene-vinyl alcohol copolymer with 32% (mol. %) ethylene content (EVOH32) supplied by Nippon Gohsei Corp., Japan, was used for preparation of the cast films. Polymer beads were dissolved in 2-propanol:water (70:30 w/w) in the ratio 8:92 (w/w) at 100ºC under reflux. The dissolved polymer was cooled down to approx. 60ºC and a suitable amount of silver nitrate (Sigma-Aldrich) was added to the solution as to achieve EVOH films with 0.00001% - 10% silver nitrate weight in dry conditions. The solution was cast in a glass Petri-dish with an adhesive PTFE sheet to prevent sticking to the bottom. The solvent was allowed to dry at 60ºC for 3h. The thickness of the dry films as measured with a micrometer was of 70±15 μm. Films were stored 20-24h in a 0% relative humidity desiccators protected from light with aluminium wrapping before undergoing testing.

2.2. Elemental Microanalysis (SEM Measurements)

The distribution of silver in the cast films was examined by energy-dispersive X-ray microanalysis (EDS) using a Si (Li) detector (EDAX, Mahwah, NJ, USA) with super ultrathin Be window. Three spectra were collected from each surface employing an area scan mode under 20kV accelerating voltage, 10 μA beam current at 2000× magnification, 1000-1500 counts/sec, a dead time of 30% and 500 s acquisition time. The study of the distribution along the fracture line of the polymer was performed with the line scan mode microanalysis at 1200× magnification and a dwelling time of 400 msec. The SEM microphotographs (S4100, Hitachi, Osaka, Japan) were taken with the same accelerating voltage of 20 keV on the sample surface just before performing the elemental analysis.

2.3 Water Sorption

The vapor water sorption capacities of EVOH with 0 wt.-%, 0.1 wt.-% and 10 wt.-% silver at different relative humidities (RH) were obtained by storing the

100±10 mg of each sample in 26%, 53% and 100% RH desiccators and following water uptake gravimetrically with an analytical balance model Voyager V11140 (precision of 0.01 mg) until equilibrium was reached. Samples were measured three times in triplicate.

2.4. FT-IR Analysis

Transmission FT-IR experiments were recorded within a N_2 purged environment using a Bruker model Tensor 37 equipment (Darmstadt, Germany) with a resolution of 1 cm^{-1}, 20 scan runs and a typical acquisition time of 60 s.

2.5. Differential Scanning Calorimetry (DSC)

Thermal properties were studied by differential scanning calorimetry (DSC) using a Perkin–Elmer DSC-7 calorimeter (Perkin–Elmer Cetus Instruments, Norwalk, CT). The rate of both heating and cooling runs was 10°C/min, where a typical sample weight was around 4 mg. The values of glass transition temperature (T_g), melting point (T_m), specific heat (ΔC_p) and melting enthalpy (ΔH_m) were taken from the second heating run. Calibration was performed using an indium sample. All tests were carried out in duplicate.

2.6. Release Study

A voltammetric method was used to determine the release of free silver ions (FSI) from the films to a slightly acidic aqueous environment. With this purpose, 1g of the cast films with 0.1 wt.-% silver content was immersed in 100 mL slightly acidified (1mM HNO_3 to stabilize silver in its ionic form) distilled water at 5ºC, 25ºC and 50ºC for 24h without stirring except before each measurement. For each measurement, 1 mL from the samples was collected and the amount removed was replaced with fresh water applying a correction factor (*) as follows:

$$Correction\ \ factor = \left(\frac{100}{100-1} \right)^{n-1} \qquad \text{Eq. 1}$$

where "n" is the sequential sample number. The FSI content for each measurement was determined by differential pulse anodic stripping voltammetry (ASV) with an Autolab III (EcoChemie) potentiostat setup under conditions stated in Metrohm application bulletin nº 207/2e "Analysis of silver by stripping voltammetry". The FSI working range was 0.004 – 0.4 ppm and a calibration curve was prepared daily for each set of measurements. All experiments were carried out in duplicate

2.7. Bacterial Strains and Growth Conditions

Bacterial strains *Listeria monocytogenes* CECT 5672 and *Salmonella enterica* CECT 554 (Spanish Type Culture Collection, Valencia, Spain) were selected as food related Gram positive and Gram negative model bacteria. These strains were grown overnight in Tryptic Soy Broth (TSB) (Conda Laboratories, Madrid, Spain) and an aliquot was again transferred to TSB and grown at 37ºC to the mid-exponential phase of growth having an absorbance value of 0.20 for *Salmonella enterica* and 0.15 for *L. monocytogenes* as determined by optical density at 600 nm by ultraviolet visible (UV) spectroscopy using a SP-2000 UV spectrometer (Spectrum Instruments, Shanghai, China). These cultures were centrifuged at 4ºC and 1888G for 20 min. The pellet was resuspended in a solution containing 10% TSB and 10% glycerol. This suspension was transferred to cryotubes and stored at -85ºC as stock cultures until needed. Previous to each study, cryotubes were thawed and diluted in 0.1% buffered peptone water as to achieve the suitable concentration for inoculation. Previous studies evinced no significant differences in cell viability could be found between the ultrafreezed and thawed bacterial stock cultures and the original fresh ones.

2.8. Antimicrobial Assays in Laboratory Conditions

For antimicrobial assays against *L. monocytogenes* and *Salmonella enterica*, M9 minimal medium supplemented with 1mM methionine (M9-Met) was used as liquid broth medium. M9 is a minimal medium without any protein sources or components and glucose as a sole carbon source. Although, the medium is not

suitable for the growth of Gram positive bacteria, previous findings evinced *L. monocytogenes* CECT 5672 can grow well if only methionine is supplemented. Susceptibility tests were performed employing the macro-dilution method M26-A described by the Clinical and Laboratory Standards Institute (CLSI) with modification. The effectiveness of the antimicrobial EVOH-Ag films was assessed by introducing 100 mg strips of approximately the same size and thickness into tubes with 10 mL M9-Met. A bacterial suspension in mid-log phase was then inoculated in each test tube to achieve an initial inoculum size of approximately 5 x 10^5 CFU/mL and incubated at 37ºC for 24 h. Then, 0.1 mL of each M9 sample was sub-cultivated on TSA plates for viable count after incubation at 37ºC for 24 h. These results were compared with EVOH32 samples without silver and samples containing different concentrations of aqueous silver nitrate. Each of these experiments was performed in triplicate.

2.9. Challenge Tests

For antimicrobial challenge tests, food samples were differentiated into two groups according to their protein content. Samples were cut in pieces of 2x2 cm irradiated with UV in the safety cabinet for 10 min and then 25microL of a *L. monocytogenes* bacterial suspension in M9 medium was spread on the sample as to achieve bacterial concentrations of about 10^5 CFU/cm^2 for food samples with high protein content (chicken with and without skin, marinated pork loin and cheese slices) and 10^8 CFU/cm^2 for food samples with low protein content (lettuces, apple peels and eggshells). After inoculation, samples were held for 10 min to allow sorption of the microorganisms tested. Then, film pieces of 2x2 cm with 0.1%, 1% and 10% silver were put on the surface of the food samples and the set was incubated at 12ºC for at least 48 hours. Incubation temperature was set to 12ºC as to reflect temperature abuse in refrigerated samples (EU regulation 2073/2005). To follow antimicrobial activity of the films, samples were removed every 12 hours and homogenized in a stomacher (Pulsifier, Microgen, UK) with 50 mL peptone water (PW) for 2 min. Serial dilutions in 0.1% PW were made and the microorganism suspensions were plated on Oxford

selective agar (Conda Laboratories, Madrid, Spain) for viable count after incubation at 37ºC for 24 h.

2.10. Release Study upon Contact with Food Samples

Additionally, and as to ascertain release under more realistic conditions, the amount of silver released from EVOH films with 1wt. % silver to food samples was determined voltammetrically by immersing the films in ultrapure water slightly acidified (1mM HNO_3 to stabilize silver in its ionic form) after having been incubated at 12ºC for 24h in contact with apple peels at ambient relative humidity and 100% relative humidity as above. All experiments were carried out in triplicate.

2.11. Color Analysis of Treated Samples

The change in color of the films after 24h contact with the food matrix was determined using a handheld Minolta Chromameter CR300 (Minolta Camera Co., Ltd., Osaka, Japan) set to D65 illuminant/10° observer. Film specimens were placed on a white standard plate, and the CIELAB color space was used to determine the parameters L*, a*, and b*. L* value ranges from 0 (black) to 100 (white); a* value ranges from -80 (green) to 100 (red); and b* value ranges from -80 (blue) to 70 (yellow). Samples were evaluated per triplicate and three measurements were taken at random locations on each of the studied films. ΔE* was calculated as a global parameter (eq. 2) using films stored for the same period but without silver and without contact with food as the reference samples.

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{(Eq. 2)}$$

2.12. Statistical Analysis

The statistical significance of differences in molecular organization, thermal properties, color changes and challenge tests with high protein food samples was determined on the ranks with a one-way analysis of variance (ANOVA) and

Tukey's multiple comparison tests. In all cases, a value of $p < 0.05$ was considered to be significant.

3. Results and Discussion

3.1. Distribution of the silver ions in the samples

As the antimicrobial effect occurs via a sorption-induced release upon contact with the water medium, it is crucial that the biocide is homogeneously distributed in the film. Elemental analysis offers the possibility of semi-quantitatively determine the composition of the surface of the matrix and additionally mapping the presence and distribution of each element in the same area. In the SEM micrographs of samples with 10 wt.-% silver, homogeneously

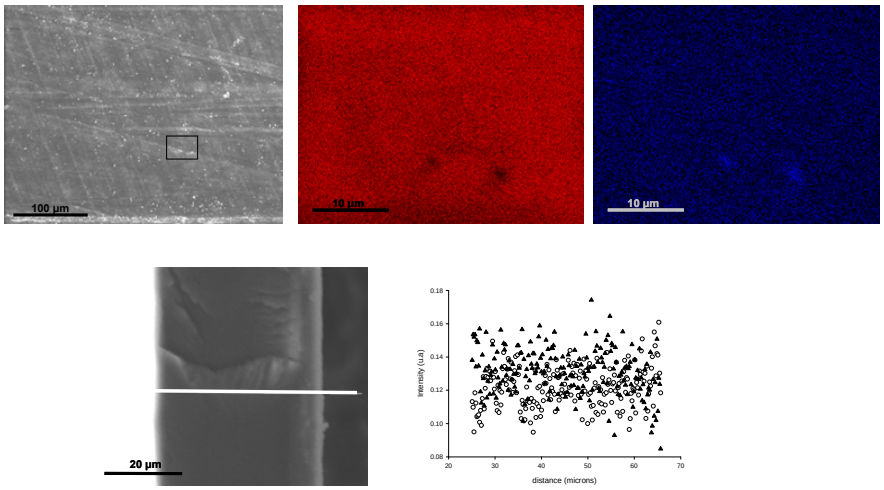

Figure 1. Distribution of silver in EVOH -films with 10% silver: a) Typical SEM image of the sample; b) carbon mapping analysis of the magnified area indicated in "a"; c) silver mapping analysis of the same area; d) SEM image of the cryofracture; e) intensity values (counts/sec) of silver/carbon (empty circles) and oxygen/carbon (filled triangles) along the fracture line

distributed particles of sizes between 1-20 microns are seen (**Figure 1**). These may correspond to precipitated silver salt and/or to reduced silver particles resulting from the beam exposure. During beam exposure the samples became

black and hence the silver reduction argument. When the carbon and oxygen images are compared to the respective elemental mapping analyses for silver, it is revealed that these particles are mostly silver agglomerates consisting of a small fraction of the added silver, while most of it remains homogeneously distributed, covering the whole mapping area. This indicates that silver ions were well dispersed on the surface matrix. The silver/carbon intensity ratio plotted scanning a line in the cryofractured polymer reveals that the distribution is also homogeneous along the thickness of the matrix. Accordingly, it is demonstrated that the incorporation of aqueous solutions of silver nitrate to EVOH by the casting technique enables the silver ions to be well dispersed along and across the films.

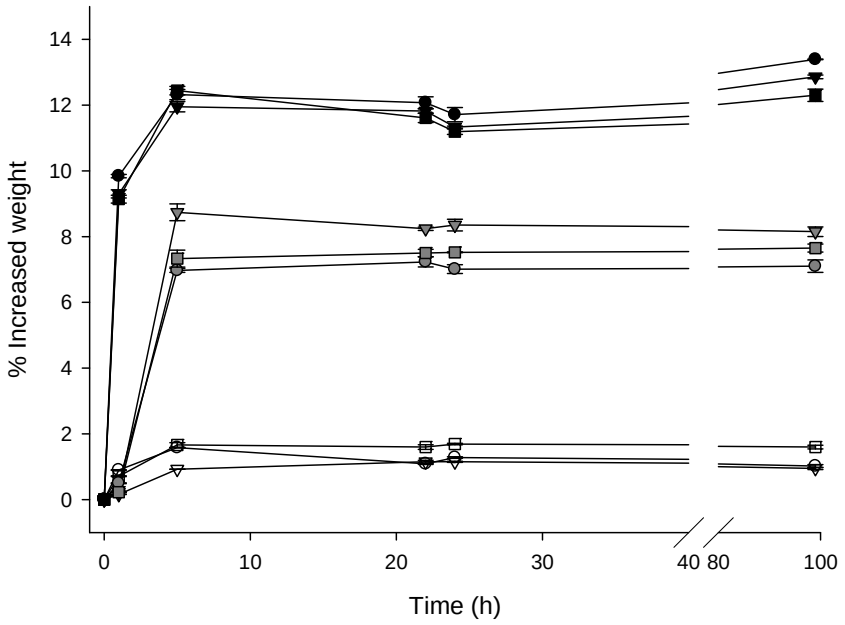

Figure 2. Weight uptake of EVOH32 loaded with 0 wt.-% (circles), 0.1 wt.-% (triangles) and 10 wt.-% (squares) silver at relative humidities of 26% (white), 53% (grey) and 100% (black) n=3.

3.2. Water Uptake

As the release of the silver ions is dependant on the water uptake capacity of the material, it is important to ascertain if these capacities are affected by the incorporation of the biocide. **Figure 2** shows water uptake capacities of EVOH copolymers loaded with 0.1 wt.-% and 10 wt.-% silver compared to an unloaded control for relative humidities (RH) of 26%, 53% and 100%. The sensitivity of the polymer to moisture is denoted with a drastic increase in weight, namely, 1-2% increase for 26% RH, 7-8% for 53% RH and up to approximately 12% weight uptake for 100% RH. Equilibrium is reached in all cases within the first 5 hours. When looking at materials with different silver content in the same moisture conditions, weight differences of up to approx. 1% can be observed. However, higher sorption values do not seem to be related with the silver content, and may be more likely related to material variations in the casting process. Accordingly, EVOH copolymers might undergo a severe plasticization in a moistured environment which does not seem to be affected even if very high concentrations of silver are incorporated in the material.

3.3. FT-IR Analysis

Infrared spectra of the EVOH samples with different silver content were analyzed to evaluate possible changes in molecular organization due to the incorporation of silver. In particular, differences in the crystalline content were investigated by comparing the intensities for the bands at 1410 cm^{-1} and at 1092 cm^{-1}. The band at 1410 cm^{-1} arises from all-trans conformation crystallizable chain segments, the vast majority of which are presumed to exist within a crystalline environment, while the broad envelope at 1092 cm^{-1} arises from the contribution of at least one amorphous band at 1115 cm^{-1} [46]. No significant differences were found in the ratio of these bands among samples with silver contents up to 10% (**Table 1**). This indicates that the amount of crystalline fraction in the polymer may not be altered even if high concentrations of silver are incorporated.

Table 1. Intensities ratio of the FTIR bands at 1410 cm^{-1} and at 1092 cm^{-1} for samples with different silver content (n=3).

Ag^+ content (%)	Absorbance ratio[a]
0	0.933 A
0.01	0.939 A
0.1	0.936 A
1	0.943 A
10	0.943 A

[a] Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests

3.4. Differential Scanning Calorimetry (DSC)

Thermal properties during the second heating run of samples with increasing silver content as measured by DSC are shown in **Table 2**. The values of T_g were taken at the midpoint of a stepwise increase in specific heat associated with the glass transition, whereas the ΔC_p was calculated as the jump in specific heat. The melting point and fusion enthalpies were calculated from the maximum temperature and peak area, respectively, of the peak associated with the melting process. No significant differences are observed for samples with silver contents of equal or less than 0.1 wt.-%. When the silver content increases from 0.1 wt.-% to 10 wt.-%, a significant increase in the amorphous phase fraction and in its stiffness can be deduced from the increase in ΔC_p and T_g, respectively. Additionally, an inhibition of the crystallization process occurs for samples with

Table 2. Thermal properties of the cast films with different silver contents as measured by DSC (n=2).

Ag+ (wt.-%)	**T_g (ºC)**	**ΔCp (J/g ºC)**	**T_m (ºC)**	**ΔH_m (J/g)**
0	58.16 A	0.040 A	183.82 A	46.24 A
0.001	58.55 A	0.041 A	183.70 A	46.25 A
0.01	58.78 A	0.039 A	183.78 A	45.83 A
0.1%	58.64 B	0.040 B	181.42 A	41.66 A
1%	61.66 B	0.048 B	179.37 A	30.58 B
10%	63.73 B	0.089 B	152.50 B	16.08 B

[a] Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests

1% and 10% silver, as evidenced by the significant decrease in T_m and ΔH_m. These changes do not correlate with the results from the infrared analysis or the water uptake capacities, where no changes were observed between unloaded and highly loaded samples, and could possibly be attributed to the reduction of silver ions to elemental silver particles during the heating process in a N_2 purged environment. The appearance of an intense yellow color in the treated food samples, characteristic of the plasmon resonance of tiny silver particles would be an indication of this reduction[47].

3.5. Release Study

As a means to preliminary assess the release kinetics of silver from the films, samples were immersed in slightly acidified bidistilled water, to prevent possible reduction of silver by aging or other ambient conditions. The water sorption-induced release of the silver ions from the materials was monitored by ASV for temperatures of 5ºC, 25ºC and 50ºC to simulate release at refrigeration, average room or extreme hot temperature conditions. The silver content selected for the release study was 0.1 wt.-%, as it was the lowest concentration for which sensitivity and reproducibility was feasible considering the threshold values of the technique under the tested conditions. Once the material is immersed in the aqueous environment, the release takes place within 30 minutes, all samples reaching equilibrium before the first hour (**Figure 3**). This could indicate that plasticization of the polymer occurs instantaneously after the polymer is immersed. Consequently, it swells, sorbing water and allowing the silver ions to migrate to the aqueous solution[48-49]. Samples at 25ºC and 50ºC release the 100% of its content in 10 and 5 min, respectively, whereas in refrigerated samples it takes about 30 min to only release 50% of their silver content. The increase in the release capacities at higher temperatures is most probably related to this process being thermally activated and having higher diffusion coefficients, as reported by[50]. The reason why only 50% of the silver content is released in refrigerated samples must be related to the polymer reducing strongly its molecular motions. In fact, it has been determined that for a fully plasticized sample, the T_g of the polymer goes slightly below but not far from 5ºC. Once

equilibrium is achieved, the silver ions remain stable in solution for at least 24h except in samples at 50ºC, where the FSI concentration gradually decreases, dropping to 62% after 24h. This decrease in FSI could be attributed to the heat induced formation of elemental silver particles.

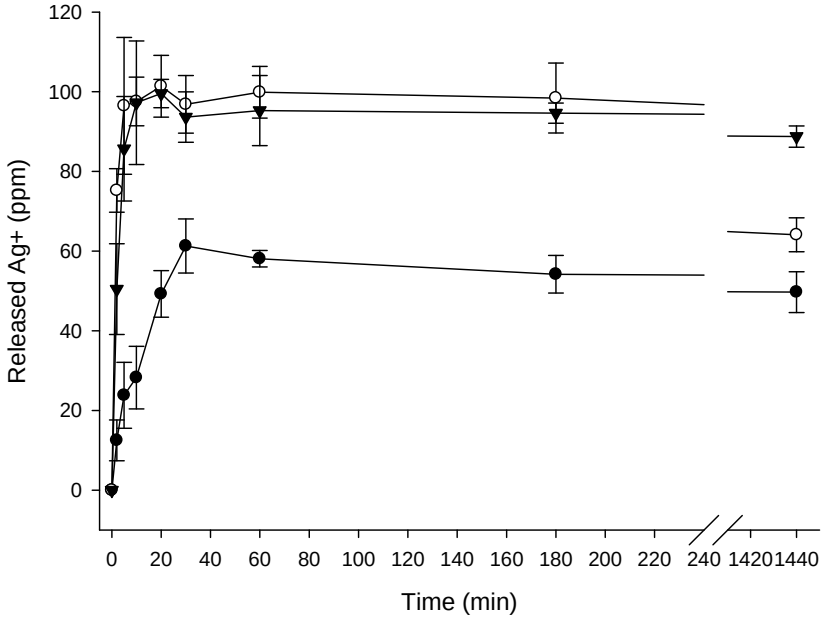

Figure 3. Sorption-induced release of free silver ions from EVOH copolymer loaded with 0.1 wt.-% silver at 5ºC (black circles), 25ºC (triangles) and 50ºC (white circles).

3.6. Antimicrobial Assays in Laboratory Conditions

Silver is known to be easily inactivated by complexes such as the sulphur groups in proteins[51-52]. To reduce inactivation of the active silver ions and, therefore, assess the potential of the silver loaded materials under favorable conditions, M9 minimal medium was selected for the susceptibility assays in nutrient broth. Logarithmic increases of silver nitrate concentrations were tested in the form of aqueous solutions and in the form of silver loaded EVOH, assuming a 100% release from the polymers, against *L. monocytogenes* (**Figure 4a**) and *Salmonella enterica* (**Figure 4b**). The graph shows how bacterial concentration

decreases with increasing silver content in both forms while EVOH without silver does not exhibit any antimicrobial effect as compared to the control. The minimal inhibitory concentration (MIC) is defined as the amount of biocide which inhibits culture growth during 24h, the bacterial counts remaining approx. equal to the initial inoculum size. For *L. monocytogenes*, this effect is achieved

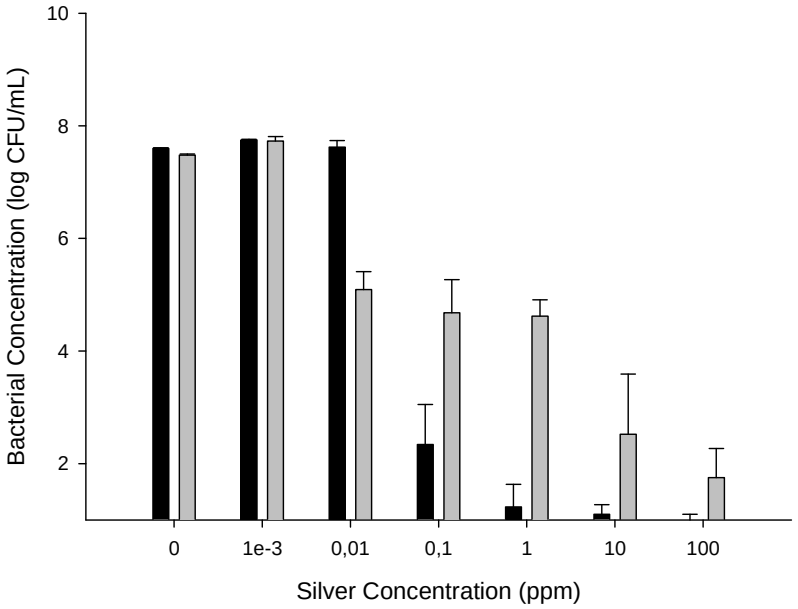

Figure 4a. Susceptibility assays in M9-Met minimal medium of *L. monocytogenes* at 37ºC to increasing concentrations of silver aqueous solutions ■ and silver loaded EVOH copolymers □ assuming a 100% release (n=3).

with silver concentrations of 0.1ppm and 0.01ppm for silver added as aqueous solution and as loaded EVOH, respectively. For *Salmonella enterica*, the same effect is achieved with silver concentrations of 0.01ppm in both forms of release. However, a decrease in the proliferation of *Salmonella enterica* is noted for concentrations as low as 0.001 ppm only in samples where the silver is released from the copolymer. This points out that for both Gram positive and Gram negative bacteria the effectiveness of antimicrobial silver is enhanced when incorporated in the copolymer as compared to pure silver nitrate, indicating the

potential of antimicrobial silver is being more efficiently exploited. This phenomenon could be associated to the inactivation of silver by the nutrient broth or by the microorganisms themselves. In solution, the entirety of the silver ions would be potentially capable of being instantaneously inactivated, whereas in the polymer, the release extends along a half-hour. This would increase the time of contact between the bacteria and the active silver ions, accordingly preventing proliferation more efficiently.

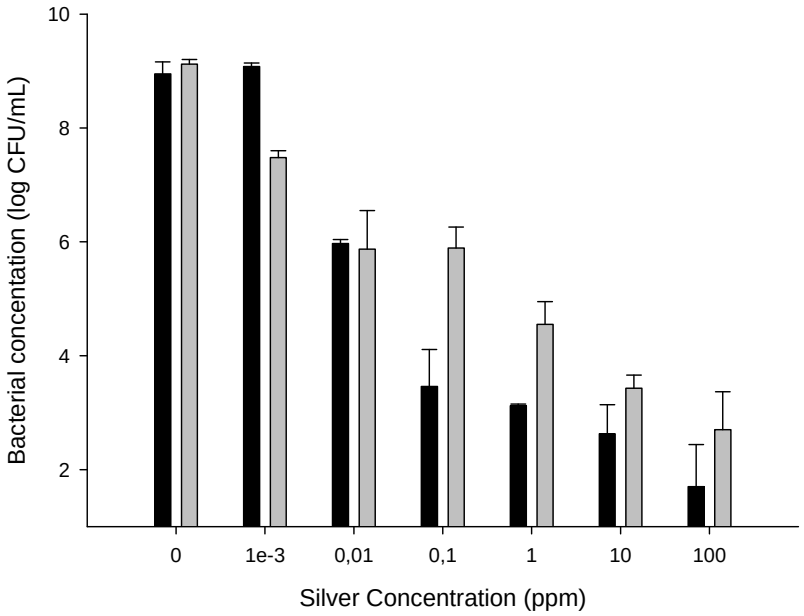

Figure 4b. Susceptibility assays in M9-Met minimal medium of S. *spp* at 37ºC to increasing concentrations of silver aqueous solutions and silver loaded EVOH copolymers assuming a 100% release (n=3).

However, this tendency is inverted when examining the effect of higher concentrations of silver. For *L. monocytogenes*, a bactericidal effect (defined as a decrease of 3 magnitudes in the bacterial load) (MBC) is achieved with silver concentrations in the range of 0.1-1 ppm and 10-100 ppm for silver added as aqueous solution or as EVOH films, respectively. For *Salmonella enterica*, the

bactericidal effect is reached with 10-100 ppm if aqueous silver nitrate is added to the broth, whereas this effect is not yet reached with 100 ppm silver when released by EVOH copolymer. This indicates about 100 times more silver is necessary to exert a bactericidal effect with the polymer compared to pure silver nitrate. It also evidences that the difference between MIC and MBC is extremely high, more so for *Salmonella enterica* than for *L. monocytogenes*. The wide range of concentrations in the survival curves where bacteria remain viable but are not able to proliferate, could be related to the mechanism of action of silver and/or explained in terms of solubility. Silver damages bacteria by unspecific binding to membrane and respiratory enzymes[23,53-54]. This unspecific binding could, in minute concentrations, result in a sub-lethal damage for which bacteria would not be able to proliferate but would still remain viable, until a certain concentration is reached where the damage would overtake their repair mechanisms and cause irreversible damage and cell death[55]. In addition, the possible formation of insoluble silver chloride, (observed as a visible white precipitate when 10wt. % silver is incorporated), might also reduce the availability of free silver ions to exert the antibacterial effect. The solubility constant (log K) of silver chloride in water is according to the ref. [52] about 9.8. This would imply a soluble concentration of 6.3 ppb silver chloride, above which saturation is reached and the equilibrium is gradually shifted towards silver chloride complexes, a very low % of silver ions remaining active in solution, according to mathematical modeling reported previously[56]. As a result, the bactericidal concentration would be above the solubility constant and much higher quantities would be needed to increase the fraction of free silver ions.

Either inside the polymer or in aqueous solutions, the amount of silver necessary to affect bacterial growth as compared to the control is in the range of 0.001-0.1 ppm. Other authors found bactericidal effects for silver ions or silver nanoparticle concentrations in the range of 0.01-1 ppm[57-59]. However, these studies were made in water or salt buffers, which do not support bacterial proliferation. Studies performed on nutrient growth media, like TSB, Luria Bertani or Müller-Hinton broth gave MBC values of 10-500 ppm[60-64]. In the

present study, M9 medium is put forth as a growth medium which supports bacterial growth while fully exploiting the antimicrobial potential of silver ions (minimizing inactivation). Accordingly, this medium could be used as a suitable substrate of reference to assess the full potential of silver based antimicrobial systems.

In addition, a few studies recently published have dealt with the incorporation of silver as antimicrobial for food packaging applications. One approach has been the inclusion of the silver ions in inorganic mineral substrates like montmorillonites as carrier in a food packaging material like polylactic acid (PLA)[65]. The same polymer has been used for incorporation of silver nanoparticles as antibacterial filler[66]. In these cases, however, filler contents were in the range of 1-10%. Kubakka et al. reported the use of EVOH loaded with TiO_2-Ag nanoparticles[67]. Silver is known to enhance the UV-induced antibacterial effect of TiO_2 by electron transfer to TiO_2[68]. However, the susceptibility tests were again performed on liquid media, and the antibacterial effect of silver alone was not considered. In the present work, EVOH copolymer was selected due to its exceptional capacity of undergoing plasticization in order to enhance the release capacities of the material and minimize silver content in the polymer. In this study optimization of experimental conditions lead to an antimicrobial effect with filler content of $\leq 0.01\%$. With this low filler contents, the very stringent restrictions limits applied by the EFSA could be fulfilled without the need for other additional filler or carrier for the active silver species.

3.7. Challenge Tests

Challenge tests on different food types were carried out to ascertain the antimicrobial effectiveness on real food samples. As inactivation of silver is favored by the presence of proteins and other biomolecules, two sets of experiments were carried out, one involving samples with a high protein content, like chicken, pork loin or cheese, and ones in which the interaction with proteins would be minimized such as with lettuces, apple peels and eggshells. The extent of inactivation and consequent loss in antimicrobial efficacy was approached

with preliminary studies so concentrations could be set to values were difference in bacterial counts might be noticed. Viable counts of *L. monocytogenes* after 24h and 72h incubation at 12ºC on food samples with high protein content and EVOH with 1% and 10% silver content are displayed in **Table 3**. Controls of chicken wings, chicken breasts and pork loin are able to increase their number in about 1.5 log and 3-3.5 log after 24 and 72h incubation, respectively. Controls on cheese slices, however, were not able to grow properly and viable counts decreased with the time, indicating this substrate is not suitable for the growth of *L. monocytogenes*. Samples with high amounts of silver added to the food either with the polymer or in aqueous solution exhibited count values up to 1 log lower than the controls without silver, the aqueous solution having slightly more effect than equivalent silver quantities in the polymer.

Table 3. Viable counts of *L. monocytogenes* in food samples with high protein content after incubation at 12ºC (n=3)

Food sample	Silver amount	*L. monocytogenes* (Log CFU/cm^2)	
		After 24h	After 72h
Chicken wings	Control	6.75 ± 0.07 A	8.68 ± 0.06 A
	10% $AgNO_3$ (aq)	5.90 ± 0.12 B	7.74 ± 0.09 B
	EVOH-Ag^+ 1%	6.33 ± 0.25 A	8.84 ± 0.12 A
	EVOH-Ag^+ 10%	6.22 ± 0.04 A	7.80 ± 0.04 AB
Chicken breasts	Control	6.37 ± 0.54 A	8.06 ± 0.15 A
	10% $AgNO_3$ (aq)	5.54 ± 0.41 B	6.92 ± 0.04 C
	EVOH-Ag^+ 1%	6.10 ± 0.07 A	7.57 ± 0.06 AB
	EVOH-Ag^+ 10%	5.85 ± 0.21 AB	7.01 ± 0.12 B
Marinated pork loin	Control	6.48 ± 0.14 A	8.31 ± 0.04 A
	10% $AgNO_3$ (aq)	5.53 ± 0.12 B	7.24 ± 0.11 B
	EVOH-Ag^+ 1%	6.27 ± 0.16 A	7.96 ± 0.06 A
	EVOH-Ag^+ 10%	6.21 ± 0.23 A	7.74 ± 0.05 A
Cheese slices	Control	5.72 ± 0.24 A	5.34 ± 0.02 A
	10% $AgNO_3$ (aq)	5.12 ± 0.31 A	5.04 ± 0.21 A
	EVOH-Ag^+ 1%	5.58 ± 0.07 A	5.41 ± 0.13 A
	EVOH-Ag^+ 10%	5.34 ± 0.20 A	5.12 ± 0.02 A

[a] Mean values with different letters in the same food sample and same incubation time represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests

When the challenge tests are performed on food matrices with low protein content (**Figure 5**), viable counts remained stationary during the whole experiment except for samples on eggshells, where bacterial counts decreased with time. Aqueous solutions of either 1% or 10% silver produced a decrease in microbial population of 4-5 log. The contact with silver loaded EVOH films exerted similar efficacy only with the highest content (10%). Polymers loaded with 0.1% or 1% silver did not achieve a bactericidal effect, producing a decrease of about 2 log after 24h contact. This evidences that the antimicrobial behavior of silver on food samples is drastically decreased compared to its efficiency in the liquid medium. This enormous decrease is noted in both forms of silver, either alone or when incorporated into the polymer. Therefore, this decrease can not be only due to release issues but should mostly be attributed to inactivation of the active silver ions. In addition, silver ions readily react with sulphur groups in proteins forming very stable complexes[52]. Also, silver ions are easily reduced in the presence of weak reducing environments to elemental silver, which does not exert an antimicrobial effect except in the nanoscale, or by gradually re-oxidizing to ions[47]. Both chemical processes could explain the low antimicrobial effect for high protein content samples, where only one log reduction is achieved for any aqueous or film samples. In food samples with low protein content, inactivation of the silver ions might occur to a lesser extent, so high bactericidal effect is achieved for 10% EVOH films or 1% aqueous solution and about 2 log reductions is reached for films with 0.1% silver. However, even when low protein samples are selected for the assays, a 0.1% silver content in the films would still imply surpassing the restriction limits recommended by the EFSA. To the best of our knowledge, successful application on food matrices of EVOH-silver ion releasing technologies has not yet been reported in the literature. In this study, extreme differences in antibacterial efficacy depending on the experimental conditions are evidenced, putting forth the need for standardization of silver biocide tests. Therefore, the present work represents a step forward in the application of silver based antimicrobial systems, still mostly bounded to clinical applications, to the food industry.

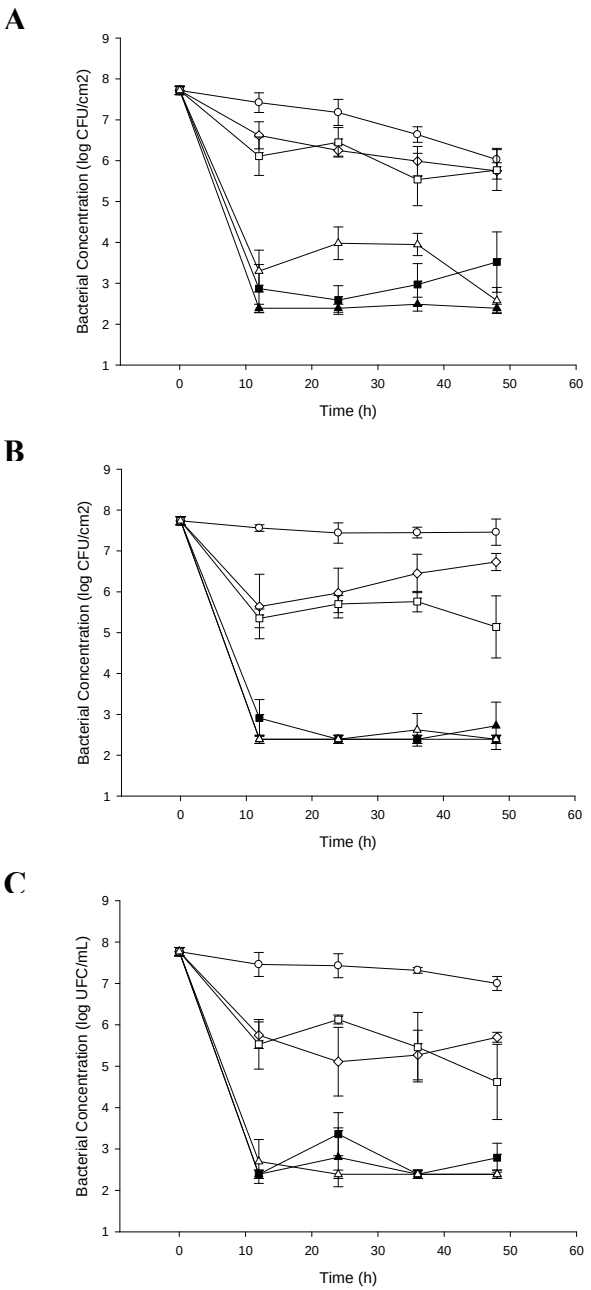

Figure 5. Viable counts in the challenge test at 12ºC on a) eggshells, b) lettuces c) apple peels of *L. monocytogenes* versus silver aqueous solutions (black) and silver loaded EVOH (white) with 0 wt.-% (circles), 0.1 wt.-% (diamonds), 1 wt.-% (squares) and 10 wt.-% (triangles) silver content (n=3).

3.8. Release Study upon Contact of Films with 0.1% Silver with Food Samples

The release of silver ions from the polymer upon contact with food samples can be calculated from the remaining quantity of silver in the polymer after incubation. Apple peels were selected to minimize loss of free silver ions due to complexation and simulate relatively dry food samples at ambient humidity conditions. Therefore, the equivalent samples were stored at 100% RH to investigate the influence of moisture on the release. The fraction of silver released to the food sample was indirectly calculated subtracting the fraction remaining inside the films to the total (100%). Values after 20 min and 24 hours contact under the stated conditions are shown in **Table 4**. Control samples not in

Table 4. Fraction of the silver content released upon contact of polymer samples of 1% silver with apple peels at ambient RH and 100% RH after 20min and 24h.

Sample	% Silver ions released	
	After 20 min	After 24 h
Control	2.4 ± 4.0	1.2 ± 3.1
Apple peel at room RH	19.4 ± 9.7	24.3 ± 8.6
Apple peel at 100% RH	19.4 ± 3.6	45.6 ± 3.5

contact with food give a release fraction of 2% which is inside the standard deviation of the measurement. After 20 minutes in contact with the food matrix, about 20% of the silver content has been released from the polymer regardless of RH. 24h contact results in the release of approx. 25% of the silver content for samples at room ambient RH whereas 45% of the total silver is released in samples at 100% RH. This suggests the existence of two mechanisms in the release of silver ions. The first one would rapidly take place as the film surface gets in contact with the moisturized food surface, producing an immediate sorption and burst release of a certain fraction of the silver content within 20 min. During this mechanism, the fraction released would depend on the moisture of the food sample. The second would imply the plasticization of the whole

polymer and the slow migration of the ions into the foods. This process would be governed by the humidity conditions in the environment. As seen in **Figure 2**, water-induced plasticization of the films at 100% RH reaches equilibrium after 3 h. Consequently, after 24 h of contact this process would be fulfilled and release of the FSI would be enhanced at higher RH. As a result, the silver fractions released to the peels are similar in the first 20 minutes and increase after 24h depending on the humidity.

3.9. Appearance of the Films after Food Contact

It is desirable that the films are transparent and colorless in the application for consumer acceptance. Color measurements contribute to objectively differentiate and evaluate changes in the color of the films. Except for samples with 10 wt.-% silver and in contact with chicken breasts, the film specimens were highly transparent (**Figure 6**). **Table 5** reveals color changes as a function of silver content in the polymer and contact with chicken breasts or apple peels, as

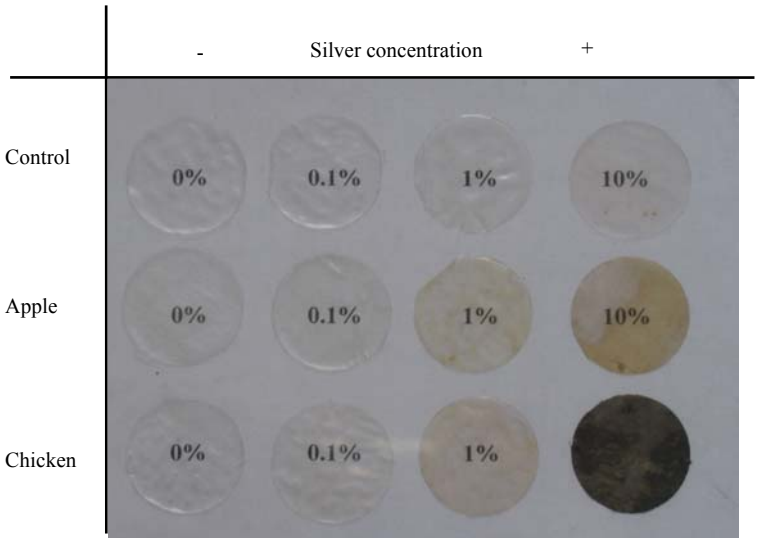

Figure 6. Images of the EVOH samples with different silver content after 24h contact with low (apple) and high (chicken) protein content food samples.

examples of high protein and low protein food samples, respectively. As it can be observed from the results, the films presented good transparency as indicated by high lightness values (L) (97-99), except for samples with 10% silver in contact with apple peels (92.13) and chicken breasts (42.38). Samples in contact with apple peels show a decrease in transparency, an increase in yellowness and

Table 5. Color measurements of the as-prepared films (control) and after contact with low and high protein content food samples.

Sample	Color measurements			
	L^*	a^*	b^*	ΔE^*
Control				
0%	98.97 A	0.07 BC	0.00 A	0.00 A
0.1%	99.06 A	0.02 BC	0.52 A	0.53 A
1%	99.11 A	0.13 BC	0.89 AB	0.88 A
10%	97.68 A	0.18 BC	2.37 AB	2.74 A
Apple peels				
0%	98.81 A	-0.08 BC	0.24 A	0.82 A
0.1%	98.61 A	-0.06 BC	0.82 AB	0.99 A
1%	98.40 A	0.05 BC	1.48 AB	1.63 A
10%	92.13 B	1.12 A	8.41 C	10.94 C
Chicken breast				
0%	99.01 A	0.04 BC	0.46 A	0.50 A
0.1%	98.83 A	-0.17 C	1.80 AB	1.82 A
1%	97.31 A	-1.23 D	7.05 C	7.38 B
10%	46.38 C	0.53 AB	3.70 B	52.82 D

[a] Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests

a slight increase in redness only significant for 10% silver content. In contact with chicken breasts, samples show an increasing yellowness and a slight increase in greenness with increasing silver content. This is significant only in samples with 1 wt.-% silver. Samples with 10 wt.-% silver radically change to opaque shiny silvery colored films. L values in these samples are not due to transparency of the films, but are attributed to the reflectance of the light by the shiny metallic surface of the samples (**Figure 6**). Silver ions are readily reduced

to elemental silver in the presence of weak reducing environments. Therefore, the increase in yellowness can probably be attributed to plasmon resonance of fine elemental silver particles formed after reduction by food components sucked in during the water sorption process. The remarkable appearance of films with 10 wt.-% silver after contact with high protein content could be due to complexation with sulphide in proteins (deep black), as well as to reduction of these agglomerates into metallic silver.

4. Acknowledgements

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5. References

1. Odumeru, J. A.; Mitchell, S. J.; Alves, D. M.; Lynch, J. A.; Yee, A. J.; Wang, S. L.; Styliadis, S.; Farber, J. M., Assessment of the microbiological quality of ready-to-use vegetables for health-care food services. *J Food Protect* **1997,** *60*, (8), 954-960.

2. Farber, J. M.; Wang, S. L.; Cai, Y.; Zhang, S., Changes in populations of Listeria monocytogenes inoculated on packaged fresh-cut vegetables. *J of Food Protect.* **1998,** *61*, (2), 192-195.

3. Valero, M.; Francés, E., Synergistic bactericidal effect of carvacrol, cinnamaldehyde or thymol and refrigeration to inhibit Bacillus cereus in carrot broth. *Food Microbiol.* **2006,** *23*, (1), 68-73.

4. Brody, A. L., Packaging for nonthermally & minimally processed foods. *Food Technol* **2005,** *59*, (10), 75-77.

5. Appendini, P.; Hotchkiss, J. H., Review of antimicrobial food packaging. *Innov. Food Sci Emerg. Technol.* **2002,** *3*, (2), 113-126.

6. López-Rubio, A.; Almenar, E.; Hernandez-Muñoz, P.; Lagarón, J. M.; Catalá, R.; Gavara, R., Overview of active polymer-based packaging technologies for food applications. *Food Rev. Int.* **2004,** *20*, (4), 357-387.

7. Ouattar, B.; Simard, R. E.; Piett, G.; Bégin, A.; Holley, R. A., Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. *Int. J Food Microbiol* **2000,** *62*, (1-2), 139-148.

8. Han, J. H.; Floros, J. D., Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. *J Plast Film Sheet* **1997,** *13*, (4), 287-298.

9. Weng, Y. M.; Chen, M. J., Sorbic Anhydride as Antimycotic Additive in Polyethylene Food Packaging Films. *LWT - Food Sci Tech* **1997,** *30*, (5), 485-487.

10. Cutter, C. N., The effectiveness of triclosan-incorporated plastic against bacteria on beef surfaces. *J Food Protect* **1999,** *62*, (5), 474-479.

11. Padgett, T.; Han, I. Y.; Dawson, P. L., Incorporation of food-grade antimicrobial compounds into biodegradable packaging films. *J Food Protect* **1998,** *61*, (10), 1330-1335.

12. Ming, X.; Weber, G. H.; Ayres, J. W.; Sandine, W. E., Bacteriocins applied to food packaging materials to inhibit Listeria monocytogenes on meats. *J Food Sci* **1997,** *62*, (2), 413-415.

13. Scannell, A. G. M.; Hill, C.; Ross, R. P.; Marx, S.; Hartmeier, W.; Arendt, E. K., Development of bioactive food packaging materials using immobilised bacteriocins Lacticin 3147 and Nisaplin(®). *Int J Food Microbiol* **2000,** *60*, (2-3), 241-249.

14. Sebti, I.; Coma, V., Active edible polysaccharide coating and interactions between solution coating compounds. *Carbohyd Pol* **2002,** *49*, (2), 139-144.

15. Gutiérrez, L.; Batlle, R.; Sánchez, C.; Nerín, C., New approach to study the mechanism of antimicrobial protection of an active packaging. *Foodborne Path Dis* **2010,** *7*, (9), 1063-1069.

16. Fernandez-Saiz, P.; Lagaron, J. M.; Ocio, M. J., Optimization of the biocide properties of chitosan for its application in the design of active films of interest in the food area. *Food Hydrocol* **2009,** *23*, (3), 913-921.

17. Coma, V., Bioactive packaging technologies for extended shelf life of meat-based products. *Meat Sci* **2008,** *78*, (1-2), 90-103.

18. Quintavalla, S.; Vicini, L., Antimicrobial food packaging in meat industry. *Meat Sci* **2002,** *62*, (3), 373-380.

19. Russell, A. D.; Hugo, W. B., Antimicrobial activity and action of silver. *Prog Med Chem* **1994,** 31, 351-370.

20. Williams, R. L.; Doherty, P. J.; Vince, D. G.; Grashoff, G. J.; Williams, D. F., The biocompatibility of silver. *Crit Rev Biocomp* **1989,** *5*, (3), 205+221-243.

21. Dowsett, C., The use of silver-based dressings in wound care. *Nurs Stand* **2004,** 19, (7), 56-60.

22. Klasen, H. J., A historical review of the use of silver in the treatment of burns. II. Renewed interest for silver. *Burns* **2000,** *26*, (2), 131-138.

23. Guggenbichler, J. P.; Böswald, M.; Lugauer, S.; Krall, T., A new technology of microdispersed silver in polyurethane induces antimicrobial activity in central venous catheters. *Infection* **1999,** *27*, (SUPPL. 1).

24. Bechert, T.; Böswald, M.; Lugauer, S.; Regenfus, A.; Greil, J.; Guggenbichler, J. P., The Erlanger silver catheter: In vitro results for antimicrobial activity. *Infection* **1999,** *27*, (SUPPL. 1).

25. Tobin, E. J.; Bambauer, R., Silver Coating of Dialysis Catheters to Reduce Bacterial Colonization and Infection. *Ther Apheresis* **2003,** *7*, (6), 504-509.

26. Hetrick, E. M.; Schoenfisch, M. H., Reducing implant-related infections: Active release strategies. *Chem Soc Rev* **2006,** *35*, (9), 780-789.

27. Yuranova, T.; Rincon, A. G.; Bozzi, A.; Parra, S.; Pulgarin, C.; Albers, P.; Kiwi, J., Antibacterial textiles prepared by RF-plasma and vacuum-UV mediated deposition of silver. *J Photoch Photobio A* **2003,** *161*, (1), 27-34.

28. Jeong, S. H.; Yeo, S. Y.; Yi, S. C., The effect of filler particle size on the antibacterial properties of compounded polymer/silver fibers. *J Mat Sci* **2005,** *4*0, (20), 5407-5411.

29. Han, D. W.; Lee, M. S.; Lee, M. H.; Uzawa, M.; Park, J. C., The use of silver-coated ceramic beads for sterilization of Sphingomonas sp. in drinking mineral water. *World J Microbiol Biotech* **2005,** *21*, (6-7), 921-924.

30. Kampmann, Y.; De Clerck, E.; Kohn, S.; Patchala, D. K.; Langerock, R.; Kreyenschmidt, J., Study on the antimicrobial effect of silver-containing inner liners in refrigerators. *J Appl Microbiol* **2008,** *104*, (6), 1808-1814.

31. Keleher, J.; Bashant, J.; Heldt, N.; Johnson, L.; Li, Y., Photo-catalytic preparation of silver-coated TiO2 particles for antibacterial applications. *World J Microbio Biotech* **2002,** *18*, (2), 133-139.

32. Galeano, B.; Korff, E.; Nicholson, W. L., Inactivation of vegetative cells, but not spores, of Bacillus anthracis, B. cereus, and B. subtilis on Stainless steel surfaces coated with an antimicrobial silver- and zinc-containing zeolite formulation. *Appl Env Microbiol* **2003,** *69*, (7), 4329-4331.

33. Bouwmeester, H.; Dekkers, S.; Noordam, M. Y.; Hagens, W. I.; Bulder, A. S.; de Heer, C.; ten Voorde, S. E. C. G.; Wijnhoven, S. W. P.; Marvin, H. J. P.; Sips, A. J. A. M., Review of health safety aspects of nanotechnologies in food production. *Regul Toxicol Pharm* **2009,** *53*, (1), 52-62.

34. Beiersdorf, B. Nivea spray silver protect. www. nivea.com/products/show/25795 (02/28/2011),

35. Silver Care Plus Self Sanitizing Toothbrush. www.dentist.net/silver-care-plus.asp (08/06/2010),

36. Gupta, A.; Silver, S., Silver as a biocide: Will resistance become a problem? *Nat Biotech* **1998,** *16*, (10), 888.

37. Rai, M.; Yadav, A.; Gade, A., Silver nanoparticles as a new generation of antimicrobials. *Biotech Adv* **2009,** 27, (1), 76-83.

38. Chen, X.; Schluesener, H. J., Nanosilver: A nanoproduct in medical application. *Toxicol Let* **2008,** *176*, (1), 1-12.

39. De Gusseme, B.; Sintubin, L.; Baert, L.; Thibo, E.; Hennebel, T.; Vermeulen, G.; Uyttendaele, M.; Verstraete, W.; Boon, N., Biogenic silver for disinfection of water contaminated with viruses. *Appl Env Microbiol* **2010,** *76*, (4), 1082-1087.

40. Lara, H. H.; Ayala-Nuñez, N. V.; Ixtepan-Turrent, L.; Rodriguez-Padilla, C., Mode of antiviral action of silver nanoparticles against HIV-1. *J Nanobiotech* **2010,** *8*.

41. Cowan, M. M.; Abshire, K. Z.; Houk, S. L.; Evans, S. M., Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel. *J Ind Microbiol Biotechnol* **2003,** *30*, (2), 102-106.

42. Challener, C., Smart coatings continue to develop. *JCT CoatingsTech* **2008,** *5*, (2), 38-42.

43. Hussain, T., Silver-based antimicrobials. *Pakistan Text J* **2007,** *56*, (7), 56-57.

44. Podhajny, R. M. In *Efficacy of antimicrobially treated substrates*, 2004; 2004; pp 667-679.

45. Cabedo, L.; Lagarón, J.; Cava, D.; Saura, J. J.; Giménez, E., The effect of ethylene content on the interaction between ethylene-vinyl alcohol copolymers and water-II: Influence of water sorption on the mechanical properties of EVOH copolymers. *Polymer Testing* **2006,** *25*, (7), 860-867.

46. López-Rubio, A.; Lagaron, J. M.; Giménez, E.; Cava, D.; Hernandez-Muñoz, P.; Yamamoto, T.; Gavara, R., Morphological alterations induced by temperature and humidity in ethylene-vinyl alcohol copolymers. *Macromol* **2003,** *36*, (25), 9467-9476.

47. Sharma, V. K.; Yngard, R. A.; Lin, Y., Silver nanoparticles: Green synthesis and their antimicrobial activities. *Adv Col Interf Sci* **2009,** *145*, (1-2), 83-96.

48. Cava, D.; Sammon, C.; Lagaron, J. M., Sorption-induced release of antimicrobial isopropanol in EVOH copolymers as determined by ATR-FTIR spectroscopy. *J Appl Pol Sci* **2007,** *103*, (5), 3431-3437.

49. Cava, D.; Sammon, C.; Lagaron, J. M., Water diffusion and sorption-induced swelling as a function of temperature and ethylene content in ethylene-vinyl alcohol copolymers as determined by attenuated total reflection Fourier transform infrared spectroscopy. *App Spectrosc* **2006,** *60*, (12), 1392-1398.

50. Zhang, Z.; Britt, I. J.; Tung, M. A., Water Absorption in EVOH Films and Its Influence on Glass Transition Temperature. *J Pol Sci B* **1999,** *37*, (2-7), 691-699.

51. Liau, S. Y.; Read, D. C.; Pugh, W. J.; Furr, J. R.; Russell, A. D., Interaction of silver nitrate with readily identifiable groups: Relationship to the antibacterial action of silver ions. *Lett Appl Microbiol* **1997,** *25*, (4), 279-283.

52. Choi, O.; Clevenger, T. E.; Deng, B.; Surampalli, R. Y.; Ross Jr, L.; Hu, Z., Role of sulfide and ligand strength in controlling nanosilver toxicity. *Water Res* **2009,** *43*, (7), 1879-1886.

53. Feng, Q. L.; Wu, J.; Chen, G. Q.; Cui, F. Z.; Kim, T. N.; Kim, J. O., A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. *J Biomed Mat Res* **2000,** *52*, (4), 662-668.

54. Matsumura, Y.; Yoshikata, K.; Kunisaki, S. I.; Tsuchido, T., Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Appl Env Microbiol* **2003,** *69*, (7), 4278-4281.

55. Woo, K. J.; Hye, C. K.; Ki, W. K.; Shin, S.; So, H. K.; Yong, H. P., Antibacterial activity and mechanism of action of the silver ion in Staphylococcus aureus and Escherichia coli. *Appl Env Microbiol* **2008,** *74*, (7), 2171-2178.

56. Ward, T. J.; Kramer, J. R., Silver speciation during chronic toxicity tests with the mysid, Americamysis bahia. *Comp Biochem Physiol C* **2002,** *133*, (1-2), 75-86.

57. Kim, T. N.; Feng, Q. L.; Kim, J. O.; Wu, J.; Wang, H.; Chen, G. C.; Cui, F. Z., Antimicrobial effects of metal ions (Ag^{+}, $Cu^{2+,}$ Zn^{2+}) in hydroxyapatite. *J Mat Sci-Mater M* **1998,** 9, (3), 129-134.

58. Hwang, M. G.; Katayama, H.; Ohgaki, S., Inactivation of Legionella pneumophila and Pseudomonas aeruginosa: Evaluation of the bactericidal ability of silver cations. *Water Res* **2007,** 41, (18), 4097-4104.

59. Bjarnsholt, T.; Kirketerp-Møller, K.; Kristiansen, S.; Phipps, R.; Nielsen, A. K.; Jensen, P. Ø.; Høiby, N.; Givskov, M., Silver against Pseudomonas aeruginosa biofilms. *APMIS* **2007,** 115, (8), 921-928.

60. Thomas, V.; Yallapu, M. M.; Sreedhar, B.; Bajpai, S. K., A versatile strategy to fabricate hydrogel-silver nanocomposites and investigation of their antimicrobial activity. *J Colloid Interf Sci* **2007,** *315*, (1), 389-395.

61. Hamilton-Miller, J. M. T.; Shah, S., A microbiological assessment of silver fusidate, a novel topical antimicrobial agent. *Int J Antimicrob Ag* **1996,** 7, (2), 97-99.

62. Nomiya, K.; Yoshizawa, A.; Tsukagoshi, K.; Kasuga, N. C.; Hirakawa, S.; Watanabe, J., Synthesis and structural characterization of silver(I), aluminium(III) and cobalt(II) complexes with 4-isopropyltropolone (hinokitiol) showing noteworthy biological activities. Action of silver(I)-oxygen bonding complexes on the antimicrobial activities. *J Inorg Biochem* **2004,** *98*, (1), 46-60.

63. Sondi, I.; Salopek-Sondi, B., Silver nanoparticles as antimicrobial agent: A case study on E. coli as a model for Gram-negative bacteria. *J Colloid Interf Sci* **2004,** *275*, (1), 177-182.

64. Ruparelia, J. P.; Chatterjee, A. K.; Duttagupta, S. P.; Mukherji, S., Strain specificity in antimicrobial activity of silver and copper nanoparticles. *Acta Biomat* **2008,** *4*, (3), 707-716.

65. Busolo, M. A.; Fernandez, P.; Ocio, M. J.; Lagaron, J. M., Novel silver-based nanoclay as an antimicrobial in polylactic acid food packaging coatings. *Food Addit Contam A* **2010,** *27*, (11), 1617-1626.

66. Fortunati, E.; Armentano, I.; Zhou, Q.; Iannoni, A.; Saino, E.; Visai, L.; Berglund, L. A.; Kenny, J. M., Multifunctional bionanocomposite films of poly(lactic acid), cellulose nanocrystals and silver nanoparticles. *Carbohyd Pol* **2012,** *87*, (2), 1596-1605.

67. Kubacka, A.; Cerrada, M. L.; Serrano, C.; Fernández-García, M.; Ferrer, M.; Fernández-Garcia, M., Plasmonic nanoparticle/polymer nanocomposites with enhanced photocatalytic antimicrobial properties. *J Phys Chem C* **2009,** *113*, (21), 9182-9190.

68. Li, Q.; Mahendra, S.; Lyon, D. Y.; Brunet, L.; Liga, M. V.; Li, D.; Alvarez, P. J. J., Antimicrobial nanomaterials for water disinfection and microbial control: Potential applications and implications. *Water Res* **2008,** *42*, (18), 4591-4602.

Chapter IV

INFLUENCE OF SPECIATION IN THE RELEASE PROFILES AND ANTIMICROBIAL PERFORMANCE OF ELECTROSPUN ETHYLENE VINYL ALCOHOL COPOLYMER (EVOH) FIBERS CONTAINING IONIC SILVER IONS AND SILVER NANOPARTICLES

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ABSTRACT

In the present study, tailor-made ethylene-vinyl alcohol copolymer (EVOH) fibers containing different amounts of antimicrobial silver ions and nanoparticles were developed by electrospinning and subsequent thermal annealing. The morphology of the fibers was examined by scanning and transmission electron microscopy and thermal properties were characterized by differential scanning calorimetry. Speciation and controlled release of silver from the fibers was monitored by anodic stripping voltammetry and energy dispersive X-ray spectroscopy. Before aging, 100% of the silver recovered from the electrospun structures was in ionic form to be instantly released in contact with moisture with varying temperature-dependent kinetics. Thermal annealing of the fibers at 100ºC for 1, 2 and 4 days prompted the gradual transformation of 70%, 93-94% and 98-99% of the total silver into nanoparticles homogeneously distributed along the fibers, which were mostly retained within them, producing a substantial decrease in their release capacity. Speciation and release profiles from the fibers were correlated with their antibacterial performance against *L. monocytogenes* and *Salmonella enterica.* This study is a step forward in the understanding of silver-based electrospun antimicrobial polymers and puts forth the suitability of EVOH for the development of targeted delivery systems in a number of applications.

Keywords: Antimicrobials, EVOH, Nanoparticles, Silver, electrospinning

1. Introduction

Electrospinning is a simple technique to continuously generate ultrafine fibrous mats with fiber diameters ranging from tenths of nanometers to several microns. Their distinct characteristics, like a very high specific surface and porosity, and the suitability of the technique for impregnating other materials within the fibers at the nanoscale level have prompted their use in a wide range of applications [1].

Silver is particularly important due to its unique physical and chemical properties at the nanoscale and high antimicrobial effect. The incorporation of silver in electrospun fibers has enabled the development of new materials with useful features. Ultrafine fibers with enhanced photoconductivity [2], tensile-strength [3] or photocatalytic properties [4-5], materials which act as bio-batteries [6] or biosensors [7] have been produced, as well as fibrous membranes with antibacterial properties with applications in water filtration [8], protective clothing [9], wound dressings, implant materials or tissue engineering [10].

The nanoparticles can be either purchased in its reduced form, or produced from silver salts by physical, chemical or biological reduction [11]. This reduction can take place without addition of any further reducing agents by heat or irradiation treatments after the electrospinning process [12-16]. The annealing of the fibers by heat treatment or UV irradiation also offers the possibility of tuning the size of the nanoparticles by changing the irradiation time [17]. Although many studies have been devoted to the incorporation of silver and silver nanoparticles into polymeric matrices, its speciation is usually neglected in the content analysis or the release studies. In most cases, silver is partially reduced to nanoparticles either during the solvent preparation or the electrospinning process [13,16,18], delivering materials with uncertain content in ions and nanoparticles without a further annealing process. This can affect silver speciation and also the release kinetics of silver ions and silver nanoparticles as active silver species, subsequently altering the antimicrobial performance of the produced fibers.

Moreover, if the presence of nanoparticles is inevitable, application of these materials in restrictive legislation frames could be severely limited. It is therefore crucial to investigate speciation and the factors governing the formation of nanoparticles inside the polymer matrices throughout their fabrication and aging processes. Controlling these factors may not only contribute to estimate the release and subsequent antimicrobial effectiveness, but also to assist in tailoring these parameters to suit a concrete application.

Among the polymers which have been selected for the incorporation of silver by electrospinning we find polyethylene oxide (PEO) [19,10], polyacrylonitriles (PAN) [13,15,20], nylon-6 [21-22] PU, PCL and others [23-24]. These studies are either focused on its suitability for a final aplication, like in implants or scaffolds [18,25,23] or on a thorough chracterization of their morphology [26,19,27-29]. Furthermore, when the release profiles are adressed, only the total content is calculated neglecting speciation [30,15,31-32]. One of the most predominantly used polymer for the incorporation of silver is polyvinylalcohol (PVOH) which has been proposed to be used for both medical [33,32,27,34] and catalytic applications [7,35]. The main advantages for this polymer to be used are good biocompatibility, facile use, easy reduction of the silver ions at low temperatures [17], and controlled release upon contact with moisture. However, it has been shown to readily reduce silver to nanoparticles even before and during the electrospinning process. Furthermore, the high sensitivity to water of this polymer could limit the range of possible applications since it completely dissolves in contact with aqueous media.

Ethylene-vinyl-alcohol copolymers (EVOH) are a family of biodegradable, semicrystalline random copolymers widely used in the food-packaging sector due to their outstanding gas barrier properties, chemical resistance and high transparency. EVOH is non-soluble in water but severely plasticizes upon its contact, which may allow a complete release of its content [36], while maintaining matrix integrity. Electrospun EVOH fibers have also been shown to support the culturing of smooth muscles and fibroblasts [37] and can be used as

a general scaffold for cell growth [38-39]. To the best of our knowledge, only one study has dealt with the incorporation of silver into EVOH fibers [40]. In this study, however, attention was focused on the optimization of the electrospinning parameters and the release after degradation of the polymer on the long term.

In the present study, we report about the fabrication of antimicrobial electrospun EVOH fibrous membranes containing silver ions (EVOH-Ag^{+}). These fibers were thermally annealed at various intervals to yield fibers with increasing content in silver nanoparticles (EVOH-Ag^{o}) with annealing time. The fibers were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and differential scanning calorimetry (DSC). A combination of a voltammetric method and elemental energy dispersive X-ray spectroscopy (EDS) analysis was followed to monitor speciation and release of silver from the fibers with different silver content and at different temperatures. The antimicrobial efficacy of different quantities of the EVOH-Ag^{+} and EVOH-Ag^{o} fibers was evaluated against *L. monocytogenes* and *Salmonella enterica* with a modified broth dilution test as compared to pure silver nitrate. Antimicrobial performance was correlated to speciation and release profiles as to evaluate antibacterial effectiveness of the different active silver species.

2. Materials and Methods

2.1. Preparation of the fibers: Electrospinning process.

An EVOH copolymer with 32% ethylene content was supplied by Nippon Gohsei Corp (Japan). Polymer pellets were dissolved in 2-propanol: water (70:30 w/w) in the ratio 8:92 (w/w) at 100ºC under reflux. The dissolved polymer was cooled down to approx. 60ºC and a suitable amount of silver nitrate (Sigma-Aldrich) was added to the solution as to achieve EVOH fibers with 0.01% - 1% silver nitrate weight in dry conditions. For electrospinning, a FluidNatek® equipment, trademark of the engineering division of BioInicia S.L. (Valencia, Spain), was used. This equipment has a variable high voltage (0-30 kV) power supply. All experiments were carried out in air at 21ºC in a controlled relative humidity chamber at 40 %RH. Electrospinning was performed using a stainless-steel needle with internal diameter 0.9 mm that was connected through a PTFE wire to the EVOH-based solutions kept in a 5 ml syringe. All solutions were electrospun at 13 kV under a steady flow-rate of 0.6 ml/h and the distance between the needles and the collector was set to 10 cm. Resultant electrospun structures were collected on an aluminum foil sheet attached to collector.

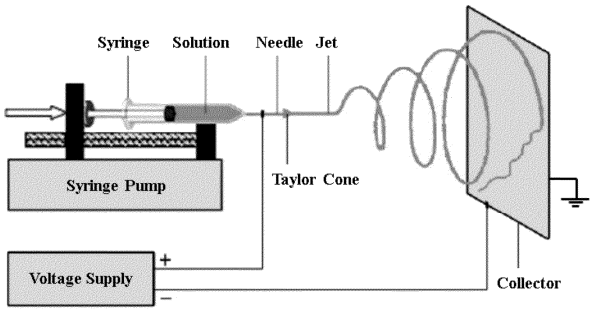

Figure 1. Schematic representation of the electrospinning set-up

2.2. Thermal annealing.

The silver ions incorporated in the fibers were reduced to silver nanoparticles by thermal annealing, in an oven at 100ºC and 0% relative humidity (RH). Thermal treatment was prolonged for 1, 2 and 4 days to produce EVOH-Agº-1d, EVOH-Agº-2d and EVOH-Agº-4d fibers, respectively. The reduction was monitored colorimetrically with a handheld Minolta Chromameter CR300 (Minolta Camera Co., Ltd., Osaka, Japan) set to D65 illuminant/10° observer and using the CIELAB color space (L*, a* and b*). , where L*, a* and b* values represent luminosity, green-red and blue-yellow scale. ΔE* was calculated as a global parameter of color alteration (eq. 1).

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{(Eq. 1)}$$

All fibers were stored in a dessiccator at 0% RH and room temperature until needed and to a maximum of 10 days.

2.3. Bacterial strains and growth conditions.

Bacterial strains *Listeria monocytogenes* CECT 5672 and *Salmonella enterica* CECT 554 (Spanish Type Culture Collection, Valencia, Spain) were selected as Gram positive and Gram negative model bacteria. These strains were grown overnight in Tryptic Soy Broth (TSB) (Conda Laboratories, Madrid, Spain) and an aliquot was again transferred to TSB and grown at 37º C to the mid-exponential phase of growth having an absorbance value of 0.20 for *Salmonella* enterica and 0.15 for *L. monocytogenes* as determined by optical density at 600 nm by ultraviolet visible (UV) spectroscopy using a SP-2000 UV spectrometer (Spectrum Instruments, Shanghai, China). This culture served as the inoculum for antimicrobial assays starting with approximately $5 \cdot 10^5$ CFU/mL.

2.4. Susceptibility tests

For antimicrobial assays against *L. monocytogenes* and *Salmonella enterica*, M9 minimal medium supplemented with 1mM methionine (M9-Met) was used as liquid broth medium. M9 is a minimal medium without any protein sources or

components and 20mM glucose as a sole carbon source. Although the medium is not suitable for the growth of Gram positive bacteria, previous findings evinced *L. monocytogenes* CECT 5672 can grow well if only methionine is supplemented [36]. Susceptibility tests were performed employing the macro-dilution method M26-A described by the Clinical and Laboratory Standards Institute (CLSI) with modification. A bacterial suspension in mid-log phase was inoculated into tubes with 10 mL M9-Met to achieve an initial inoculum size of approximately 5 x 10^5 CFU/mL. The antimicrobial effectiveness of the EVOH-Ag^+ and EVOH-Agº fibers was assessed by introducing 0.5-50 mg of fibers with 0.01-1% silver into the test tubes and incubating them at 37ºC for 24 h Then, 0.1 mL of each M9 sample was sub-cultivated on TSA plates for viable count. These results were compared with EVOH-Ag^+ and EVOH-Agº fibers electrospun under the same conditions but without silver and also with samples containing different concentrations of aqueous silver nitrate. Each of these experiments was performed in triplicate.

2.5. Surface morphology and particle size

The morphology of the fibers was examined using SEM. The SEM images were taken with an Hitachi S-4100 electron microscope using a gold–palladium mixture under vacuum for sputtering. Experiments were carried out at an accelerating voltage of 15 kV. The morphology and distribution of the nanoparticles in the fibers was studied by means of TEM, using a JEOL 1010 at an accelerating voltage of 20kV. Estimation of the fiber and nanoparticle dimensions was done by means of the ImageJ software from 200 fibers or 500 nanoparticles at random.

2.6. Thermal properties

Thermal properties were studied by DSC using a Perkin–Elmer DSC-7 calorimeter (Perkin–Elmer Cetus Instruments, Norwalk, CT). The rate of heating was 10°C/min from 30ºC to 220ºC, where a typical sample weight was around 2 mg. Peak height and peak area in the thermogramm were ascribed to the melting

point (T_m) and melting enthalpy (ΔH_f), respectively. Calibration was performed using an indium sample. All tests were carried out in triplicate.

2.7. Release study

A voltammetric method was followed to determine the release of free silver ions (FSI) from the fibers to an aqueous environment. The silver content selected for the release study was 0.1%, as it was the lowest concentration for which sensitivity and reproducibility were assured for release percentages above 1%. To this end, 100 mg of the EVOH-Ag^+ and EVOH-Agº fibers with 0.1% silver content were immersed in 10 mL distilled water at 2ºC and 22ºC for 24h in semi-static conditions. For each measurement, 0.1 mL from the samples was collected and the concentration adjusted for the remaining solution volume. The FSI content for each measurement was determined by differential pulse ASV with an Autolab III (EcoChemie) potentiostat setup under conditions stated in Metrohm application bulletin nº 207/2e "Analysis of silver by stripping voltammetry". The FSI working range was 0.004 – 0.4 ppm and a calibration curve was prepared daily for each set of measurements. All experiments were carried out in duplicate.

2.8. Silver content analysis

The total contents of silver in the fibers were semiquantitatively determined by EDS using a Si (Li) detector (EDAX, NJ, USA). Three spectra were collected from each surface employing an area scan mode under 20 kV accelerating voltage, 10 μA beam current, 1000-1500 counts/sec and 100 s acquisition time.

3. Results and Discussion

3.1. Susceptibility tests of EVOH-Ag+ fibers

Silver is known to be easily inactivated by complexing with different compounds present in natural organic matter (NOM), like for example sulphur groups in proteins [41-42]. Previously published reports have proposed M9 minimal medium as a suitable substrate of reference to assess the full potential of silver based antimicrobial systems, preventing silver inactivation by NOM while allowing bacterial proliferation of the tested strains [43]. Under these conditions, the antimicrobial effectiveness of increasing amounts of EVOH-Ag^+ fibers with 0.01%, 0.1% and 1% silver content was measured against *L. monocytogenes* (Fig. 2a) and *Salmonella enterica* (Fig. 2b).

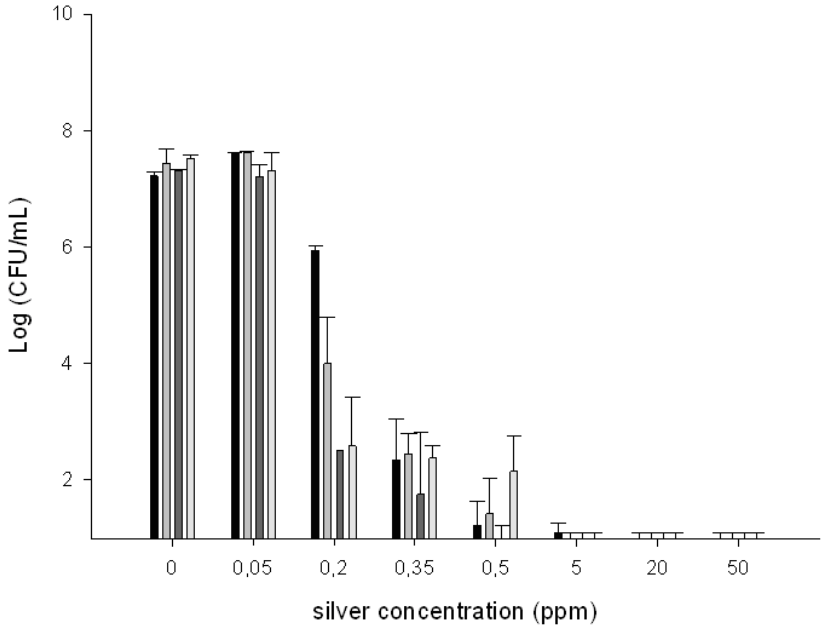

Figure 2a. Susceptibility assays at 37ºC in M9-Met minimal medium of *L. monocytogenes* to increasing concentrations of silver aqueous solutions and EVOH-Ag^+ fibers with 0.01% , 0.1% and 1% silver assuming a 100% release (n=3).

Results are presented as a function of silver nitrate concentration in solution assuming a 100% release from the fibers. Effectiveness of aqueous silver nitrate solutions is also given for comparison purposes and is in line with other reports on silver efficacy in synthetic water or salt buffers [44-46] Controls without silver are able to grow to a maximum of about 10^7 and 10^9 CFU/mL for *L. monocytogenes* and *Salmonella enterica* under the stated conditions, respectively. Viable bacterial concentration decreases with increasing silver content for all EVOH-Ag^+ fibers as well as for silver nitrate solutions.

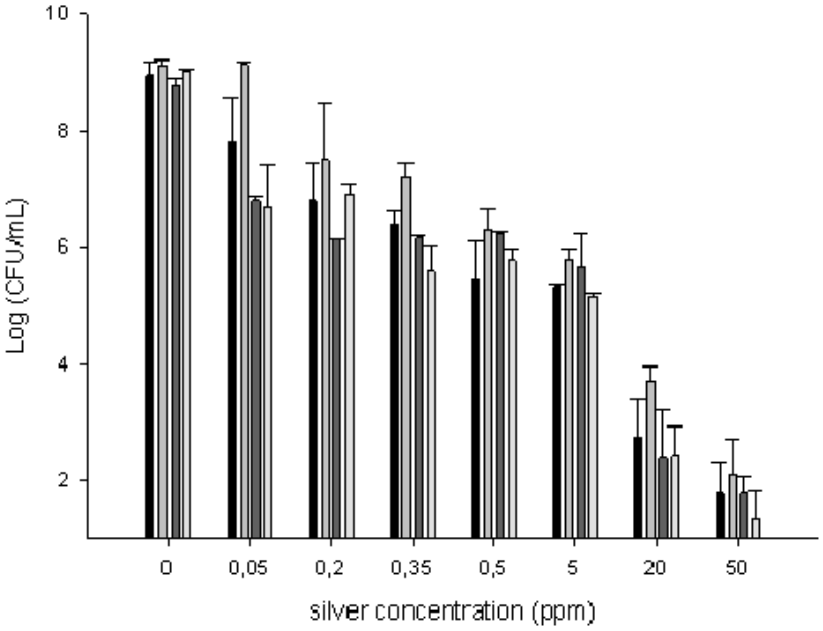

Figure 2b. Susceptibility assays at 37ºC in M9-Met minimal medium of *S. enterica* to increasing concentrations of silver aqueous solutions and EVOH-Ag^+ fibers with 0.01% , 0.1% and 1% silver assuming a 100% release (n=3).

For *L. monocytogenes*, no noticeable differences were observed between samples with the same silver concentration, either in aqueous form or in form of EVOH-Ag^+ fibers, except for 0.2ppm silver. This evidences that the different load of silver or the amount of fiber introduced in the tubes does not influence antimicrobial efficacy, this being mostly dependant on the total amount of silver

present in any of the samples tested. A bactericidal effect, that is, a reduction of 3 log units compared to the initial inoculum according to the Clinical and Laboratory Standards Institute (CLSI), is achieved with concentrations ≥0.35 ppm, indicating high efficiency of the fibers against *L. monocytogenes*. Interestingly, at the threshold concentration of 0.2 ppm, the antimicrobial effectiveness is greater in EVOH-Ag^+ fibers, producing a decrease in viable counts of 1-3 log, as compared to pure silver nitrate solutions, which only prevents proliferation reaching similar viable counts as the initial inoculum. This indicates the potential of antimicrobial silver is being more efficiently exploited when incorporated in the electrospun copolymer. This phenomenon could be associated to the inactivation of silver by the nutrient broth or by the microorganisms themselves, as proposed previously [43].

In the case of *Salmonella*, again, viable counts are similar among the different EVOH-Ag^+ fibers or aqueous silver nitrate solutions with equal silver content. However, inhibitory effect is only achieved with concentrations above 0.35 ppm silver and no decrease in viable counts is noted with concentrations ≤5 ppm as compared to the initial inoculum. In both bacteria, but much more so with the Gram negative *Salmonella*, the reduction of viable counts with increasing concentrations is rather gradual. As an example, 0.05 ppm of silver in some of the forms tested are already able to inhibit the growth of *Salmonella* cultures to some extent as compared to the controls. However, after increasing the silver concentration by a 100-fold, the difference in viable counts is nearly negligible in some cases. This event is probably related to an unspecific mechanism of action and emphasizes the difficulty in establishing breakpoints for silver-based antibacterial products as stated by [47].

3.2. Susceptibility tests of EVOH-Ag^0 fibers

The same procedure was applied for testing the antibacterial efficacy of the fibers thermally annealed for 1, 2 and 4 days (EVOH-Ag^0-1d, EVOH-Ag^0-2d and EVOH-Ag^0-4d fibers, respectively) with 0.1% silver content (Fig. 3). Results for aqueous silver nitrate solutions and for as electrospun EVOH-Ag^+

fibers are given for comparison as to evaluate how the incorporation and prolonged thermal treatment of the fibers may affect their biocidal properties. As before, a gradual decrease in viable counts is noted as silver concentration in any of the forms tested increases. However, a slight decrease in the antimicrobial efficacy of the EVOH-Ag^0 fibers is observed as the thermal treatment is

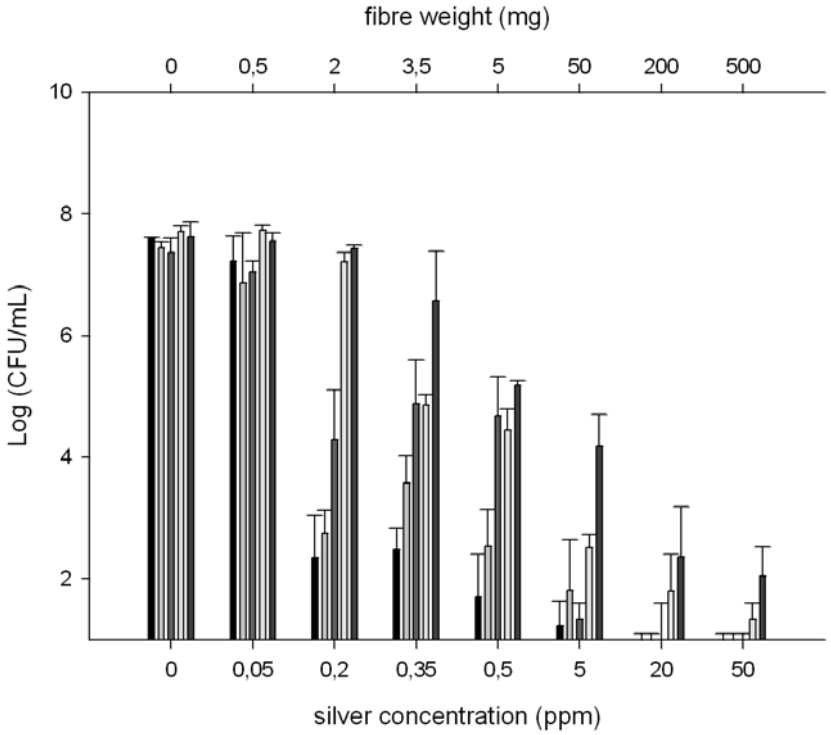

Figure 3a. Susceptibility assays in M9-Met minimal medium of *L. monocytogenes* to equal amounts of silver in form of aqueous solutions ■ (bottom axis) and EVOH-Ag^+ ▭, EVOH-Ag^0-1d ▬, EVOH-Ag^0-2d ▭ and EVOH-Ag^0-4d ▬ fibers (top axis) (n=3).

prolonged as compared to EVOH-Ag^+ fibers. This effect is most predominantly observed with *L. monocytogenes* (Fig. 3a), for instance, when looking at concentration pairs of 100-fold difference like 0.2 and 20 ppm or 0.5 and 50 ppm. In these cases, viable counts with EVOH-Ag^0-4d fibers are similar to those

achieved with 100-fold less concentration when EVOH-Ag^+ or pure silver nitrate is tested. This evidences antibacterial efficacy of the EVOH-Ag^0 fibers gradually decreases with thermal annealing. This decrease is probably related to the formation of silver particles from the ions, which most likely do not significantly migratefor the relatively short time tested [48]. In longer terms, gradual degradation of the EVOH matrix may allow these nanoparticles to sustainably migrate, as suggested by [40]. This second mechanism of migration may be very useful in the preparation of materials with a sustained release of the antimicrobial in addition to the initial burst release.

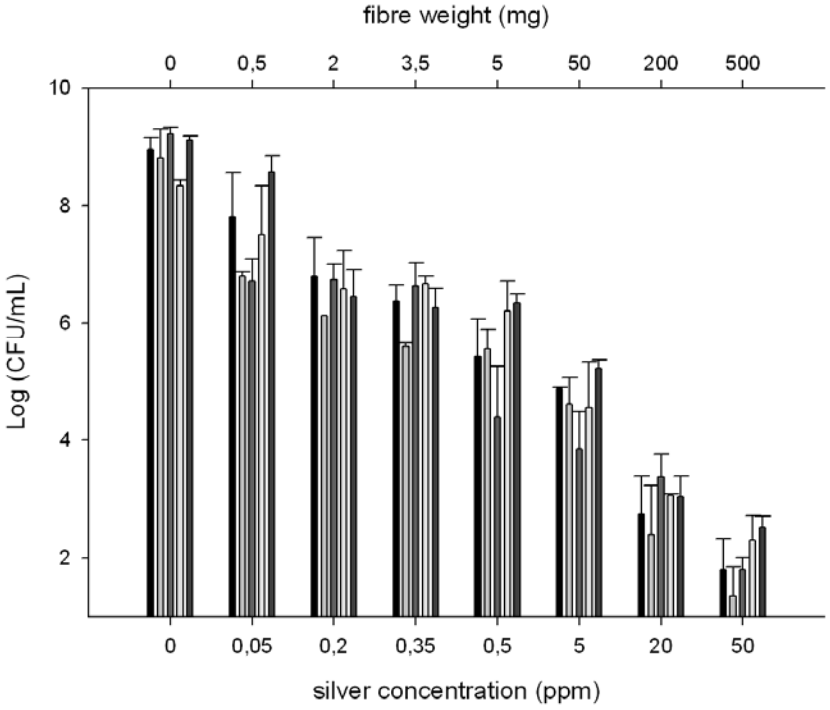

Figure 3b. Susceptibility assays in M9-Met minimal medium of *S. enterica* to equal amounts of silver in form of aqueous solutions (bottom axis) and EVOH-Ag^+ , EVOH-Ag^0-1d , EVOH-Ag^0-2d and EVOH-Ag^0-4d fibers (top axis).

3.3. Color measurements

It is known that silver exhibits surface plasmon resonance, a unique property attributed to collective excitation of the electron gas in the particles with a periodic change in electron density at the surface. When the colloidal particles are smaller than the wavelength of visible light, the impregnated materials can therefore acquire a yellow to brownish color. Color analysis can consequently be

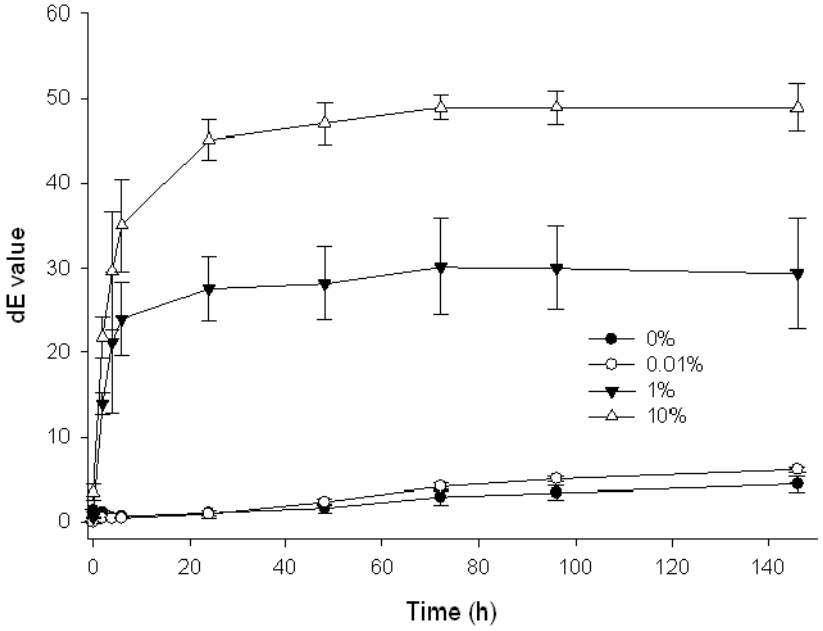

Figure 4. Color alteration of neat EVOH-Ag fibers and fibers with 0.01%, 1% and 10% silver as a function of annealing time (n=3).

an efficient and easy tool to monitor the reduction process. The color patterns of as electrospun EVOH-Ag^+ fibers without and with 0.01%, 1% and 10% silver nitrate was examined before and throughout the annealing process using the CIELAB color space. Fig. 4 illustrates the evolution of color alteration of the samples (ΔE). For visual orientation, digital images of EVOH-Ag^+ and EVOH-Ag^0-4d with 0%, 1% and 10% silver are shown in Fig. 4. According to L*, a*

and b* values, all as electrospun fibers display no significant differences among each other, indicating that silver ions were not significantly reduced during the preparation and electrospinning process of the fibers. Furthermore, the incorporation of a high load of silver ions did not affect the appearance of the polymer fibers (Fig. 5). Yellowness, as the main color indicator for reduction of silver, greatly increases in samples with higher (0.1%, 1% and 10%) load of silver, at the expense in luminosity, producing a considerable change in the visual appearance of the fibers (Fig 4).

Figure 5. Typical photographs of fiber mats of EVOH-Ag^{+} (up) and EVOH-Ag^{0}-4d (below) with (left to right) 0%, 0.1%, 1% and 10% silver.

In parallel, the aging of the fibers at high temperatures produces an additional slight increase in yellow and green color and a decrease in luminosity in all fibers, either with or without silver. This slight discoloration, visually not appreciable, could be attributed to degradation of the fibers during the annealing process, which is in line with thermal analysis (see below). Color alteration (ΔE) in samples with silver is mainly governed by the increase in yellowness, attributed to the reduction of silver ions to nanoparticles. As can be observed in

Fig. 4, this process is hyperbolic, which implies that ions are easily reduced at the beginning, but the reduction of the remnant of ions becomes more difficult as their presence decreases.

3.4. Surface morphology

Efficient electrospinning depends on many different factors, such as viscosity, conductivity or surface tension. One of the most important factors which govern these aspects is the polymer concentration in the electropinning solution. Although EVOH32 is not soluble in the 2-propanol/water solutions below 70ºC, it was possible to carry out the electrospinning process at room temperature, owing to the slow kinetics of precipitation of EVOH32 in the solvent mixture. It has been suggested that this thermodynamic instability of the polymer provides an additional, early stabilization of the jet to form the fibers, allowing to easily and efficiently electrospin EVOH copolymers [37]. It is generally accepted that increasing the concentration of the polymer produces an increase in the diameter of the fibers [37]. According to preliminary assays, above polymer concentrations of 9-10% average diameter of the fibers surpassed the micron size. Additionally, precipitation was significantly increased above 9% concentration, considerably decreasing the yield of electrospun fibers. On the other hand, if the viscosity is too low to offset the surface tension, the formation of beads on the fibers will predominate. Considering these factors, concentration of the polymer for electrospinning was set to 8% weight, as to obtain continuous fibers with few o no beads and in the submicron diameter range.

SEM images of the EVOH-Ag^{+} and EVOH-Ag^{0}-4d fibers without and with 10% silver and their corresponding average diameters are shown in Fig. 6. A significant decrease in the diameter of the fibers is observed when silver is incorporated. This effect has been previously described on other materials, and is attributed to the increase in charge density and conductivity, which produces an increase in the stretching forces in the jet, consequently decreasing the fiber diameter [49,18,29,50-51]. Although to a lesser extent, the heat treatment also decreases the diameter of the fibers significantly. This effect has been attributed to evaporation of solvent and moisture around the fibers [32,13].

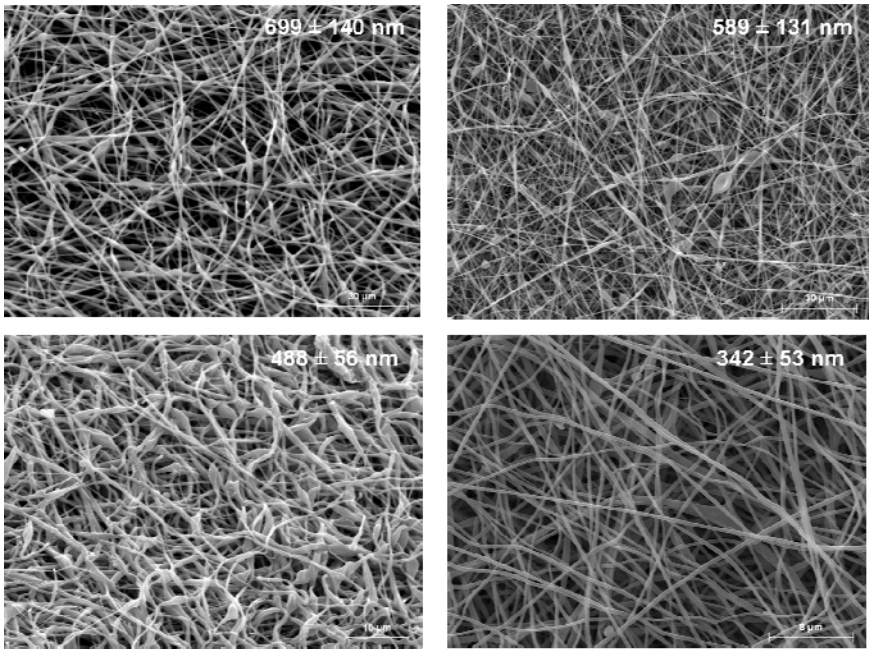

Figure 6. SEM morphologies of EVOH-Ag^{+} (left) and EVOH-Ag^{0}-4d fibers (right) without (above) and with 10% (below) silver content. The numbers refer to the average diameter of the fibers. Scale bars correspond to 30 μm (above), 10 μm (left) and 8 μm (right).

3.5. Silver morphology

The distribution, size and size distribution of silver nanoparticles inside the fibers as examined by TEM is illustrated in Fig. 7. No particles can be observed in neat electrospun fibers (a), whereas spherical nanoparticles of different sizes are detected on all EVOH-Ag fibers. It is generally accepted that rapid high energy reducing treatments, either physical or chemical, produce smaller particles, while prolonged heat or UV treatment induces the gradual growth of the produced nanoparticles [17,12-13,10]. In as electrospun EVOH-Ag^{+} fibers, very small nanoparticles of 3.03±1.05 nm average diameter in a narrow size distribution are homogeneously dispersed along the polymer matrix (b, c). Silver nanoparticles of similar characteristics have already been observed in as

electrospun fibers of other materials. The presence of reduced nanoparticles in these materials is mainly attributed to a reduction of silver in the solvent mixture previous to electrospinning, which is in all cases associated with a yellowing of the solution and of the resulting fibers. This phenomenon is reported when solvents with a relative reducing capacity, like dimethylformamide (DMF) were used [13,20,18,16], although it has also been observed in PVOH or PVP aqueous solutions [52], perhaps because the polymer itself has some relative reducing capacity. In this last case, this inconvenient reduction has been surmounted by decreasing the pH, yielding white colored fibers.

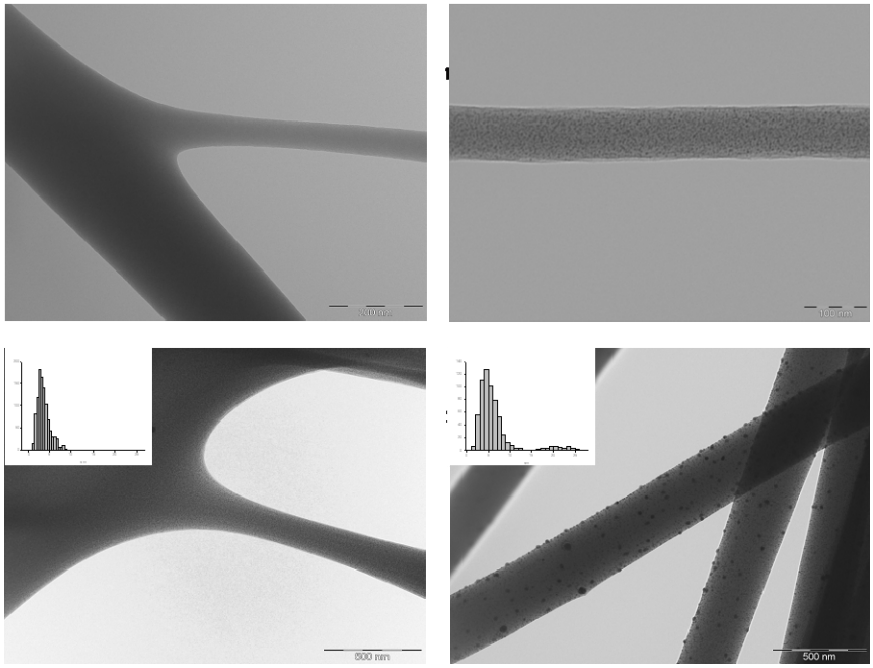

Figure 7. TEM images of electrospun EVOH without silver (a), EVOH-Ag^{+} (b, c) and EVOH-Ag^{0}-4d fibers (d) with 10% silver content. The images insets display the size distribution of the nanoparticles. Scale bars correspond to 200 nm (a), 100 nm (b) and 500 nm (c, d)

We found, however, no visually or colorimetrically significant differences between as electrospun fibers with or without silver. Therefore, the possibility of

a rapid reduction of ions by the highly energetic electron beam during the irradiation process could also be taken into consideration, as it would correlate with subsequent described results. In EVOH-Ag^0 fibers, however, two different populations of nanoparticles are observed (d). The first group consists of nanoparticles of about the same size as the ones observed in EVOH-Ag^+ fibers (4.12±1.72 nm; for visual comparison, EVOH-Ag^+ and EVOH-Ag^0 fibers are shown at the same magnification in c and d, respectively). This group is probably also ionic silver that has been reduced during the TEM experiments. The second population of particles is of about 20.95±2.94 nm diameter. These much bigger particles may possibly be the result of reduced particles that have been grown during the annealing process. The increase in the size of silver nanoparticles as a result of thermal annealing or irradiation with UV light has been reported previously [16-17,31,13]. This evidences thermal annealing can be an easy and efficient process to produce homogeneously dispersed silver nanoparticles with controlled size.

3.6. Thermal properties

Thermal properties as measured by DSC of EVOH-Ag^+ and EVOH-Ag^0-4d fibers with increasing silver content are shown in Table 1. The melting point (T_m) and melting enthalpies (ΔH_m) were calculated from the maximum temperature and peak area, respectively, of the peak associated with the melting process. In EVOH-Ag^+ fibers, no significant differences are observed among samples with silver contents of ≤1%. Values for EVOH-Ag^+ fibers with 10% silver could not be accurately calculated by means of DSC because the exothermic peak ascribed to the reduction of silver possibly overlaps with the melting endotherm of the polymer. When thermal annealing is applied to the fibers, the melting points slightly decrease, and there is a considerable increase in the melting enthalpies of the materials. The decrease in T_m could be attributed to partial degradation of the fibers during the thermal treatment, which is in line with colorimetric results. This increase in ΔH_m is probably due to an increase in the crystalline fraction during the annealing process and is noted in all thermally treated fibers regardless of its silver content. Therefore, no significant differences in T_m and

ΔH_m were observed among samples annealed with silver content ≤1%. EVOH-Ag^0-4d fibers with 10% display significantly lower T_m and ΔH_m. This, however, could either be associated to inhibition of crystallization in the fibers at such high loads of silver, or/and to the effect of reduction of the remaining ions during the heating run, which would overlap and distort the peak associated with the melting of the polymer.

Table 1. Thermal properties of EVOH-Ag^+ and EVOH-Ag^0-4d fibers (n=2).

Sample	*% silver content*	T_m (ºC)	ΔH (J/g)
EVOH-Ag^+ fibers	0	184.6 A[a]	70.4 A
	0.01	184.5 A	69.8 A
	0.1	183.1 AB	67.5 A
	1	182.5 AB	70.6 A
EVOH-Ag^0 -4d fibers	0	181.5 B	77.8 B
	0.01	181.0 B	77.6 B
	0.1	182.3 BC	76.7 B
	1	182.4 BC	78.1 B
	10	176.2 C	56.0 C

[a] Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples as determined with a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests

3.7. Release study

The antibacterial efficacy of silver-based products relies on the release of active silver species to the target aqueous environment where bacteria dwell. ASV has been shown to be a useful tool to determine trace amounts of FSI in solution [53-54]. Reduced silver particles are on the other hand voltammetrically undetectable. Accordingly, ASV offers the possibility to investigate the release profiles of silver from the fibers, while at the same time providing an insight on silver speciation throughout the annealing process. The release profiles of FSI was examined in EVOH-Ag^+ and all EVOH-Ag^0 fibers after being immersed in ultrapure water (Fig. 8). EVOH-Ag^+ fibers are able to release about 95-100% of their content in form of FSI almost instantly upon immersion. This very rapid release is probably the result of, first, the instantaneous plasticization of the

polymer after it is immersed and, second, the enormous surface to volume ratio of the electrospun fiber mats. The latter explains the higher release capacities of the fibers compared to the material in form of cast films [36]. Other hydrophilic materials with good release yields, like PVOH, have the disadvantage of being very sensitive to moisture. This inconvenience is usually surmounted with a thermal annealing of the fibers. In most cases, however, ions will be partially reduced to elemental silver in the preparation or thermal annealing process, reducing the quantity of the active silver ions available for release, hence altering the release profiles as well as the antimicrobial efficacy of the materials. Moreover, concerns about nanoparticle migration could severely limit application of these materials in restrictive legislation frames. The unique release profile of EVOH-Ag^+ fibers offers the possibility of assessing their whole silver content in form of antimicrobial silver ions to be instantly delivered after targeted release in contact with moisture. This release mechanism may be useful in a number of applications, such as tissue engineering, food packaging or other targeted delivery systems.

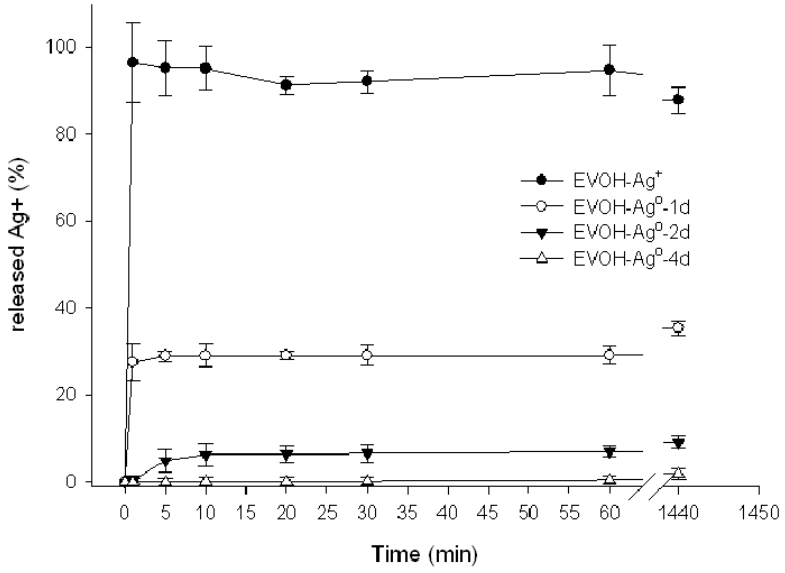

Figure 8. Release profiles of silver ions in the various samples (n=2).

When thermal annealing is applied to the fibers, ions are partially reduced to silver particles, which cannot be detected voltammetrically. Therefore, the % of FSI reached at equilibrium decreases with increasing annealing time. EVOH-Agº-1d fibers reach equilibrium within 5 min inmersion in water, delivering about 30% of silver in form of FSI. In the case of EVOH-Agº-2d fibers, a maximum delivery of 6-7% of the silver is reached after 10 minutes. Finally, for EVOH-Agº-4d fibers, silver ion delivery is below 0.5% within the first hour, reaching values around 1-2% over the tested period of 24h. A slight decrease in the release capacities of the fibers is noted with increasing annealing time, which could be attributed to the formation of bigger crystals during the annealing process, which would hinder the movement of the polymer chains and would accordingly slow down the release (Table 1). The increasing presence of the hydrophobic silver nanoparticles could also contribute in this direction. Considering the whole FSI content in the fibers has been released once equilibrium is reached, it can be concluded that after 1, 2 and 4 days of thermal treatment, about 70%, 94-95% and 98-99%, respectively, of the silver content in the fibers has been reduced. This once more evinces the suitability of thermal annealing as an easy and efficient process to produce silver nanoparticles. However, reduction of the remnant of ions becomes more difficult as their presence decreases, as seen in colorimetric measurements (Fig. 4), which points towards reduction kinetics of first-order. This implies that, even after prolonged thermal treatments, a residual amount of silver ions might still be present which should be taken into consideration in the design and evaluation of this kind of materials.

The influence of the temperature on the release of FSI was also studied. Preliminary assays demonstrated that increasing the temperature above 40ºC gradually produced a decrease in FSI release, probably due to the chemical reduction of the biocide. On the other hand, differences between samples at room temperature (22ºC) or refrigerated samples could be found especially at temperatures near to freezing point (data not shown). Therefore, temperatures of

1-2ºC and 22ºC were selected to analyse the release of FSI from EVOH-Ag^+ and EVOH-Ag^0-2d as examples for untreated and treated fibers (Fig. 9). Whereas at 22ºC the maximum release of FSI was about 95-100% and 6-7% for EVOH-Ag^+ and EVOH-Ag^0-2d, respectively, the same samples are only able to release about 70% and 4-6% of the FSI, respectively at 1-2ºC. Furthermore, the kinetics of release decreased at 2ºC as compared to samples at room temperature, reaching equilibrium in 5-10 min and 2-3 hours at 2ºC , respectively, instead of <1 min and 10-20 min at 22ºC. These differences may be explained by the lower diffusion and solubility coefficients of aqueous solutions in EVOH with decreasing temperature as reported previously [55].

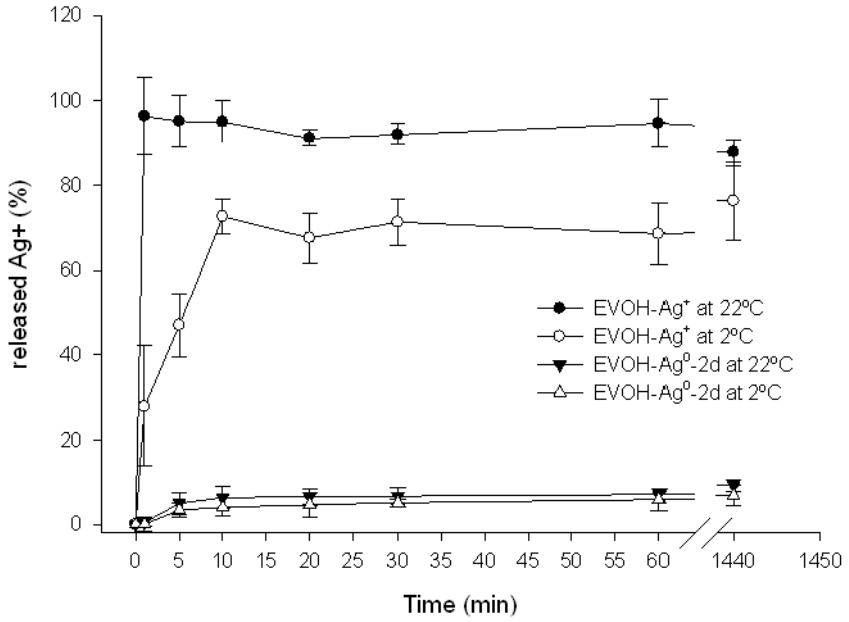

Figure 9. Release profiles of silver ions from EVOH-Ag^+ and EVOH-Ag^0-2d fibers at 2ºC and 22ºC temperature (n=2).

3.8. Silver content analysis

The total silver content, regardless if it is in its ionic or reduced form, in EVOH-Ag^+ and EVOH-Ag^0-4d fibers was semiquantitatively determined by EDS before and after the release study in order to compare the total amount of silver with the

amount of ions released and have an insight on speciation of silver in the samples. In control fibers without silver, the signal was negligible (0.009%). In as electrospun EVOH-Ag^+ fibers, 97± 3.8 % % of the theoretically incorporated silver was retrieved. However, after the fibers had been immersed in water for 24h, no signal for silver was detected (0.013%), indicating the whole content had been released to the aqueous solution. EVOH-Agº-4d before and after immersion retained 93.7 ± 8.8 % and 82.8 ± 2.3 % silver, respectively. Differences between these samples were not found significant according to Tukey's comparison test ($p< 0.05$).

Considering both voltammetric and elemental EDS microanalysis results, it is evidenced that the entirety of silver in EVOH-Ag^+ fibers is in ionic form. These ions are very rapidly released once in contact with an aqueous environment, which ensures the outstanding antimicrobial potential of silver ions is being fully exploited. At the same time, the absence of elemental silver confirms that EVOH could be a very useful carrier of antimicrobial silver ions for targeted delivery in application frames restricting silver at the nanoscale. On the other hand, in EVOH-Agº-4d silver is predominantly in form of reduced nanoparticles, a very small remnant of ions remaining in the inside. The release of solid nanoparticles from polymeric substrates which do not dissolve is very unlikely in that relatively short time [48]. As a result, almost the whole silver content is retained in the fibers after these have been immersed in water for 24h. These results could explain the decrease in antimicrobial efficacy of the fibers with increasing annealing time. As silver nanoparticles are not able in principle to migrate from the fibers to the growth medium, their antimicrobial effect is expected to be the result of the released ionic species, which are incredibly active even at very low concentrations [36]. The above results correlate well with the antimicrobial performance presented in Fig. 3a. Wang et al. reported that the degradation of EVOH in longer terms could trigger the controlled and sustaind release of the nanoparticles. This would allow the fabrication of materials with two release mechanisms, an initial burst release activated upon targeted contact with moisture and a second mechanism based on sustasined migration of nanoparticles upon gradual degradation of the polymer.

4. Concluding Remarks

Although many studies have been devoted to the incorporation of silver and silver nanoparticles into polymeric matrices, its speciation is usually neglected in the content analysis or the release studies. This work aimed at the development and characterization of antimicrobial EVOH fibers with silver ions and silver nanoparticles, emphasizing the evaluation of silver speciation and controlled release. 95-100% of the silver was recovered inside the fibers in its ionic form after the electrospinning process without further treatments. The entirety of these ions was found to be instantly delivered after targeted release in contact with moisture.In thermally annealed fibers, silver ions were partially transformed into nanoparticles depending on the time of treatment. The thermal treatment increased the crystallinity of the materials while their ion release capacities drastically decreased. Silver nanoparticles were found to be mostly mostly retained in the polymer matrix although a delayed and sustained release of these may be possible upon degradation of the polymer. Nevertheless, thermally treated fibers also exhibited high antimicrobial efficacy which was ascribed to the remaining silver ions in the fibers, in agreement with the antimicrobial tests. Hence, the presence of a very small fraction of silver ions in these materials may overestimate the antimicrobial effectiveness of silver nanoparticles. These results point out the importance of speciation in the release and efficiency profiles of silver based antibacterial systems. Additional research on the controlled release of nanoparticles on the long term is required before practical use.

5. Acknowledgements

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6. References

1. Li D, Xia Y (2004) Electrospinning of nanofibers: Reinventing the wheel? Advanced Materials 16 (14):1151-1170

2. Wu H, Lin D, Zhang R, Pan W (2007) Facile synthesis and assembly of Ag/NiO nanofibers with high electrical conductivity. Chemistry of Materials 19 (8):1895-1897

3. Francis L, Giunco F, Balakrishnan A, Marsano E (2010) Synthesis, characterization and mechanical properties of nylon-silver composite nanofibers prepared by electrospinning. Current Applied Physics 10 (4):1005-1008

4. Jin M, Zhang X, Nishimoto S, Liu Z, Tryk DA, Emeline AV, Murakami T, Fujishima A (2007) Light-stimulated composition conversion in TiO₂-based nanofibers. Journal of Physical Chemistry C 111 (2):658-665

5. Patel AC, Li S, Wang C, Zhang W, Wei Y (2007) Electrospinning of porous silica nanofibers containing silver nanoparticles for catalytic applications. Chemistry of Materials 19 (6):1231-1238

6. Baptista AC, Martins JI, Fortunato E, Martins R, Borges JP, Ferreira I (2011) Thin and flexible bio-batteries made of electrospun cellulose-based membranes. Biosensors and Bioelectronics 26 (5):2742-2745

7. Wu H, Hu L, Rowell MW, Kong D, Cha JJ, McDonough JR, Zhu J, Yang Y, McGehee MD, Cui Y (2010) Electrospun metal nanofiber webs as high-performance transparent electrode. Nano Letters 10 (10):4242-4248

8. Botes M, Cloete TE (2010) The potential of nanofibers and nanobiocides in water purification. Critical Reviews in Microbiology 36 (1):68-81

9. Pant HR, Bajgai MP, Nam KT, Seo YA, Pandeya DR, Hong ST, Kim HY (2011) Electrospun nylon-6 spider-net like nanofiber mat containing TiO₂ nanoparticles: A multifunctional nanocomposite textile material. Journal of Hazardous Materials 185 (1):124-130

10. An J, Zhang H, Zhang J, Zhao Y, Yuan X (2009) Preparation and antibacterial activity of electrospun chitosan/poly(ethylene oxide) membranes containing silver nanoparticles. Colloid and Polymer Science:1-10

11. Tolaymat TM, El Badawy AM, Genaidy A, Schekel KG, Luxton TP, Suidan M (2010) An evidence-based environmental perspective of manufactured silver nanoparticle in syntheses and applications: A systematic review and critical appraisal of peer-reviewed scientific papers. Science of the Total Environment 408 (5):999-1006

12. Chun JY, Kang HK, Jeong L, Kang YO, Oh JE, Yeo IS, Jung SY, Park WH, Min BM (2010) Epidermal cellular response to poly(vinyl alcohol) nanofibers containing silver nanoparticles. Colloids and Surfaces B: Biointerfaces 78 (2):334-342

13. Rujitanaroj PO, Pimpha N, Supaphol P (2010) Preparation, characterization, and antibacterial properties of electrospun polyacrylonitrile fibrous membranes containing silver nanoparticles. Journal of Applied Polymer Science 116 (4):1967-1976

14. Li Z, Huang H, Shang T, Yang F, Zheng W, Wang C, Manohar SK (2006) Facile synthesis of single-crystal and controllable sized silver nanoparticles on the surfaces of polyacrylonitrile nanofibres. Nanotechnology 17 (3):917-920

15. Sichani GN, Morshed M, Amirnasr M, Abedi D (2010) In situ preparation, electrospinning, and characterization of polyacrylonitrile nanofibers containing silver nanoparticles. Journal of Applied Polymer Science 116 (2):1021-1029

16. Jeon HJ, Kim JS, Kim TG, Kim JH, Yu WR, Youk JH (2008) Preparation of poly(ε-caprolactone)-based polyurethane nanofibers containing silver nanoparticles. Applied Surface Science 254 (18):5886-5890

17. Jin WJ, Jeon HJ, Kim JH, Youk JH (2007) A study on the preparation of poly(vinyl alcohol) nanofibers containing silver nanoparticles. Synthetic Metals 157 (10-12):454-459

18. Sheikh FA, Barakat NAM, Kanjwal MA, Chaudhari AA, Jung IH, Lee JH, Kim HY (2009) Electrospun antimicrobial polyurethane nanofibers containing silver nanoparticles for biotechnological applications. Macromolecular Research 17 (9):688-696

19. Penchev H, Paneva D, Manolova N, Rashkov I (2010) Hybrid nanofibrous yarns based on N-carboxyethylchitosan and silver nanoparticles with antibacterial activity prepared by self-bundling electrospinning. Carbohydrate Research 345 (16):2374-2380

20. Lee DY, Lee KH, Kim BY, Cho NI (2010) Silver nanoparticles dispersed in electrospun polyacrylonitrile nanofibers via chemical reduction. Journal of Sol-Gel Science and Technology 54 (1):63-68

21. Dong H, Wang D, Sun G, Hinestroza JP (2008) Assembly of metal nanoparticles on electrospun nylon 6 nanofibers by control of interfacial hydrogen-bonding interactions. Chemistry of Materials 20 (21):6627-6632

22. Park SW, Bae HS, Xing ZC, Kwon OH, Huh MW, Kang IK (2009) Preparation and properties of silver-containing nylon 6 nanofibers formed by electrospinning. Journal of Applied Polymer Science 112 (4):2320-2326

23. Grasl C, Bergmeister H, Stoiber M, Schima H, Weigel G (2010) Electrospun polyurethane vascular grafts: In vitro mechanical behavior and endothelial adhesion molecule expression. Journal of Biomedical Materials Research - Part A 93 (2):716-723

24. Kanjwal MA, Barakat NAM, Sheikh FA, Baek W, Khil MS, Kim HY (2010) Effects of silver content and morphology on the catalytic activity of silver-grafted titanium oxide nanostructure. Fibers and Polymers 11 (5):700-709

25. Lin D, Wu H, Zhang R, Pan W (2009) Enhanced photocatalysis of electrospun Ag-ZnO heterostructured nanofibers. Chemistry of Materials 21 (15):3479-3484

26. Costa RGF, Ribeiro C, Mattoso LHC (2010) Preparation and characterization of PVA-Ag nanocomposite fibers with antibacterial activities. Science of Advanced Materials 2 (2):157-162

27. Son B, Yeom BY, Song SH, Lee CS, Hwang TS (2009) Antibacterial electrospun chitosan/poly(vinyl alcohol) nanofibers containing silver nitrate and titanium dioxide. Journal of Applied Polymer Science 111 (6):2892-2899

28. Lee S (2009) Multifunctionality of layered fabric systems based on electrospun polyurethane/zinc oxide nanocomposite fibers. Journal of Applied Polymer Science 114 (6):3652-3658

29. An J, Zhang H, Zhang J, Zhao Y, Yuan X (2009) Preparation and antibacterial activity of electrospun chitosan/ poly(ethylene oxide) membranes containing silver nanoparticles. Colloid and Polymer Science 287 (12):1425-1434

30. Horzum N, Boyaci E, Eroğlu AE, Shahwan T, Demir MM (2010) Sorption efficiency of chitosan nanofibers toward metal ions at low concentrations. Biomacromolecules 11 (12):3301-3308

31. Hong KH (2007) Preparation and properties of electrospun poly (vinyl alcohol)/silver fiber web as wound dressings. Polymer Engineering and Science 47 (1):43-49

32. Hong KH, Park JL, Hwan Sul IN, Youk JH, Kang TJ (2006) Preparation of antimicrobial poly(vinyl alcohol) nanofibers containing silver nanoparticles. Journal of Polymer Science, Part B: Polymer Physics 44 (17):2468-2474

33. Park JH, Karim MR, Kim IK, Cheong IW, Kim JW, Bae DG, Cho JW, Yeum JH (2010) Electrospinning fabrication and characterization of poly(vinyl alcohol)/montmorillonite/silver hybrid nanofibers for antibacterial applications. Colloid and Polymer Science 288 (1):115-121

34. Liu S, He J, Xue J, Ding W (2009) Efficient fabrication of transparent antimicrobial poly(vinyl alcohol) thin films. Journal of Nanoparticle Research 11 (3):553-560

35. Barakat NAM, Woo KD, Kanjwal MA, Choi KE, Khil MS, Kim HY (2008) Surface plasmon resonances, optical properties, and electrical conductivity thermal hystersis of silver nanofibers produced by the electrospinning technique. Langmuir 24 (20):11982-11987

36. Martínez-Abad A, Lagaron JM, Ocio MJ (2012) Development and characterization of silver-based antimicrobial ethylene-vinyl alcohol copolymer (EVOH) films for food-packaging applications. Journal of Agricultural and Food Chemistry 60 (21):5350-5359

37. Kenawy ER, Layman JM, Watkins JR, Bowlin GL, Matthews JA, Simpson DG, Wnek GE (2003) Electrospinning of poly(ethylene-co-vinyl alcohol) fibers. Biomaterials 24 (6):907-913

38. Tian S, Ogata N, Shimada N, Nakane K, Ogihara T, Yu M (2009) Melt electrospinning from poly(L-lactide) rods coated with poly(ethylene-co-vinyl alcohol). Journal of Applied Polymer Science 113 (2):1282-1288

39. Shimada N, Tsutsumi H, Nakane K, Ogihara T, Ogata N (2010) Poly(ethylene-co-vinyl alcohol) and nylon 6/12 nanofibers produced by melt electrospinning system equipped with a line-like laser beam melting device. Journal of Applied Polymer Science 116 (5):2998-3004

40. Wang B, Xu C, Xu F, Lu T (2011) Electrospinning of poly(ethylene-co-vinyl alcohol) nanofibres encapsulated with Ag nanoparticles for skin wound healing. Journal of Nanomaterials 2011

41. Liau SY, Read DC, Pugh WJ, Furr JR, Russell AD (1997) Interaction of silver nitrate with readily identifiable groups: Relationship to the antibacterial action of silver ions. Letters in Applied Microbiology 25 (4):279-283

42. Choi O, Clevenger TE, Deng B, Surampalli RY, Ross Jr L, Hu Z (2009) Role of sulfide and ligand strength in controlling nanosilver toxicity. Water Research 43 (7):1879-1886

43. Martínez-Abad A, Sánchez G, Lagaron JM, Ocio MJ (2012) On the different growth conditions affecting silver antimicrobial efficacy on Listeria monocytogenes and *Salmonella enterica*. International Journal of Food Microbiology 158 (2):147-154

44. Kim TN, Feng QL, Kim JO, Wu J, Wang H, Chen GC, Cui FZ (1998) Antimicrobial effects of metal ions (Ag⁺, Cu²⁺, Zn²⁺) in hydroxyapatite. Journal of Materials Science: Materials in Medicine 9 (3):129-134

45. Hwang MG, Katayama H, Ohgaki S (2007) Inactivation of Legionella pneumophila and Pseudomonas aeruginosa: Evaluation of the bactericidal ability of silver cations. Water Research 41 (18):4097-4104

46. Bjarnsholt T, Kirketerp-Møller K, Kristiansen S, Phipps R, Nielsen AK, Jensen PØ, Høiby N, Givskov M (2007) Silver against Pseudomonas aeruginosa biofilms. APMIS 115 (8):921-928

47. Chopra I (2007) The increasing use of silver-based products as antimicrobial agents: A useful development or a cause for concern? Journal of Antimicrobial Chemotherapy 59 (4):587-590

48. Šimon P, Chaudhry Q, Bakoš D (2008) Migration of engineered nanoparticles from polymer packaging to food - A physicochemical view. Journal of Food and Nutrition Research 47 (3):105-113

49. Saquing CD, Manasco JL, Khan SA (2009) Electrospun nanoparticle-nanofiber composites via a one-step synthesis. Small 5 (8):944-951

50. Son WK, Youk JH, Lee TS, Park WH (2004) Preparation of antimicrobial ultrafine cellulose acetate fibers with silver nanoparticles. Macromolecular Rapid Communications 25 (18):1632-1637

51. Lee HK, Jeong EH, Baek CK, Youk JH (2005) One-step preparation of ultrafine poly(acrylonitrile) fibers containing silver nanoparticles. Materials Letters 59 (23):2977-2980

52. Dong G, Xiao X, Liu X, Qian B, Liao Y, Wang C, Chen D, Qiu J (2009) Functional Ag porous films prepared by electrospinning. Applied Surface Science 255 (17):7623-7626

53. Joyce-Wöhrmann RM, Münstedt H (1999) Determination of the silver ion release from polyurethanes enriched with silver. Infection 27 (SUPPL. 1)

54. Ward TJ, Kramer JR (2002) Silver speciation during chronic toxicity tests with the mysid, Americamysis bahia. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 133 (1-2):75-86

55. Zhang Z, Britt IJ, Tung MA (1999) Water Absorption in EVOH Films and Its Influence on Glass Transition Temperature. Journal of Polymer Science, Part B: Polymer Physics 37 (2-7):691-699.

Chapter V

ASSESSING SILVER RELEASE AND ANTIMICROBIAL CAPACITY OF IONIC SILVER LOADED POLY(L-LACTIDE) FILMS OF INTEREST IN FOOD COATING APPLICATIONS

Journal of Applied Polymer Science (under review)

Antonio Martínez Abad[a], José M. Lagaron[a], Maria J. Ocio[a, b]

ABSTRACT

In the present study, silver ions were incorporated into a poly-(L-lactide) (PLA) matrix by a solvent casting technique using different solvents and glycerol as plasticizer. The effect of the different formulations on the morphology, thermal, mechanical and color properties were first evaluated. Additionally, the release of silver ions to an aqueous environment was also monitored over time by anodic stripping voltammetry and correlated with the antimicrobial performance against *S. enterica*. The incorporation of silver contents of up to 1wt. % did not affect morphology, thermal or mechanical properties of the films. Sustainable, antibacterial effectiveness was found for films containing silver loading between 0.01wt. % and 1wt.%. In all cases, an initial burst release was observed which arrested with time. These results give new unreported insight into the behavior of ionic silver loaded PLA films and about their potential application in antimicrobial food coatings.

Keywords: Silver ions, poly(lactic acid) or poly(L-lactide), controlled release, antimicrobial food packaging, antimicrobial coatings.

1. Introduction

Although the antimicrobial efficacy of silver has been recognized since ancient times[1], it is during the last decade that its use has become more and more popular. Due to its unspecific mechanism of action, silver ions are active against a very broad spectrum of bacteria, yeasts, fungi and viruses and are not toxic to human cells[2-3]. Therefore, a wide variety of materials used in daily life are recently incorporated with silver or silver salts as key components to control microbial proliferation, ranging from textile clothing[4], stainless steel coatings in home appliances[5] and food-contact materials [6-7] (see *8-10* for review). In the U.S., the Center for Food Safety and Applied Nutrition in the Food and Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled waters[11] and in the EU, silver is accepted under directive 94/36/EC as a colouring agent (E-174) with no restrictions[12]. Additionally, the European Food Safety Authority (EFSA) has provided positive opinions for silver zeolites, silver zirconium phosphates, and silver containing glasses in its provisional list of additives for food contact materials with a general restriction of ≤0.05 mg/kg food for the whole group[13]. Regardless of the stringent regulations, silver still remains the most widely used antimicrobial polymer additive in food applications[14-15].

The globally increasing demand for minimally processed, easily prepared and ready-to-eat 'fresh' food products has encouraged manufacturers to develop new technologies as an alternative to thermal processing. These new alternative technologies such as lower thermal, pulsed electric fields or high pressure treatments may in some instances allow pathogenic bacterial growth. However, even if foodborne pathogens are totally eliminated by efficient thermal treatments, microbial recontamination of the food surface could take place during the post-processing steps, when the risk of cross-contamination is elevated. As a result, a reduction in food shelf-life is observed and the risk of foodborne illnesses is greatly increased. Therefore, new preservation techniques,

such as incorporation of antibacterial substances into the food products or the packaging or the surfaces where food contact takes place, are currently being investigated and applied. As bacterial contamination occurs primarily on the surface, the incorporation of the biocide in a film or as a coating has many advantages due to the high exposure areas for the biocide[16-17]. Additionally, targeted, sustained or controlled of the antimicrobial would allow the use of less quantities of the antimicrobial while assuring the desired effect over the shelf-life of the product.

The approach of the use of silver in the food industry has been mostly bounded to silver zeolites[18-20] and silver-zirconium ion-exchange resins, which are subsequently incorporated as a coating on predominantly stainless steel surfaces. These systems rely on the sustained release of silver ions via a moisture dependent ion exchange mechanism. However, the very low migration rates of the silver ions from these materials imply the need for the incorporation of silver filler contents of up to 5wt.%. This high silver content could limit the application of these systems in antimicrobial packaging, due to possible permeability and dispersion problems, surpassing the migration limits during the shelf-life of the product or a negative environmental impact. For the correct development and final application of silver in the food industry, it is crucial to elucidate the threshold of biocide action and optimize the silver system so that tiny contents are required and the potential is fully realized. Additionally, the extreme instability of silver ions in the presence of complex organic matter, like foods, advices the need for a sustained and targeted release of silver ions[21-22]. A feasible approach to this challenge inside the range of food packaging polymers might be the use of poly-lactide (PLA) based polymers. PLA is a biodegradable and compostable polymer that can be derived from renewable resources such as starch. Consumers' demands, and the requirements by regulatory authorities, to pursue more environmentally friendly and less polluting packages, have prompted interest into PLA materials, owing to its high mechanical strength and transparency, easy processability and relatively low prices in the market compared to other biopolymers[23].

In the present paper, different amounts of silver were incorporated into PLA by a solvent casting technique. The influence on the use of two different solvents for the solubilization of the polymer as well as the addition of glycerol as a plasticizer to the films was evaluated. A characterization of the morphology, thermal properties, tensile tests and colour alteration of the films was carried out. Sustained release of silver ions from the films was monitored after successive daily and weekly washings by ASV. The antibacterial performance against *S. enterica* was accurately correlated to the release patterns, as to establish breakpoints for silver under these conditions.

2. Materials and Methods

2.1. Film preparation

PLA supplied by Natureworks Llc was used for preparation of the cast films. The polylactide with a D-isomer content of approximately 2% had a number-average molecular weight (M_n) of ca. 130,000 g/mol, and an average molecular weight (M_w) of ca. 150,000 g/mol as reported by the manufacturer. Polymer pellets were dissolved in two different solvents: tetrahydrofuran (THF) and a mixture of THF and dimethylformamide (DMF) in the ratio 3:1 (w/w). 5 g of polymer were dissolved in 95g of THF and THF:DMF at 50 ºC under stirring to generate PLA-THF and PLA-DMF films, respectively. Glycerol (Panreac, Barcelona, Spain) was added with the pellets to a 10wt.% dry weight to produce PLA-THF-G and PLA-DMF-G films. After dissolution, the suitable amount of silver nitrate (Sigma-Aldrich) was added to the solution as to achieve films with 0wt.%, 0.01wt.%, 0.1wt.%, 1wt.% and 5wt.% silver nitrate weight in dry conditions. The solution was cast onto glass Petri dishes to obtain a 30-50 μm thick films after solvent evaporation for 3h at 50ºC and 60ºC for PLA-THF and PLA-DMF films, respectively. The remaining solvent in the films was further allowed to diffuse out in a vacuum oven at 50 ºC for 18 h. Films were stored in a 0% relative humidity (RH) desiccators protected from light with aluminium wrapping before undergoing testing and to a maximum of 14 days.

2.2. Morphology

To investigate the morphology of the films, SEM microphotographs (XL30 ESEM, Phillips) were taken with an accelerating voltage of 20 keV on the sample surface and on the thickness of the films after cyofracturing the samples immersed in liquid nitrogen. SEM coupled energy-dispersive X-ray microanalysis (EDX) in mapping mode was used to identify the element silver on the film surface. Further optical images were taken with an optical miroscope Eclipse 90i (Nikon).

2.3. FT-IR Analysis

Transmission FTIR and ATR-FTIR using the GoldenGate of Specac Ltd. (Orpington, UK) experiments were recorded within a N_2 purged environment using a Bruker model Tensor 37 equipment (Darmstadt, Germany) with a resolution of 1 cm^{-1}, 20 scan runs and a typical acquisition time of 60 s.

2.4. Differential Scanning Calorimetry (DSC)

Thermal properties were studied by differential scanning calorimetry (DSC) using a Perkin–Elmer DSC-7 calorimeter (Perkin–Elmer Cetus Instruments, Norwalk, CT). Samples with a typical weight of 3-4 mg were held at 60ºC for 5 minutes in the nitrogen purged chamber to eliminate humidity, then cooled down to 0ºC and finally heated to 180ºC. The rate of both heating and cooling runs was 10°C/min. The values of glass transition temperature (T_g), melting points (T_m), and melting enthalpy (ΔH_m) were taken from this heating run. Calibration was performed using indium and dodecane as reference samples. All tests were carried out in triplicate.

2.5. Mechanical properties

Tensile tests were performed according to ASTM Standard D 638 in stamped dogbone-shaped specimens of the samples. An Instron Testing Machine (Model 4469; Instron Corp., Canton, MA) was used, with a crosshead speed of 10 mm/min, at ambient conditions of typically 23ºC and 60%RH. At least, four specimens of each film were tensile tested as to obtain statistically meaningful results.

2.6. Color Analysis of Treated Samples

The change in color of the films after 24h contact with the food matrix was determined using a handheld Minolta Chromameter CR300 (Minolta Camera Co., Ltd., Osaka, Japan) set to D65 illuminant/10° observer. Film specimens were placed on a white standard plate, and the CIELAB color space was used to determine the parameters L*, a* and b*. L* value ranges from 0 (black) to 100 (white); a* value ranges from -80 (green) to 100 (red); and b* value ranges from

-80 (blue) to 70 (yellow). Samples were evaluated per triplicate and four measurements were taken at random locations on each of the studied films. ΔE* was calculated as a global parameter of colour alteration according to the following equation (eq. 1):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{(Eq. 1)}$$

2.7. Release Study

A voltammetric method was used to determine the release of free silver ions (FSI) over time from the films to a slightly acidic aqueous environment. For this purpose, 1g of the cast films with different silver contents was immersed in 100 mL slightly acidified (1mM HNO_3 to stabilize silver in its ionic form) distilled water at 25ºC and stored without stirring before testing. The FSI content for each measurement was determined on an aliquot extracted from the polymer film containing solution by differential pulse anodic stripping voltammetry (ASV) with an Autolab III (EcoChemie) potentiostat setup under conditions stated in Metrohm application bulletin nº 207/2e "Analysis of silver by stripping voltammetry". The ASV working range was 0.001 – 0.1 ppm of silver. After each ASV measurement, the film containing solution was sterilized by filtration and set apart before its use to determine antimicrobial capacity. Subsequently, the films were reimmersed in new fresh slightly acidified water and reincubated again under the same conditions for a subsequent release measurement. This procedure was correlatively repeated in two sets of batches, each day for 14 days to measure release and antimicrobial performance and each week for 10 weeks to evaluate antimicrobial performance on the long term. All experiments were carried out in duplicate.

2.8. Antimicrobial testing

Salmonella enterica CECT 554 strain was obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain) and stored in Phosphate Buffer Saline (PBS) with 10% Tryptone Soy Broth (TSB, Conda Laboratories, Madrid,

Spain) and 10% glycerol at -80 ºC until needed. For experimental use, the stock culture was maintained by regular subculture to Tryptone Soy Agar (TSA) slants at 4 ºC and transferred monthly.
Previous to each study, a loopful of bacteria was transferred to 10 mL of TSB and incubated at 37 ºC overnight. A 100 µL aliquot from the overnight culture was again transferred to TSB and grown at 37 ºC to the mid-exponential phase of growth. This culture served as the inoculum for antimicrobial assays.

To assess the effectiveness of PLA-THF and PLA-DMF films, susceptibility tests were carried out employing the macro-dilution method M26-A described by the Clinical and Laboratory Standards Institute (CLSI) with modification. Briefly, 8 mL of the aqueous solution containing the silver released over each consecutive day or week were added into 2mL 5x M9 medium. A bacterial suspension in mid-log phase was then inoculated in each test tube to achieve an initial inoculum size of approximately 5 x 10^5 CFU/mL and incubated at 37ºC for 24 h. Then, 0.1 mL of each sample was sub-cultivated on TSA plates for viable count after incubation at 37ºC for 24 h. Samples treated analogously but without silver were used as controls. Each of these experiments was performed in duplicate.

3. Results and Discussion

3.1. Morphology

SEM images of the cryofractured films as well as optical microscopy images of the samples were taken to evaluate the effect of the solvent, the incorporation of silver or the presence of glycerol in the morphology of the films. No differences in morphology were found for silver contents below 0.1 wt.%, indicating that small silver concentrations do not measurably affect the morphology of the cast films (data not shown). PLA-THF films with 1 wt.% silver showed the presence of homogeneously distributed star-like crystals (Fig. 1B) which were not present in films without silver (Fig. 1A). These crystals were confirmed to contain silver as measured by EDX (data not shown) and are thought to be made of cristallized silver nitrate aggregates. Silver nitrate is highly soluble in water but it is not soluble in organic solvents, while biopolyesters, on the contrary, are mostly soluble in solvents with low polarity, like chloroform or THF. Silver nitrate was found to be soluble in THF only at very low concentrations. Therefore, a combination of DMF and THF (1:3) was further selected, as it was the combination which yielded the highest solubility of the active compound without compromising the film-forming capacity of PLA. PLA-DMF films presented a rough surface with a granular pattern, indicating that the casting conditions do not allow a continuous microstructure to be well formed (Fig. 1C). No such silver aggregates were found in PLA-DMF films with silver, indicating that the higher solubility of the antimicrobial in the second solvent mixture resulted in a better distribution of the biocide. When glycerol was added into both PLA-DMF and PLA-THF films, this was not found to be miscible with the polyester as expected[24]. Instead, it was found to be confined in segregated phases homogeneously distributed along the surface and thickness of the films (Fig. 1D and Fig. 1E). Crystals of the silver salt were not found in the films when glycerol was added and it was not possible to locate silver compounds in PLA-THF-G or PLA-DMF-G by EDX mapping. Therefore, as an alternative approach to ascertain if silver had more affinity for any of the two phases, PLA-

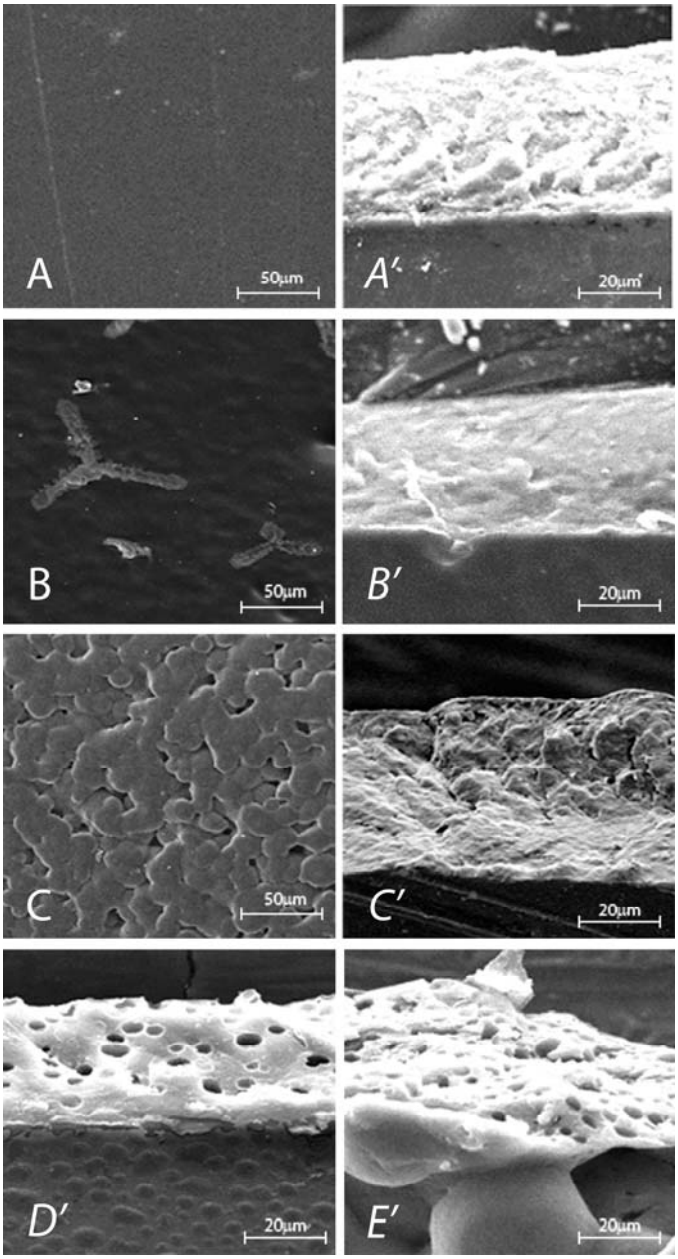

Figure 1. SEM images of the surface (bold) and thickness (in italics) of PLA-THF (A,B), PLA-THF-G (D), PLA-DMF (C) and PLA-DMF-G (E') films without (A) and with 1wt.% silver (B,C, *D'* and *E'*).

THF-G and PLA-DMF-G were irradiated with UV light at 254 nm for 5 hours. Silver was, thus, reduced to elemental silver, yielding a characteristic brown colour. As it can be seen from the observation of Fig. 2, silver was found to be mainly confined within the glycerol phases, probably due to its higher solubility in the more polar component.

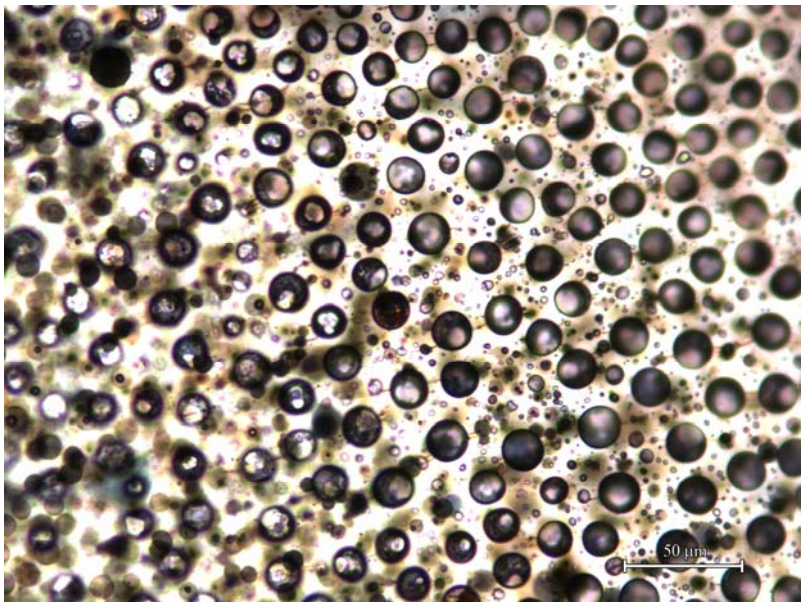

Figure 2. Optical microscopy image of PLA-THF-G films with 1wt.% silver after 5 hours UV irradiation at 254nm.

3.2. FTIR analysis

Infrared spectra of PLA-THF and PLA-DMF samples with different silver contents were analyzed to evaluate possible changes in molecular organization due to the incorporation of silver. In particular, differences in the crystalline content were investigated due to its distinct IR absorption patterns of amorphous and crystalline components in characteristic bands. All of the PLA samples showed a band around 921 cm^{-1}, which is well assigned to the coupling of the C-C backbone stretching with the CH_3 rocking mode and sensitive to the 10_3 helix chain conformation of PLA α crystals[25]. The presence of β-crystals can be ruled

out by the absence of the characteristic band at 908 cm^{-1} in all spectra[26]. On the other hand, the amorphous fraction in PLA can be ascribed to the band at 955 cm^{-1} [25, 27-28].

Table 1. Absorbance ratio of Transmission FT-IR bands at 955 and 922cm-1 as a function of silver content in the different PLA cast films (n=3).

Silver content (wt.%)	Absorbance ratio ($955cm^{-1}/922cm^{-1}$)	
	PLA-THF	PLA-DMF
0	0.985 aA	0.997 aA
0.01	0.930 aA	0.984 aA
0.1	0.949 aA	0.983 aA
1	0.992 aA	0.997 aA
5	0.997 aA	1.159 bB

* Mean values for all replicates of samples with silver content between 0-5wt.%
[a] Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests

As intensity of the band at 921 cm^{-1} increases while that of the band at 955 cm^{-1} decreases during the crystallization process, and their changes are synchronized, it was possible to determine the relative crystallinity of neat PLA pellets, PLA-THF and PLA-DMF film samples by using the ratio of the two bands[25, 29]. No significant differences were found in the ratio of these bands among samples with silver contents up to 1wt.%, either in PLA-THF or in PLA-DMF samples (Table 1). This indicates that the amount of crystalline fraction in the polymer may not be altered even if relatively high concentrations of silver are incorporated. The cast films, however, displayed a significantly higher crystallinity fraction as compared to the PLA pellets (absorbance ratio of 1.12). This could be explained by a faster cooling of neat PLA during extrusion and pelletization, which is expected to difficult polymer crystallization.

3.3. Thermal properties

In order to evaluate further the influence of the casting conditions, the presence of glycerol or the incorporation of an increasing load of silver on the different polymer formulations, the material thermal properties were analyzed by DSC.

Figure 3 shows typical thermograms of the polymer pellets before film preparation, PLA-THF films and PLA-DMF films both with 1wt.% silver content. The values of the glass transition temperature (T_g), melting point (T_m), and melting enthalpy (ΔH_m) for all samples are shown in table 2. Strong differences are observed between the polymer pellets and the cast films. In the low temperature frame, cast films show significantly lower T_g values and a substantially lower increase in specific heat change associated with the glass transition (ΔCp) (Fig. 3).

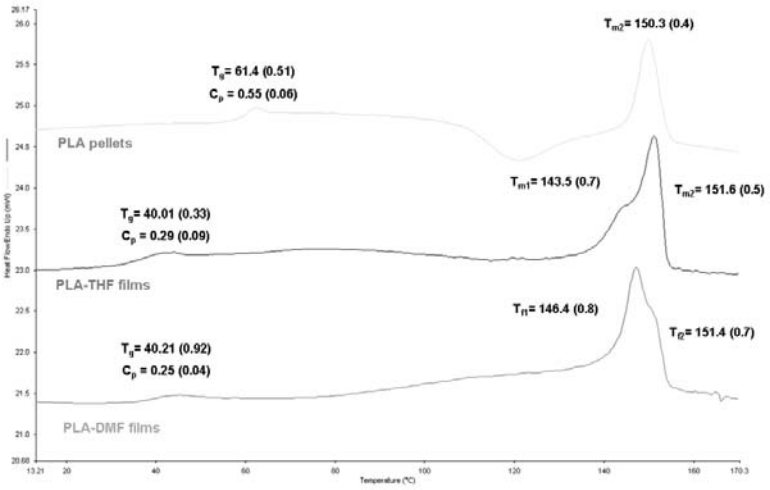

Figure 3. Typical thermogramms of PLA pellets, PLA-THF and PLA-DMF films. Values for the different parameters are embebbed in the image.

The lower ΔCp points to a higher degree of cristallinity, while the lower rigidity of the amorphous phase might be associated with a plasticization of the polymer by possible remnants of casting solvents within the films. At higher temperatures, a cold crystallization exotherm could not be observed in the cast films, and two melting peaks appear instead of one. This phenomenon can be explained by the melt-recrystallization model ascribed to the PLA melts as well as to other semicrystalline polymers[30]. The model suggests that small and imperfect crystals

change successively into more stable crystals through a melt-recrystallization mechanism. That is, the melting and recrystallization are competitive in the heating process. Accordingly, an endothermic peak appears when the rate of melting overwhelms that of recrystallization, and the exothermic peak appears when the rate of recrystallization overwhelms that of melting. Because the recrystallization proceeds slowly, it is gradually suppressed with increasing cooling or heating rates.

Table 2. Thermal properties of the cast films with different silver contents as measured by DSC (n=2).

Sample	T_g (ºC)	T_{m1} (ºC)	T_{m2} (ºC)	ΔH_m (J/g)
Polymer pellets	63.79 A[a]	-	150.29 A	6.59 A
PLA-THF 0%	41.24 B	144.87 A	152.03 A	31.93 B
PLA-THF 0.01%	40.05 B	143.15 A	150.67 A	29.45 B
PLA-THF 0.1%	40.34 B	144.10 A	151.10 A	28.09 B
PLA-THF 1%	40.88 B	143.14 A	151.25 A	28.35 B
PLA-THF 5%	37.86 BC	143.87 A	153.03 A	25.17 B
PLA-THF-G 0%	41.54 B	141.58 A	148.70 B	27.46 B
PLA THF-G 0.1%	42.40 B	141.11 A	148.87 B	31.54 B
PLA-THF-G 1%	39.74 B	142.25 A	146.12 B	26.76 B
PLA-THF-G 5%	41.86 B	141.08 A	146.87 B	28.99 B
PLA-DMF 0%	40.91 B	145.92 A	151.59 A	29.29 B
PLA-DMF 0.01%	43.22 B	145.89 A	152.22 A	29.88 B
PLA-DMF 0.1%	42.84 B	145.50 A	151.64 A	27.31 B
PLA-DMF 1%	41.37 B	145.48 A	151.70 A	30.87 B
PLA-DMF 5%	37.36 C	143.53 A	149.28 A	29.17 B
PLA-DMF-G 0%	44.72 B	142.11 A	149.20 B	31.50 B
PLA-DMF-G 0.1%	45.35 B	141.95 A	149.05 B	35.10 B
PLA-DMF-G 1%	49.24 B	140.89 A	148.20 B	28.47B
PLA-DMF-G 5%	47.66 B	144.20 A	148.65 B	34.88 B
PLA- THF	39.97 b	143.97 b	151.62 a	28.79 a
PLA-THF-G	41.38 b	141.51 c	147.64 b	28.47 a
PLA-DMF	41.14 b	146.37 a	151.29 a	29.51 a
PLA-DMF-G	46.77 a	140.14 c	148.78 b	32,49 a

[a]Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests

In their fabrication, polymer pellets are rapidly cooled down after extrusion. This hinders crystallization, and allows the polymer to crystallize from early stages throughout the heating run until crystals are finally melt in the form of one single endotherm (T_{m2}). In cast films, solvent is evaporated slowly, which might allow a higher degree of crystallization to be achieved. As melting of crystals overwhelms crystallization in these cases, no visible exotherms are observed during the heating run, while two melting endotherms appear instead, the second (T_{m2}) being thought to be the final melting of crystallites formed throughout the heating process. For all polymer formulations, the addition of increasing amounts of silver did not significantly alter the thermal properties of the cast films, except for PLA-THF and PLA-DMF films with 5wt.% content, which display significantly lower T_g. This may be due to weakening of the amorphous phase by the presence of the silver aggregates as postulated above. The results indicate that a relatively high load of silver (up to 1 wt.%) can be incorporated in the films without altering their thermal properties. Differences can be observed, however, when the different casting methods and the addition of glycerol are evaluated. PLA-THF films display a significantly lower T_{m1} as compared to PLA-DMF films. This suggests crystallites in PLA-DMF are bigger or more perfect than in PLA-THF films. Both effects could be due to a slower evaporation of the THF:DMF solvent mixture. Because T_{m2} is the result of the final melting of recrystallized crystals, its value is almost constant with both solvents[30]. The addition of glycerol, however, produced an overall decrease in both T_{m1} and T_{m2} [24].

3.4. Mechanical properties

PLA is known to be inherently brittle, which has prompted much research on improving this by for example adding different additives[31]. Mechanical properties of PLA-THF, PLA-DMF, PLA-THF-G and PLA-DMF-G with increasing silver content were evaluated. The materials modulus, elongation at brake and maximum tensile strength are presented in Table 3. No significant differences in the elastic modulus of PLA-THF, PLA-DMF and PLA-THF-G

were observed. This suggests that incorporation of silver in these films does not alter their mechanical properties and that differences among the samples may be ascribed to the actual variations between different castings. On the other hand, PLA-DMF-G films displayed a significantly lower modulus and higher deviation among samples, which can be attributed to weakening of the film structure during casting under the stated conditions.

Table 3. Mechanical properties of the various films tested (n=4).

Sample	Modulus (Mpa)	Elongation at break (%)	Max. Tensile Strength (MPa)
PLA-THF 0%	1664 ± 165 A[a]	4.4 ± 0.4 C	42.3 ± 2.7 AB
PLA-THF 0.1%	1745 ± 141 A	4.0 ± 0.1 C	47.8 ± 2.7 AB
PLA-THF 1%	1583 ± 189 A	4.5 ± 0.5 C	40.9 ± 2.9 ABC
PLA-THF 5%	1725 ± 234 A	5.0 ± 0.3 C	44.7 ± 4.5 AB
PLA-THF-G 0%	1588 ± 38 A	28.4 ± 3.3 AB	34.9 ± 2.0 ABC
PLA THF-G 0.1%	1621 ± 95 A	28.4 ± 9.6 AB	36.4 ± 1.8 ABC
PLA-THF-G 1%	1514 ± 175 A	27.5 ± 4.0 AB	33.6 ± 0.3 ABC
PLA-THF-G 5%	1596 ± 44 A	33.2 ± 10.1 A	35.9 ± 0.2 ABC
PLA-DMF 0%	1601 ± 104 A	8.0 ± 1.1 C	45.4 ± 3.3 AB
PLA-DMF 0.1%	1682 ± 57 A	7.2 ± 1.3 C	47.1 ± 4.7 A
PLA-DMF 1%	1576 ± 81 A	5.6 ± 0.4 C	42.1 ± 2.7 AB
PLA-DMF 5%	1498 ± 123 A	9.1 ± 1.7 BC	42.0 ± 1.8 ABC
PLA-DMF-G 0%	1185 ± 49 A	12.7 ± 1.8 BC	31.4 ± 2.1 ABC
PLA-DMF-G 0.1%	671 ± 216 B	16.5 ± 5.9 ABC	31.6 ± 3.9 BC
PLA-DMF-G 1%	705 ± 178 B	14.2 ± 6.3 ABC	29.8 ± 4.5 BC
PLA-DMF-G 5%	674 ± 205 B	18.5 ± 7.4 ABC	25.6 ± 1.2 C
PLA- THF	1679 ± 206 a	4.6 ± 0.5 b	44.5 ± 4.2 a
PLA-THF-G	1580 ± 155 a	25.6 ± 10.5 a	33.9 ± 3.1 b
PLA-DMF	1595 ± 115 a	7.3 ± 1.9 b	43.6 ± 3.7 a
PLA-DMF-G	808 ± 198 b	15.6 ± 5.3 b	30.0 ± 3.7 b

[a]Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests

Elongation at break is significantly increased with the presence of glycerol, while the maximum tensile strength significantly decreases in both PLA-THF-G and PLA-DMF-G films, as would be expected[24]. PLA-DMF films seem to be somewhat more ductile than PLA-THF films although differences were not found significant. Again, the incorporation of silver did not significantly affect either parameter.

3.5. Colour analysis

Transparency is highly desirable in many applications and PLA is a highly transparent polymer. Colour measurements contribute to objectively differentiate and evaluate changes in the colour of the films. All films tested showed a high transparency, except PLA-DMF films containing silver and PLA-DMF-G films with ≥ 1 wt.% silver content (Table 4).

Table 4. Colour analysis of the films (n=3).

Sample	L*	a*	b*	ΔE
Standard plate	94.28 A[a]	0.56 A	2.83 A	-
PLA-THF 0%	93.85 A	0.67 A	3.11 A	0.78 A
PLA-THF 0.1%	94.56 A	0.66 A	3.25 A	0.52 A
PLA-THF 1%	92.27 A	0.84 A	5.40 AB	3.28 AB
PLA-THF 5%	93.44 A	0.69 A	3.35 A	1.01 AB
PLA-THF-G 0%	94.43 A	0.47 A	3.22 A	0.44 A
PLA THF-G 0.1%	94.12 A	0.51 A	3.33 A	0.68 A
PLA-THF-G 1%	94.32 A	0.52 A	3.37 A	0.56 A
PLA-THF-G 5%	94.30 A	0.17 A	4.13 A	1.37 AB
PLA-DMF 0%	93.71 A	0.61 A	3.11 A	0.64 A
PLA-DMF 0.1%	91.06 B	1.14 A	9.17 BC	7.18 AB
PLA-DMF 1%	72.59 D	4.75 B	14.25 DE	24.95 AB
PLA-DMF 5%	61.99 E	3.39 B	16.86 E	35.33 B
PLA-DMF-G 0%	93.71 A	0.45 A	3.52 A	0.93 A
PLA-DMF-G 0.1%	93.82 A	0.66 A	3.29 A	0.68 A
PLA-DMF-G 1%	84.01 BC	-0.22 A	16.95 E	17.53 AB
PLA-DMF-G 5%	81.31 C	1.12 A	12.38 CD	16.26 AB

[a]Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests

In these cases, the decrease in transparency (ΔL^*) and a significant increase in yellowness (Δb^*) was associated with the increase in silver content. In PLA-DMF samples with 1 wt.% and 5 wt.% silver content, where these changes are higher, a significant increase in redness is also observed. In PLA-THF and PLA-THF-G films, however, the slight changes in colour are not significant. Silver is known to readily reduce to elemental particles in slightly reducing environments. The silver particles formed, if small enough, generate a yellow to brownish colour depending on their size and shape due to plasmon resonance[32-34]. DMF is a weak reducing agent and the reduction of silver when in contact with this solvent has been reported previously[35-38]. However, the increase in yellowness in the films is mostly noticeable for high silver concentrations. Additionally, slightly acidified conditions during casting were found to decrease this colour alteration (data not shown). Interestingly, colour alteration by the addition of silver is attenuated if glycerol is present. This could be attributed to a better stabilization of silver which was found to be preferentially confined within the glycerol domains (Fig. 2).

3.6. Release study

For several applications of antimicrobial films in foods or other related fields, it is desirable that the release of the antimicrobial be activated upon contact with moisture and then sustainably maintained over the shelf-life of the product. To evaluate suitability for this use, the controlled release of silver from PLA-THF, PLA-DMF and PLA-DMF-G films with different silver content was measured by means of ASV over successive daily washings. ASV has been proven a useful tool to monitor the release of traces of silver from polymer matrices[39-42]. The cumulative amounts of silver recovered throughout the experiment are presented in Figure 4. In all tested films, there is an initial burst release before 24-48h, after which ions are discharged in much slower and sustained kinetics. Despite following analogous patterns, there are remarkable differences between the samples depending on the solvent used in the casting, the silver contents incorporated in the film or the addition of glycerol. With increasing silver load, a

higher initial burst release and a smaller slope of sustained release is observed in the films. This effect is far more noticeable in PLA-THF films. As an example, in films with 1wt.% silver, the amount of ions released within 24h is far greater than the cumulative release throughout the rest of the experiment.

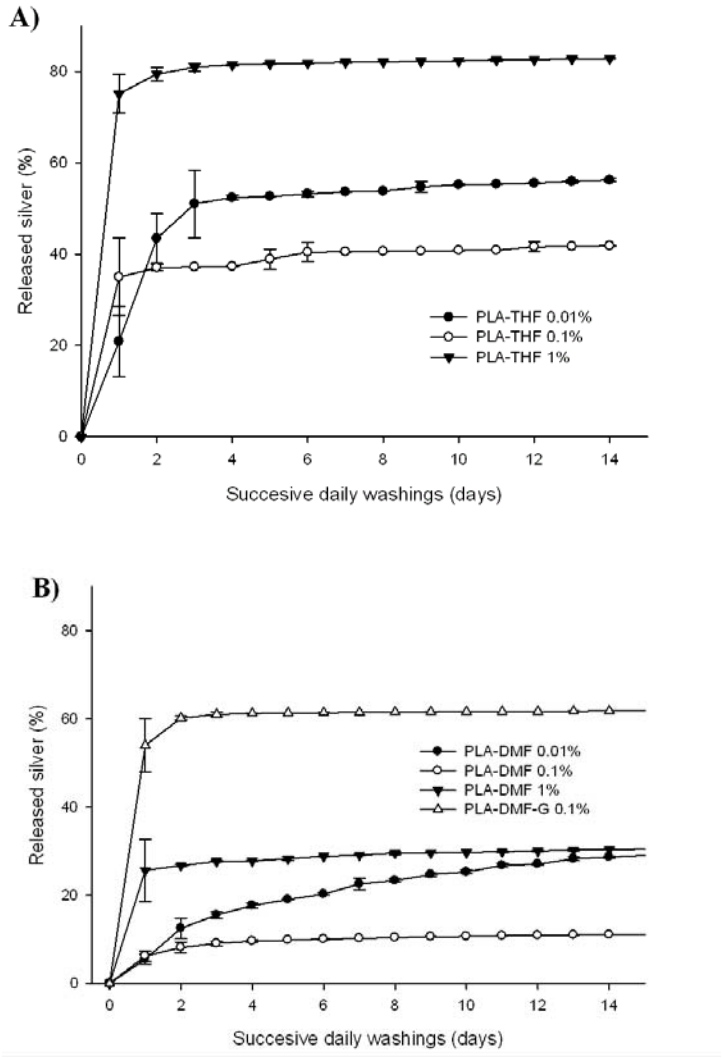

Figure 4. Total accumulated release of silver from a) PLA-THF and b) PLA-DMF and PLA-DMF-G films with increasing silver contents after successive daily washings (n=2).

In films with 0.01wt.% silver, however, this burst release regime is prolonged for 2-3 days depending on the solvent used for casting, and the amount of silver released daily afterwards is higher. On the other hand, when the use of a different solvent is evaluated, a similar effect is observed. For equal amounts of silver, PLA-THF films show stronger burst release and lower slope of sustained ion release. This is especially evident at higher silver concentrations, while no such differences are observed between solvents when 0.01wt.% silver is incorporated. These differences appear to be in good agreement with the morphological analysis and could so be explained in terms of solubility. As explained in the morphological analysis, agglomerates of silver nitrate salts were found to crystalize and precipitate during solvent evaporation depending on solubility. Upon immersion in an aqueous solution, these agglomerates would be more rapidly dissolved, producing a burst release of silver ions. Hence, a higher silver content would increase the amount of agglomerated silver available for burst release. The use of a more compatible solvent mixture, like DMF:THF, would help increase the solubility of the silver salt, which could explain the slower burst release but a faster sustained release of silver compared to PLA-THF films. Considering the more sustained release of PLA-DMF films with lower silver contents, the effect of glycerol in the release profiles of these films was further studied (Fig. 4). In this case, the addition of the plasticizer led to a drastic increase in burst release, whereas the sustained release was slightly decreased As seen in previous analysis, glycerol was not found to be miscible with the PLA matrix (Fig. 1 and Fig. 2) and did not promote substantial changes in the crystalline structure of the polymer (Table 1 and Table 2). Additionally, a significant amount of silver was observed to be confined within the glycerol phases allowing a faster release of the antimicrobial entrapped in this phase. These results evidence that the selection of different solvents or the addition of a plasticizer such as glycerol may serve as an additional tool to tailor the release capacities of silver based antimicrobial polyesters. This may be useful for implementation of these technologies in the food or in other areas. For example in food products there may be a need for an initial higher biocide capacity followed by a slower sustained release to prevent recontamination.

3.7. Long-term antimicrobial performance after weekly washings

In order to evaluate the antimicrobial effectiveness of the produced films, susceptibility assays were performed against the foodborne pathogen *Salmonella enterica* in M9. The synthetic medium contains glucose as a sole carbon source and could stand for a hypothetical environment of surface contamination[43]. First, the released silver over successive weekly washings was tested as to evaluate endurance of the antimicrobial activity of the films after a relatively long term in contact with moisture (Fig. 5). Control films without silver did not produce any changes in viable counts, indicating antimicrobial effectiveness was only due to the release of silver (data not shown). All tested films released enough silver during the first week as to decrease viable counts of *Salmonella* below the detectable threshold (10 CFU/mL; Fig. 5).

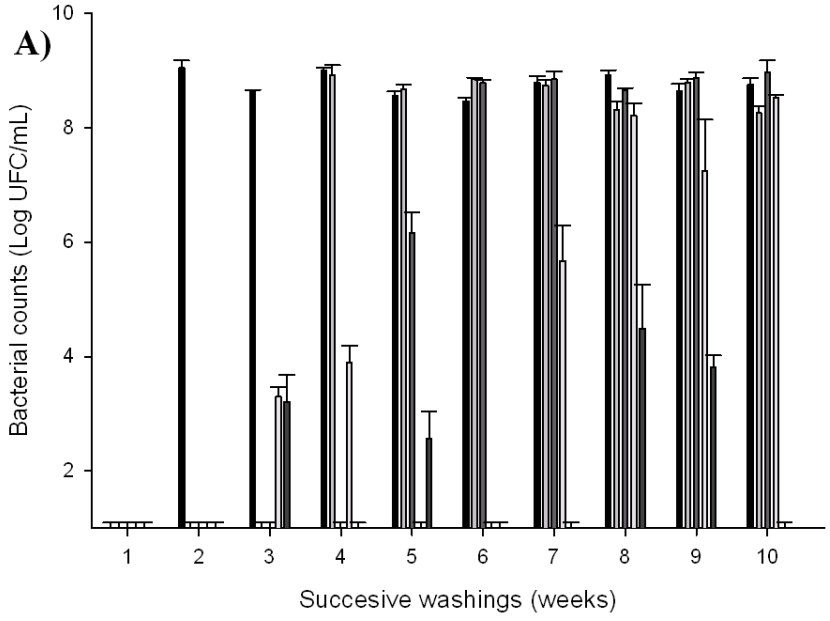

Figure 5a. Viability of *Salmonella enterica* after incubation at 37ºC with silver released from PLA-THF films with 0.01wt. % silver , 0.1wt.% silver and glycerol , 0.1wt.% silver , 1wt.% silver and glycerol , and 1wt.% silver (n=3).

Antibacterial effectiveness of PLA-THF (Fig. 5a) and PLA-DMF (Fig. 5b) films with 0.01wt.% was not sufficient from the second week on as samples were able to reach the same viable counts as the controls. In PLA-DMF-G samples with 0.1wt.% silver, a reduction of viables of at least 2 log is noted until week 4 (Fig. 5b). After that time, the antibacterial effect is gradually lost thorough weeks 5-7. When PLA-DMF films with the same silver content were evaluated, no viable counts were detected until week 4, after which efficacy is totally lost. When 1wt.% silver is incorporated in PLA-DMF and PLA-DMF-G films, no detectable counts are observed until week 4 and week 7, respectively.

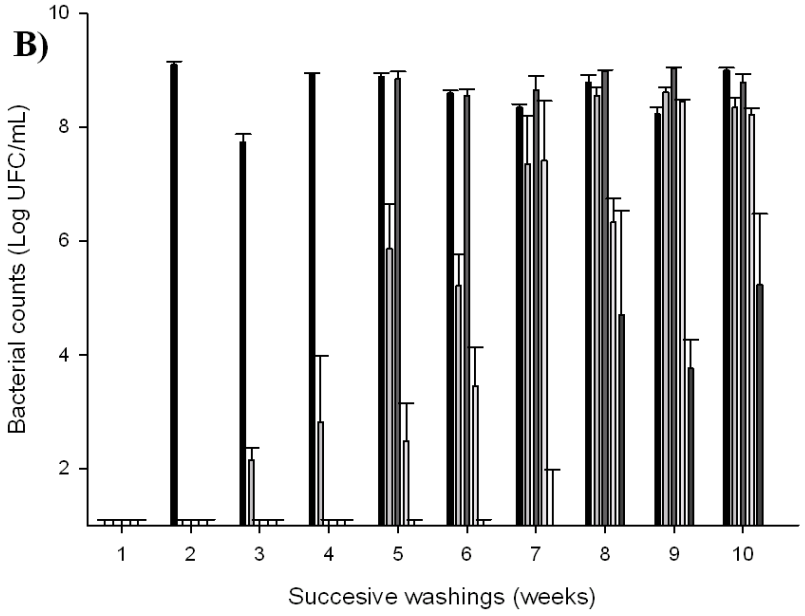

Figure 5b. Viability of *Salmonella enterica* after incubation at 37ºC with silver released from PLA-DMF films with 0.01wt. % silver , 0.1wt.% silver and glycerol , 0.1wt.% silver , 1wt.% silver and glycerol , and 1wt.% silver (n=3).

While PLA-DMF films with 1wt.% silver demonstrate antibacterial efficacy thorough the ten weeks in contact with water, this is substantially reduced or lost after week 7 for PLA-DMF-G films with the same silver content. This indicates

that the presence of glycerol does not improve the long-term efficacy of PLA-DMF films. The same effect is observed when PLA-THF-G and PLA-THF with the same amount of silver are compared (Fig. 5a). With 0.1 wt.% silver, a reduction of viables as compared to controls without silver is observed after week 3 and 5, respectively. The same films with 1 wt.% silver content exert an antibacterial effect for at least 7 and 10 weeks, respectively. Glycerol is usually added to drug releasing polymers as a plasticizer in order to increase the release capacities. However, release analysis revealed that only the burst release was increased when glycerol was incorporated in the films, whereas the sustained release was slightly decreased. Antimicrobial performance of these films in this relatively long-term study is therefore in line with the release analysis and points out that the addition of glycerol may enhance the antimicrobial performance at the burst release stages but would be detrimental if a more sustained release is desirable. When the effect of the use of a different solvent mixture in the casting is compared among films with the same characteristics, it was found that the effectiveness of films formed with only THF were somewhat less sustained and more erratic. As an example, in PLA-THF samples with 1 wt.% silver no viable bacteria were detected on weeks 1, 2 4, 6, 7 and 10, while with analogue PLA-DMF films the same effect is achieved sustainably until week 8. This behaviour can again be attributed to the different compatibility of silver with the solvent mixture used and is in line with the release results discussed above.

3.8. Antimicrobial performance after daily washings

Considering that the films demonstrated high antimicrobial efficacy immediately after contact with the solution, it is important to ascertain if this release may be sufficiently sustained as to allow a continuous effect over time in shorter terms. Therefore, a second assay was conducted evaluating the effectiveness of the released silver from PLA-THF and PLA-DMF films with 0.01wt.% and 0.1wt.% silver after daily washing. Additionally, silver in the solutions was quantified before inoculation, in order to accurately assess the required silver concentration in solution as to exert an antibacterial effect and establish breakpoints for silver under the stated conditions. As silver release was not constant in all samples, the

released silver from only one replicate of the release study was incubated per triplicate with *S. enterica*. Released silver and the corresponding viable count number detected for each sample and through 14 days are presented in Table 5. As shown in the release results, a considerable amount of the silver is released within the first one or two days, the release being more sustained in film samples with 0.01wt.% silver. Accordingly, no bacterial counts were detected for this period in any of the samples tested.

Table 5. Release and antibacterial performance of silver released from PLA-THF and PLA-DMF films with 0.01wt.% and 0.1wt.% silver over consecutive daily washings.

	PLA-THF				**PLA-DMF**				**PLA-DMF-G**	
	Silver contents (wt.%)									
Time	**0.01wt.%**		**0.1wt.%**		**0.01wt.%**		**0.1wt.%**		**0.1wt.%**	
(day)	**R[a]**	**B[b]**	**R**	**B**	**R**	**B**	**R**	**B**	**R**	**B**
1	263	<1	2742	<1	45	<1	728	<1	3991	<1
2	264	<1	257	<1	59	<1	230	<1	502	<1
3	17	5.24±0.13[c]	16	<1	31	<1	122	<1	51	<1
4	12	4.89±0.38	13	5.66±0.24	18	<1	63	<1	15	<1
5	3	8.57±0.03	≤1	8.97±0.13	12	<1	20	<1	9	6.58±0.16
6	10	5.54±0.27	241	<1	10	6.23±0.25	21	<1	6	8.92±0.03
7	4	8.97±0.10	13	<1	30	<1	15	<1	4	8.99±0.06
8	≤1	8.78±0.07	12	<1	3	8.78±0.15	17	<1	5	9.03±0.12
9	≤1	8.89±0.02	6	7.01±0.55	15	<1	16	<1	6	8.79±0.09
10	3	9.04±0.04	17	<1	3	8.87±0.07	6	8.95±0.19	3	8.98±0.02
11	≤1	8.85±0.11	2	8.89±0.03	10	5.52±0.13	13	<1	≤1	8.83±0.08
12	≤1	8.65±0.15	124	<1	2	9.01±0.08	5	8.26±0.09	4	8.73±0.15
13	5	8.54±0.21	7	8.69±0.11	11	4.73±0.31	9	5.42±0.33	7	8.79±0.12
14	≤1	7.98±0.05	18	<1	≤1	8.54±0.15	3	8.96±0.10	2	9.06±0.05

R[a]: released silver ions (ppb) B[b]: Bacterial counts (log CFU/mL) [c]: Standard deviation (n=2)

In PLA-THF films, the antibacterial effect was found to be less sustained than in PLA-DMF films. With 0.01wt.% silver, no viables are detected until day 2 and day 5, respectively, while the same effect is achieved with 0.1wt.% silver until

day 4 and day 9, respectively. Moreover, the release and subsequent antibacterial effect follows a more irregular pattern in PLA-THF than in PLA-DMF films. This behaviour again correlates with the release results as well as with the morphology of both films as discussed above. The addition of glycerol to PLA-DMF films resulted in a reduction of the sustained antimicrobial effect from 9 to 5 days as compared with the same material without glycerol. Interestingly, the silver detected on the test tubes previous to inoculation can be quite well correlated with its corresponding antimicrobial effect in any of the samples. As an example, a silver concentration of ≤8 ppb did not produce any reduction of viable counts for any of the samples tested. On the other hand, no detectable viables were found when the test tubes contained ≥18 ppb silver. For samples where about 10-20ppb silver were detected, the antibacterial effect varied substantially, which suggests that this might be the threshold range to be considered as a breakpoint for the tested strain and under the stated conditions. The very low concentrations necessary to exert an antibacterial effect evidence the outstanding potential of silver as antimicrobial and are in line with previous results in other synthetic media[22,43-45]. Although literature has extensively reported the use of polymers incorporating silver nanoparticles for their possible use in food packaging applications, nanoparticles are beginning to be considered within the frames of existing legislation in most coutries, including the EU. Antimicrobial materials based on the release of ionic silver are, on the other hand, permitted and widely used in most countries[11-13]. These materials are mostly based on silver exchange from resins or other inorganic fillers, like montmorillonites (MMT), zeolites[39, 46-47] being the most widely used of all[14]. In these cases, however, filler contents were in the range of 1-10wt.%. In the present study, the developed PLA films without further inorganic fillers led to a strong antibacterial effect with filler content of 0.01wt.% and 0.1wt.%, respectively, which could allow the possible application of silver based polymers in food packaging or food-contact surfaces. The EFSA has stated a stringent migration limit of 50 μg silver/kg food (or ppb). The results in the present study evidence that a bactericidal effect can be achieved even below this value, following a controlled and sustained release over storage time or even

after successive washing (Table 5). However, in the PLA-THF and PLA-DMF films tested, the sustained release proceeded in all cases by an initial burst release of 5-80% of the whole silver content upon immersion in water, which may impose some limitations for their use as such in direct food contact for instance. Currently, other strategies are being developed to tailor the release, such as multilayers, which will be the subject of further studies.

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5. References

1. Klasen HJ (2000) Historical review of the use of silver in the treatment of burns. I. Early uses. Burns 26 (2):117-130

2. Williams RL, Doherty PJ, Vince DG, Grashoff GJ, Williams DF (1989) The biocompatibility of silver. Critical Reviews in Biocompatibility 5 (3):205+221-243

3. Russell AD, Hugo WB (1994) Antimicrobial activity and action of silver. Progress in medicinal chemistry 31:351-370

4. Yuranova T, Rincon AG, Bozzi A, Parra S, Pulgarin C, Albers P, Kiwi J (2003) Antibacterial textiles prepared by RF-plasma and vacuum-UV mediated deposition of silver. Journal of Photochemistry and Photobiology A: Chemistry 161 (1):27-34

5. Kampmann Y, De Clerck E, Kohn S, Patchala DK, Langerock R, Kreyenschmidt J (2008) Study on the antimicrobial effect of silver-containing inner liners in refrigerators. Journal of Applied Microbiology 104 (6):1808-1814

6. Galeano B, Korff E, Nicholson WL (2003) Inactivation of vegetative cells, but not spores, of Bacillus anthracis, B. cereus, and B. subtilis on Stainless steel surfaces coated with an antimicrobial silver- and zinc-containing zeolite formulation. Applied and Environmental Microbiology 69 (7):4329-4331

7. Bouwmeester H, Dekkers S, Noordam MY, Hagens WI, Bulder AS, de Heer C, ten Voorde SECG, Wijnhoven SWP, Marvin HJP, Sips AJAM (2009) Review of health safety aspects of nanotechnologies in food production. Regulatory Toxicology and Pharmacology 53 (1):52-62

8. Gupta A, Silver S (1998) Silver as a biocide: Will resistance become a problem? Nature Biotechnology 16 (10):888

9. Rai M, Yadav A, Gade A (2009) Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances 27 (1):76-83

10. Martinez-Abad A (2010) Silver- and nanosilver-based plastic technologies In: Lagarón J, Ocio MJ, Lopez-Rubio A (eds) Antimicrobial Polymers. John Wiley and sons inc., Hoboken, New jersey, pp 287-316

11. FDA/CFSAN (2010). Listing of food additive status: Silver nitrate-172.167, U. FDA/CFSAN, Editor.

12. European Comission. European parliament and council directive *94/36*/EC of 30th June 1994 on colours for use in foodstuffs.

13. European Food Safety Authority (2011). Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request related to a 12th list of substances for food contact materials. *EFSA Journal* 2011, 395–401

14. Quintavalla S, Vicini L (2002) Antimicrobial food packaging in meat industry. Meat Science 62 (3):373-380

15. Appendini P, Hotchkiss JH (2002) Review of antimicrobial food packaging. Innovative Food Science and Emerging Technologies 3 (2):113-126

16. López-Rubio A, Almenar E, Hernandez-Muñoz P, Lagarón JM, Catalá R, Gavara R (2004) Overview of active polymer-based packaging technologies for food applications. Food Reviews International 20 (4):357-387

17. Ouattar B, Simard RE, Piett G, Bégin A, Holley RA (2000) Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. International Journal of Food Microbiology 62 (1-2):139-148

18. Cowan MM, Abshire KZ, Houk SL, Evans SM (2003) Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel. Journal of Industrial Microbiology and Biotechnology 30 (2):102-106

19. Podhajny RM Efficacy of antimicrobially treated substrates. In, 2004. pp 667-679

20. Challener C (2008) Smart coatings continue to develop. JCT CoatingsTech 5 (2):38-42

21. Ilg Y, Kreyenschmidt J (2011) Effects of food components on the antimicrobial activity of polypropylene surfaces containing silver ions (Ag +). International Journal of Food Science and Technology 46 (7):1469-1476

22. Martínez-Abad A, Sánchez G, Lagaron JM, Ocio MJ (2012) On the different growth conditions affecting silver antimicrobial efficacy on Listeria monocytogenes and *Salmonella enterica*. International Journal of Food Microbiology 158 (2):147-154

23. Auras R, Harte B, Selke S (2004) An overview of polylactides as packaging materials. Macromolecular Bioscience 4 (9):835-864

24. Zhang J, Duan Y, Sato H, Tsuji H, Noda I, Yan S, Ozaki Y (2005) Crystal modifications and thermal behavior of poly(L-lactic acid) revealed by infrared spectroscopy. Macromolecules 38 (19):8012-8021

25. Kazuyo DS, Aki Sasashige T, Kanamoto T, Hyon SH (2003) Preparation of oriented β-form poly(L-lactic acid) by solid-state coextrusion: Effect of extrusion variables. Macromolecules 36 (10):3601-3605

26. Liu D, Yuan X, Bhattacharyya D (2012) The effects of cellulose nanowhiskers on electrospun poly (lactic acid) nanofibres. Journal of Materials Science 47 (7):3159-3165

27. Vasanthan N, Ly H, Ghosh S (2011) Impact of nanoclay on isothermal cold crystallization kinetics and polymorphism of poly(L -lactic acid) nanocomposites. Journal of Physical Chemistry B 115 (31):9556-9563

28. Zhang J, Tsuji H, Noda I, Ozaki Y (2004) Structural changes and crystallization dynamics of poly(L-lactide) during the cold-crystallization process investigated by infrared and two-dimensional infrared correlation spectroscopy. Macromolecules 37 (17):6433-6439

29. Yasuniwa M, Tsubakihara S, Sugimoto Y, Nakafuku C (2004) Thermal analysis of the double-melting behavior of poly(L-lactic acid). Journal of Polymer Science, Part B: Polymer Physics 42 (1):25-32

30. Nordqvist D, Sanchez-García MD, Hedenqvist MS, Lagaron JM (2010) Incorporating amylopectin in poly(lactic Acid) by melt blending using poly(ethylene-co-vinyl Alcohol) as a thermoplastic carrier. (I) morphological characterization. Journal of Applied Polymer Science 115 (3):1315-1324

31. Sanchez-Garcia MD, Lagaron JM (2010) On the use of plant cellulose nanowhiskers to enhance the barrier properties of polylactic acid. Cellulose 17 (5):987-1004

32. Sharma VK, Yngard RA, Lin Y (2009) Silver nanoparticles: Green synthesis and their antimicrobial activities. Advances in Colloid and Interface Science 145 (1-2):83-96

33. Wiley B, Sun Y, Mayers B, Xia Y (2005) Shape-controlled synthesis of metal nanostructures: The case of silver. Chemistry - A European Journal 11 (2):454-463

34. Pal S, Kyung Tak Y, Joardar J, Kim W, Eun Lee J, Soo Han M, Myong Song J (2009) Nanocrystalline silver supported on activated carbon matrix from hydrosol: Antibacterial mechanism under prolonged incubation conditions. Journal of Nanoscience and Nanotechnology 9 (3):2092-2103

35. Rujitanaroj PO, Pimpha N, Supaphol P (2010) Preparation, characterization, and antibacterial properties of electrospun polyacrylonitrile fibrous membranes containing silver nanoparticles. Journal of Applied Polymer Science 116 (4):1967-1976

36. Jeon HJ, Kim JS, Kim TG, Kim JH, Yu WR, Youk JH (2008) Preparation of poly(ε-caprolactone)-based polyurethane nanofibers containing silver nanoparticles. Applied Surface Science 254 (18):5886-5890

37. Sheikh FA, Barakat NAM, Kanjwal MA, Chaudhari AA, Jung IH, Lee JH, Kim HY (2009) Electrospun antimicrobial polyurethane nanofibers containing silver nanoparticles for biotechnological applications. Macromolecular Research 17 (9):688-696

38. Lee DY, Lee KH, Kim BY, Cho NI (2010) Silver nanoparticles dispersed in electrospun polyacrylonitrile nanofibers via chemical reduction. Journal of Sol-Gel Science and Technology 54 (1):63-68

39. Busolo MA, Fernandez P, Ocio MJ, Lagaron JM (2010) Novel silver-based nanoclay as an antimicrobial in polylactic acid food packaging coatings. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 27 (11):1617-1626

40. Damm C, Münstedt H (2008) Kinetic aspects of the silver ion release from antimicrobial polyamide/silver nanocomposites. Applied Physics A: Materials Science and Processing 91 (3):479-486

41. Kumar R, Münstedt H (2005) Polyamide/silver animicrobials: Effect of crystallinity on the silver ion release. Polymer International 54 (8):1180-1186

42. Jiang ZM, Li HX, Ji JH (2009) Influence of crystallinity on the silver ion release in LDPE containing silver-ion antimicrobial agent. Gaofenzi Cailiao Kexue Yu Gongcheng/Polymeric Materials Science and Engineering 25 (12):55-58

43. Hwang MG, Katayama H, Ohgaki S (2007) Inactivation of Legionella pneumophila and Pseudomonas aeruginosa: Evaluation of the bactericidal ability of silver cations. Water Research 41 (18):4097-4104

44. Kim TN, Feng QL, Kim JO, Wu J, Wang H, Chen GC, Cui FZ (1998) Antimicrobial effects of metal ions (Ag^{+}, Cu^{2+}, Zn^{2+}) in hydroxyapatite. Journal of Materials Science: Materials in Medicine 9 (3):129-134

45. Martínez-Abad A, Lagaron JM, Ocio MJ (2012) Development and characterization of silver-based antimicrobial ethylene-vinyl alcohol copolymer (EVOH) films for food-packaging applications. Journal of Agricultural and Food Chemistry 60 (21):5350-5359

46. Costa C, Conte A, Buonocore GG, Del Nobile MA (2011) Antimicrobial silver-montmorillonite nanoparticles to prolong the shelf life of fresh fruit salad. International Journal of Food Microbiology 148 (3):164-167

47. Gammariello D, Conte A, Buonocore GG, Del Nobile MA (2011) Bio-based nanocomposite coating to preserve quality of Fior di latte cheese. Journal of Dairy Science 94 (11):5298-5304

Chapter VI

EVALUATION OF SILVER-INFUSED POLYLACTIDE FILMS FOR INACTIVATION OF *SALMONELLA* AND FELINE CALICIVIRUS IN VITRO AND ON FRESH-CUT VEGETABLES

International Journal of Food Microbiology 162 (1), pp. 89-94

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ABSTRACT

There is a growing trend to develop packaging materials with an active role in guarantying that the quality and safety characteristics of packaged products will remain or improve from preparation throughout shelf-life.

In the present study, 0.001-1.0 wt.-% silver ions were satisfactorily incorporated into polylactide (PLA) films by a solvent casting technique. Silver migration from the films was measured by voltamperometry and then correlated with its antimicrobial efficacy against *Salmonella enterica* and feline calicivirus (FCV), a human norovirus surrogate, by using the Japanese industrial standard (JIS Z 2801). The PLA-silver films showed strong antibacterial and antiviral activity *in vitro*, with increasing effects at higher silver concentrations. Moreover, results show that FCV was less susceptible to silver than *Salmonella*. When films were applied on food samples, antibacterial and antiviral activity was reduced as compared to *in vitro*. Antimicrobial activity was very much dependent on the food type and temperature. In lettuce samples incubated at 4°C during 6 days, 4 log CFU of *Salmonella* were inactivated for films with 0.1 wt.-% and 1.0 wt.-% and no infectious FCV were reported under the same conditions. On paprika samples, no antiviral effect was seen on FCV infectivity whereas films showed less antibacterial activity on *Salmonella*.

Keywords: Active packaging, antimicrobial activity, norovirus, *Salmonella*.

1. Introduction

The consumption of fresh-cut vegetables has increased globally as they are generally considered safe and healthy by consumers (Lynch et al., 2009). However, agricultural irrigation with wastewater that may be raw, treated and/or partially diluted, is a common practice worldwide and constitutes the main source of pathogen contamination. Several factors affect microbial quality and shelf-life of vegetables, such as intrinsic properties of the vegetables (e.g. pH, water content), processing factors (e.g. washing, cutting, blanching), extrinsic factors (e.g. storage temperature, packaging) and implicit factors (e.g. microbial characteristics) (Heard, 1999).

The increase in mass production and distribution of food products will lead to an increase in the number of multinational outbreaks. A wide range of pathogens has been associated with outbreaks related to vegetable products. Among them, the most common agents causing fresh produce-related outbreaks are human norovirus (NV) and *Salmonella* (Doyle and Erickson, 2008; EFSA, 2012). In addition, human norovirus and *Salmonella* have been listed in the top 5 highest-ranking pathogens with respect to the total cost of foodborne illness in the United States (Scharff, 2010). As a means to prevent recontamination with pathogens and allow extending shelf-life of foods, antimicrobial packaging is one of the most promising technologies in the food area. However, few studies have confronted the task of fabricating and evaluating materials with antimicrobial properties in real food applications, and very scarce information is available about packaging materials with both antibacterial and virucide properties.

Among natural antimicrobials, silver has emerged as a very efficient technology to prevent microbial proliferation on food contact surfaces in the food industry. Due to its unspecific mechanism of action, silver ions are not only active against a very broad spectrum of bacteria, but also against yeasts, fungi and even viruses, being non toxic to human cells (Russell and Hugo, 1994; Williams et al.,1989). In the U.S., the Center for Food Safety and Applied Nutrition in the Food and

Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled waters and in the EU, silver is accepted under directive 94/36/EC as a colouring agent (E-174) with no restrictions. Additionally, the European Food Safety Authority (EFSA) has provided positive opinions for silver zeolites, silver zirconium phosphates, and silver containing glasses in its provisional list of additives for food contact materials with a general restriction of ≤0.05 mg/kg food for the whole group.

As a result of its outstanding potential, and given the absence of cost-effective alternatives among other natural antimicrobials, silver is the most widely used polymer additive for food contact applications (Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002).

The approach of the use of silver in the food industry has been mostly bounded to ion exchange from mineral carriers, which are incorporated as a coating on predominantly stainless steel surfaces and plastics. These systems rely on the sustained release of silver ions via a moisture dependent ion exchange mechanism. Despite its widespread use, strong differences are found in the appraisal of silver efficacy among the different studies, due to complexity of availability of free silver ions. As silver ions are known to form non-active complexes in the presence of proteins and other organic matter, its antimicrobial activity strongly varies depending on the environment of action (Ilg and Kreyenschmidt, 2011; Martinez-Abad et al., 2012). Moreover, the very low migration rates of the silver ions from these materials imply the need of high filler contents, which could limit the application of these systems in antimicrobial packaging, due to high production costs or a negative environmental impact. Therefore, it is crucial to thoroughly investigate the release and efficacy patterns of silver ions from the selected polymer under conditions as similar to the final application as possible. Consumer' demands, and the requirements by regulatory authorities, to pursue more environmentally friendly and less polluting packages, have directed research into packaging materials that are made from renewable resources to replace some of the synthetic polymers (Kuorwel et al., 2011).

In particular, polylactides, a renewable family of polymers derived from biobased resources such as maize, are becoming increasingly popular owing to its high mechanical strength, transparency, water resistance, melt processability and relatively low prices in the market compared to other biopolymers (Auras et al., 2004).

If incorporated into PLA, the wide spectrum of action of silver ions might be useful to fight bacterial as well as viral contamination in food products.

In the previous work, silver ions were incorporated into PLA without the addition of further mineral carriers, as a means to allow a targeted and controlled release of the biocide to the food matrix upon contact with the moisture of the food. The materials were characterized and the release of ions was related to their antibacterial efficacy under laboratory conditions. In this work, the materials were evaluated under relatively realistic conditions. Furthermore, the virucide activity of PLA-silver materials was reported for the first time. The films were tested both under the Japanese industrial standard Z 2801 and with vegetable samples in the presence of *Salmonella* and feline calicivirus (FCV), as a norovirus surrogate. Effectiveness was further evaluated after subsequent washings, to additionally test the film endurance on longer times.

2. Materials and Methods

2.1. Film preparation

PLA supplied by Natureworks Llc, US was used for preparation of packaging films. Polymer pellets were dissolved in tetrahydrofuran (THF) or in a mixture of THF and dimethylformamide (DMF; 3:1 w/w) in the ratio 5:95 (w/w) at 50 ºC under stirring. Then, the suitable amount of silver nitrate (Sigma-Aldrich) was added to the solution as to achieve PLA-THF and PLA-DMF films with 0.001% - 1.0% silver nitrate weight in dry conditions. The solution was cast onto glass Petri dishes to obtain a ~30-40 μm thick films after solvent evaporation at 60 ºC for 3 h. Remaining solvent in the films was further allowed to diffuse out in a vacuum oven at 50 ºC for 5 h. Pure PLA films were also prepared by the same procedure. Films were stored in a 0% relative humidity desiccators protected from light with aluminium wrapping before undergoing testing and to a maximum of 14 days.

2.2. Determination of bactericidal activity

The *Salmonella enterica* CECT 554 strain was obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain) and stored in Phosphate Buffer Saline (PBS) with 10% Tryptone Soy Broth (TSB, Conda Laboratories, Madrid, Spain) and 10% glycerol at -80 ºC until needed. For experimental use, the stock culture was maintained by regular subculture to Tryptone Soy Agar (TSA) slants at 4 ºC and transferred monthly.

Previous to each study, a loopful of bacteria was transferred to 10 mL of TSB and incubated at 37 ºC overnight. A 100 μL aliquot from the overnight culture was again transferred to TSB and grown at 37 ºC to the mid-exponential phase of growth. This culture served as the inoculum for antimicrobial assays starting. These CFU counts were accurately and reproducibly obtained by inoculation into 10 mL growth medium of 0.1 mL of a culture having an absorbance value of 0.20 as determined by optical density at 600 nm by ultraviolet visible (UV) spectroscopy (SP-2000 UV, Spectrum Instruments, Shanghai, China).

To test the antibacterial activity of PLA composites, a modification of the Japanese Industrial Standard JIS Z 2801 was used. Briefly, *S. enterica* suspension as to achieve 5 $\times 10^5$ CFU/cm^2 was applied onto the test PLA films of 3 x 3cm and covered by a an inert piece of Low-Density Polyethylene (LDPE) of 2.5 × 2.5cm and 10 μm thickness. After incubation at 24 °C for 24 h, bacteria were recovered and then, 10-fold serially diluted in 0.1% buffered peptone water (BPW) and plated on TSA for plate counts after incubation at 37 °C for 24 h. PLA films without silver were used as a negative control.. Antibacterial activity was calculated by determining $\log_{10} (N_t/N_0)$, where N_0 is the bacterial counts recovered from PLA films without silver and Nt the bacterial counts recovered from PLA films loaded with silver. Each experimental condition was analysed in triplicate.

2.3. Determination of virucidal activity

The cytopathogenic F9 strain of FCV (ATCC VR-782) was propagated and assayed on CRFK cells (CCL-94). Semi-purified stocks were subsequently produced on CRFK cells by centrifugation of infected cell lysates at 660×g for 30 min. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose ($TCID_{50}$) with eight wells per dilution and 20 μL of inoculum per well.

Determination of the virucidal activity of PLA films containing different percentages of silver (1.0, 0.1 and 0.01%) was performed by adapting the above mentioned standard (JIS Z 2801:2000). Briefly, a suspension of FCV diluted in PBS buffer (ca. 10^5 $TCID_{50}$ /mL) was placed onto the test films and covered by a piece of LDPE. Samples were incubated at 24 °C for 24 h. Thereafter, the top film was lifted, and the virus droplet-exposed sides were recovered and 10-fold diluted with PBS. Lastly, a cell culture assay was performed to determine whether the films were effective in inactivating the virus. A PLA film without silver was used as the negative control material. Virucidal activity was calculated by comparing the number of infectious viruses on PLA films without

silver and on the test films. Each experimental condition was analysed in triplicate.

2.4. Test of antimicrobial activity after washing PLA-films

In order to evaluate the antimicrobial activity of films after washing, triplicate samples of pure PLA or PLA-silver films processed according to the JIS Z 2801 as described above were rinsed with ethanol, properly dried, and then reinoculated again with bacterial and viral suspensions (ca. 5×10^5 CFU/mL and 10^5 $TCID_{50}$ /mL). After incubation at 24 °C for 24 h, microorganisms were recovered following the same JIS Z 2801 standard described above. This procedure was correlatively repeated 6 times.

2.5. Challenge tests

Locally purchased fresh lettuce and paprika were used in this study. Vegetable samples were cut in pieces of 3x3 cm and sterilized with UV light in a safety cabinet under laminar flow for 15 min prior to inoculation of the test microorganisms. A 25 µL aliquot of *S. enterica* and FCV were independently inoculated on the food sample to achieve concentrations of about 5×10^6 CFU/cm^2 or 10^5 $TCID_{50}$/cm^2 respectively. After inoculation, vegetable samples were held for 10 min to allow sorption of the tested microorganisms. Then, vegetables were covered with pieces of 2x2 cm PLA-THF films with 0.1% and 1.0% silver and the set was incubated at 4ºC and 12 °C for 7 days. To measure antimicrobial activity of the films, samples were removed at different time interval (1, 3 and 6 days) and homogenized with 40 mL BPW in a sterile plastic bag with a lateral filter using a Pulsifier (Microgen Bioproducts, UK) for 2 min. Serial dilutions in 0.1% BPW or PBS were made in order to quantify the number of viable bacteria or infectious viruses, respectively, as described above. Each experimental condition was analysed in triplicate.

2.6. Silver migration

Voltammetric analysis of the samples was conducted to determine the release of free silver ions (FSI) from the films to an aqueous environment. Triplicate

samples of PLA containing 0.1 and 1.0 wt% of silver films were cut in pieces of 2x2 cm, 100 µL of ultrapure water slightly acidified (1mM HNO_3 to stabilize silver in its ionic form) was added and the samples were covered with LDPE, simulating the JIS Z 2801:2000 but without microorganisms. The FSI content in each sample was determined by differential pulse anodic stripping voltammetry (ASV) with an Autolab III potentiostat setup (EcoChemie B.V., The Netherlands) under conditions stated in Metrohm application bulletin nº 207/2e "Analysis of silver by stripping voltammetry". The FSI working range was 0.001 – 0.1 ppm and a calibration curve was prepared daily for each set of measurements.

2.7. Statistical analysis

The statistical significance of differences was determined on the ranks with a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. In all cases, a value of $p < 0.05$ was considered to be significant.

3. Results and Discussion

3.1. Antimicrobial activity and silver release of silver-PLA films

The Japanese industrial standard (JIS) Z 2801:2000 (Anonymous, 2000) is among the most common methods for assessing the antimicrobial activity of materials and it was applied in the current study to determine the antibacterial and antiviral activity of PLA-silver films (Table 1). After 24 h exposure, no viable counts of *Salmonella* were recorded when in contact with PLA-DMF and PLA-THF films containing 0.1 and 1.0% and 0.01, 0.1 and 1.0% of silver, respectively (Table 1).

Table 1. Silver release and antimicrobial effect of silver-PLA films on *Salmonella* viability and feline calicivirus (FCV) infectivity after 24 h contact at 24ºC following the Japanese industrial standard Z 2801 (n=3).

		Salmonella[a] (Log_{10} CFU/mL)		**FCV** (Log_{10} $TCID_{50}$/mL)		**Silver ions released**	
Solvent	**% of silver**	**Counts after 24 h**	**Reduction**	**Counts after 24 h**	**Reduction**	**Ppm**	**Percentage**[b]
DMF	0	8.0 ± 0.1 A	0	5.5 ± 0.1 A	0	NT[c]	NT
	0.001	7.8 ± 0.2 A	0.2	NT	-	NT	NT
	0.01	4.1 ± 0.4 B	3.9	5.4 ± 0.1 A	0	NT	NT
	0.1	< 2.0 C	> 6.0	3.5 ± 0.3 B	2	0.64 ± 0.03	0.11 ± 0.01
	1.0	< 2.0 C	> 6.0	< 1.1 C	> 4.4	33.0 ± 14.0	0.55 ± 0.23
THF	0	8.1 ± 0.1 A	0	4.6 ± 0.7 A	0	NT	NT
	0.001	8.1 ± 0.7 A	0	NT	-	NT	NT
	0.01	< 2.0 B	> 6.1	4.6 ± 0.09 A	0	1.56 ± 0.92	2.61 ± 1.53
	0.1	< 2.0 B	> 6.1	2.5 ±0.09 B	2.1	4.72 ± 0.13	0.79 ± 0.02
	1.0	< 2.0 B	> 6.1	< 1.1 C	> 4.4	661.6±19.6	11.03 ± 1.99

PLA-DMF films containing 0.01% of silver showed about 4 log CFU reduction in comparison with the control, i.e. PLA-DMF films without silver. These results are somewhat similar to that for the silver-based nanoclay incorporation into PLA films reported by Busolo et al. (2010), where the *Salmonella* viable

counts were reduced by more than 4 log CFU /mL when in contact with PLA films containing 1.0 % of silver nanoclay. The antiviral effect of silver against suspensions of enteric viruses have been reported (De Gusseme et al., 2010; Silvestry-Rodriguez et al., 2007), suggesting the potential to be incorporated in active packaging to control enteric viruses. In the current study, FCV, a norovirus surrogate broadly used for testing disinfectants (EPA, 2002), was exposed to PLA- silver films for 24 h (Table 1). FCV titers decreased by 2 log $TCID_{50}$/mL when treated with PLA-DMF films at concentrations of 0.1% of silver, while in films produced with THF, FCV titers decreased by 2.1 log $TCID_{50}$/mL. So far, only Bright et al. (2009) have evaluated the antiviral activity of active packaging, reporting that FCV infectivity was reduced by 5 log $TCID_{50}$/mL when in contact with plastic coupons impregnated with 10% silver-copper zeolites. However, this silver content was higher than the one evaluated in this study.

Finally, the release of silver from PLA films was monitored only in films incorporated with 1.0 and 0.1 % of silver, due to the limitations of the technique. Table 1 shows that THF films released higher amounts of silver, which correlates with higher antibacterial activity of the mentioned films. As commented in chapter 5, silver nitrate is highly soluble in water and not soluble in organic solvents, while polyesters, on the contrary, are mostly soluble in organic solvents like chloroform, acetone or THF. Silver nitrate was found to be soluble in THF only at low concentrations. Therefore, a combination of DMF and THF (1:3) was further selected, as it was the combination which yielded the highest solubility of the active compound without compromising the film-forming capacities. As solvent evaporates during casting, the active compound may agglomerate, depending on the solubility, which could explain the faster release of silver from PLA-THF films and the more sustained release from PLA-DMF films (see chapter 5). Accordingly, the selection of different solvents may serve as an additional tool to tune the release capacities of silver based antimicrobial polyesters.

Although literature has extensively reported the use of polymers incorporating silver nanoparticles for their possible use in food packaging applications,

nanoparticles are still out of the legislative frame in most countries, including the EU. Antimicrobial materials based on the release of ionic silver are, on the other hand, permitted and widely used in most countries (Anonymous, 2006; FDA-CFSAN, 2010). These materials are mostly based on silver exchange from resins or other inorganic fillers, such as monttmorillonites (MMT), and zeolites (Busolo et al., 2010; Costa et al., 2011; Gammariello et al., 2011) being the most widely used of all (Quintavalla and Vicini, 2002). So far, only a few recently published studies have dealt with the incorporation of silver as antimicrobials into PLA for food packaging applications. One approach has been the inclusion of silver ions in MMT as a carrier in PLA (Busolo et al., 2010). Fortunati et al. (2012) have also incorporated silver nanoparticles as antibacterial filler. In these cases, however, filler contents were in the range of 1.0-10%. In the present study, the developed PLA-films, without further inorganic fillers, led to a strong antibacterial and antiviral effect with filler content of 0.01% and 0.1%, respectively.

3.2. Long-term antimicrobial activity of washed PLA-films

The effectiveness of PLA-silver films against the most common foodborne pathogens, i.e. *Salmonella* and norovirus, appears promising for its potential use in applications to reduce environmental contamination of food contact surfaces.

Table 2. Reduction of *Salmonella* viability and feline calicivirus (FCV) infectivity in contact with PLA-DMF-silver films being washed.

		Number of Washing					
	% of silver	**0**	**1**	**2**	**3**	**4**	**5**
***Salmonella*[a] (log CFU/mL)**	0.01	> 6.0	3.7±3.0	0.6±0.5	0.00±0.1	-	-
	0.1	> 6.0	> 6.0	> 6.0	> 6.0	> 6.0	> 6.0
	1.0	> 6.0	> 6.0	> 6.0	> 6.0	> 6.0	> 6.0
FCV[b] (log $TCID_{50}$/mL)	0.01	-	-	-	-	-	-
	0.1	1.6±0.2	0.9±0.4	0.4±0.1	1.6±0.0	0.5±0.5	0.5±0.7
	1.0	> 4.4	3.9±0.6	> 4.4	> 4.4	3.9±0.3	3.0±0.3
% Silver ions released[c]	1.0	0.55±0.23	0.07±0.14	0.08±0.02	< 0.05	< 0.05	< 0.05

[a]Reduction of *Salmonella* counts after contact 24 h at 24ºC with films containing silver in comparison with films without silver [b]Reduction of infectious FCV titers after contact 24 h with films containing silver in comparison with films without silver [c]Fraction of the silver content released upon contact of PLA-1 % silver films. The initial *Salmonella* inoculum size was 5.7 log CFU. Experiments were performed in triplicate.

Therefore, in order to evaluate the potential use of the PLA-silver-films for food contact applications where washings and recontamination of surfaces may occur, films were rinsed, dried and reinoculated after each evaluation several times and their antibacterial, antiviral properties and the silver release evaluated (Table 2 and 3). Overall, PLA-THF films were again more effective inactivating *Salmonella* whereas not many differences were seen for FCV. Films containing 1.0 and 0.1% silver completely inactivated *Salmonella* after 5 washings, except the PLA films containing 0.01% silver. In addition, *Salmonella* counts were reduced by > 6 log CFU when in contact with PLA-THF with 0.01% silver films after 1 washing, but antimicrobial activity was progressively reduced along the subsequent washings. When films were produced with DMF, *Salmonella* counts were reduced by 3.7 log CFU after one washing, and thereafter reductions were around 2 log CFU.

Table 3. Reduction of *Salmonella* viability and feline calicivirus (FCV) infectivity in contact with PLA-THF-silver films being washed

		Number of Washing					
	% of silver	**0**	**1**	**2**	**3**	**4**	**5**
***Salmonella*[a] (log CFU/mL)**	0.01	> 6.1	> 6.1	3.0±1.8	2.0±0.2	2.4±3.2	1.8±2.2
	0. 1	> 6.1	> 6.1	> 6.1	> 6.1	> 6.1	1.6±0.5
	1.0	> 6.1	> 6.1	> 6.1	> 6.1	> 6.1	> 6.1
FCV[b] (log $TCID_{50}$/mL)	0.01	-	-	-	-	-	-
	0.1	2.3±0.3	0.0±0.0	0.0±0.1	0.0±0.2	0.1±0.00	1.9±0.9
	1.0	> 4.4	3.7±0.1	> 4.4	3.9±0.00	3.7±0.4	> 4.4
% Silver ions released[c]	1.0	11.03±1.99	6.64±8.32	0.08±0.02	< 0.05	< 0.05	< 0.05

[a]Reduction of *Salmonella* counts after contact 24 hat 24ºC with films containing silver in comparison with films without silver
[b]Reduction of infectious FCV titers after contact 24 h with films containing silver in comparison with films without silver
[c]Fraction of the silver content released upon contact of PLA-1 % silver films. The initial *Salmonella* inoculum size was 5.7 log CFU. Experiments were performed in triplicate.

Antiviral activity of films filled with 1% silver reduced FCV infectivity by more than 4 log $TCID_{50}$/mL. This highly antiviral activity was maintained even after washing the films 5 times. When films containing 0.1% of silver were evaluated,

antiviral activity was only reported without washing, evidencing FCV is less susceptible to silver than *Salmonella*. This is not entirely surprising since it is very well known than non-enveloped viruses are more resistant than gram negative bacteria to biocides (Russell, 2003). These results demonstrate controlled release may be sufficient for silver based antimicrobial PLA films to produce an antimicrobial effect over storage time or after washing, putting forth its suitability for possible application in food packaging or food contact surfaces. However, an initial burst release upon the first contact of about 0.55% for PLA-THF, followed by a much slower release capacities over time, could limit the use of these materials due to the stringent restriction limits (0.05 mg/kg food) stated by the EFSA. Therefore, technologies retarding this burst release, such as a multilayered materials or the use of other solvents to enhance solubility, should be taken into consideration.

3.3. Challenge tests

Fresh produce is an important part of a healthy diet, but concerns about its safety have been raised as they represent the 2nd leading cause of foodborne illnesses in the USA (Sivapalasingam et al., 2004). The use of active packaging during the storage could be helpful in inactivating foodborne pathogens while improving the shelf-life of these highly perishable products. Challenge tests on lettuce and paprika were carried out to ascertain the antibacterial and virucidal effectiveness of PLA-THF films containing 1.0 and 0.1% silver on real food samples. PLA with 1.0% silver content in contact with lettuce reduced *Salmonella* counts by 4 log CFU when incubated at 4 and 12º C during 6 days (Fig 1A and 1B). Films containing only 0.1% silver initially reduced *Salmonella* counts by 2 log CFU after 24 h in contact at 12 °C, however *Salmonella* counts were recovered to the levels of the control when the storage time was extended, indicating a burst release of silver during the first hours that was slowly inactivated during the storage time, and at 12°C, the remaining survival bacterial growth at the initial levels.

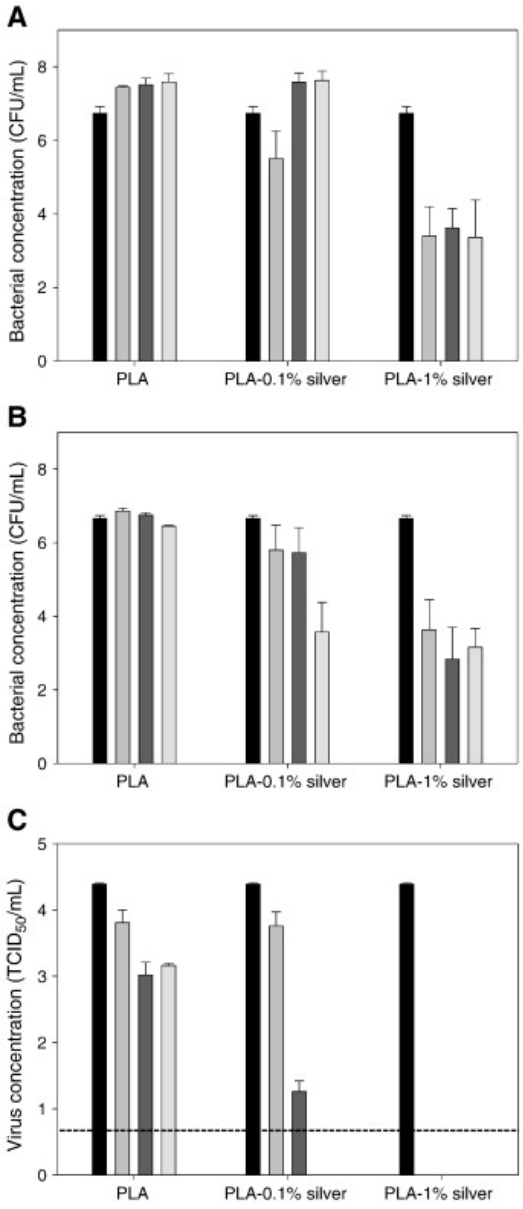

Figure 1. Challenge tests in lettuce A) *Salmonella* viable counts at 12°C, B) *Salmonella* viable counts at 4°C and C) infectious feline calicivirus (FCV) titers at day 0 (black bars), 1 day (grey bars), 3 days (dark grey bars), and 6 days (light grey bars) upon contact with PLA 0%, 0.1 or 1.0 % silver content. Dashed line depicts the detection limit. Experiments were performed in triplicate.

When the experiments were performed at 4 °C, antimicrobial activity was less pronounced, with only 1 log reduction upon contact with PLA with 0.1% silver content but then antibacterial activity was progressively increased along the storage time. Temperature is an important factor that influences the antibacterial action of silver.

Some studies have reported that at low temperatures the release of silver from its carrier material decreases (Quintavalla and Vicini 2002; Kampmann et al., 2008). Experiments with viruses were only performed at 4ºC since they are not able to grow in food in food products. When FCV was exposed to PLA films with 1.0% silver, infectivity was completely removed (Fig. 1C), whereas the use of 0.1% silver films was progressively reducing the FCV infectivity, achieving the completely reduction of infectivity at the end of the storage time. Infectious FCV inoculated on lettuce were slightly reduced along the storage time, with ca. 1 log $TCID_{50}$ reduction. FCV has previously been shown to be inactivated on the surface of vegetables (Stine et al., 2005). So it may be possible that antiviral silver effect is enhanced by the inactivation due to the storage time. When experiments were carried out on paprika, no effect was seen on FCV infectivity (Fig. 2C) whereas films showed less antibacterial activity on *Salmonella* (Fig 2A and 2B), indicating that silver was precipitating with some compounds of the paprika, hence, reducing antimicrobial activity of FSI. This indicates that application of active packaging based on silver depends very much on the food type, on the environmental factors and on the pathogen itself (Kampmann et al., 2008) Nevertheless, films containing 1.0% of silver reduced by 4 log CFU *Salmonella* viability when paprika samples were incubated at 4 °C. When incubated at 12 °C, an initial decay of viability of 2.3 log CFU was reported after 24 h, but then viable counts increased to the initial concentration after 7 days of storage (Fig 2A). So, again, the free silver ions concentration was not sufficient to inactivate the *Salmonella* present in the paprika sample, and at this temperature *Salmonella* was able to slightly grow.

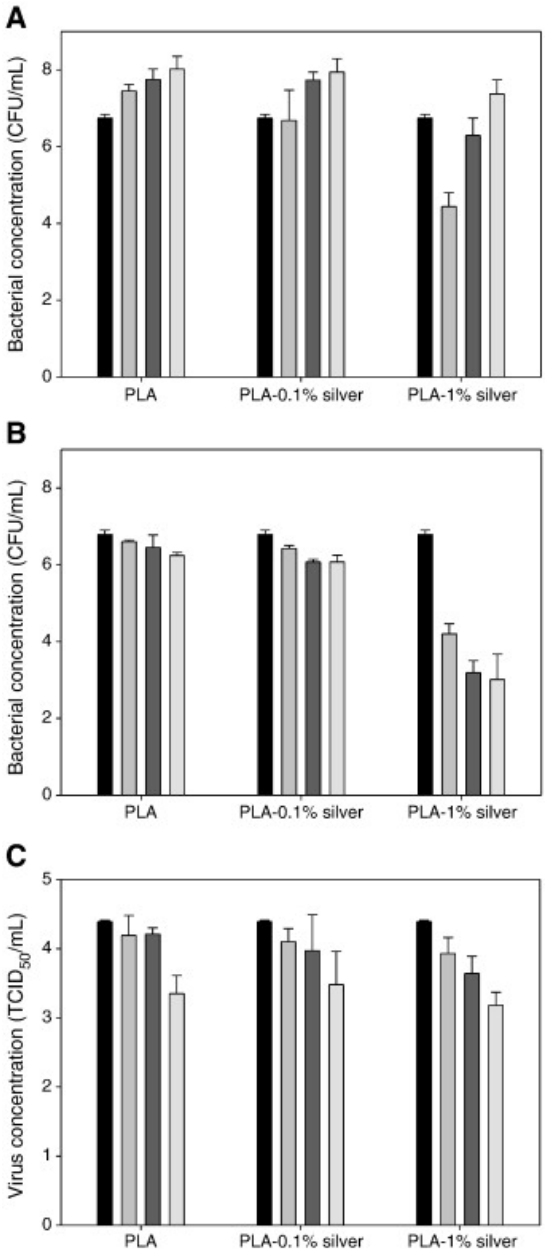

Figure 2. Challenge tests in paprika A) *Salmonella* viable counts at 12°C, B) *Salmonella* viable counts at 4°C and C) infectious feline calicivirus (FCV) titers at day 0 (black bars), 1 day (grey bars), 3 day (dark grey bars), and 6 days (light grey bars) upon contact with PLA 0%, 0.1 or 1.0 % silver content.

These studies have been carried out inoculating high levels of pathogens in order to assess the efficacy of the active packaging, however previous studies showed that bacterial pathogens in food products are mostly present in lower levels (between 10^2 and 10^4 CFU/g) (Elizaquível et al., 2011). Therefore we may expect that the films tested in this study may have a greater effect when applied in more real conditions. Levels of viruses in food products are still not well documented, and when so, (Mattison et al., 2010; Stals et al., 2011), molecular techniques have always been applied and therefore the real level of infectious viruses is not well known.

While evaluation of silver based antimicrobial polymers is mostly done in vitro by broth dilution or disk diffusion techniques, some studies on real food samples have also been performed. When chemically complex foods are selected, silver based polymers were mostly ineffective in reducing bacterial populations by further than 1 log even at very high silver concentrations. When applied to turkey and pork, silver zeolites loaded paper (4.0%) was able to somewhat reduce the bacterial growth rates at refrigeration temperatures (Lee et al., 2011). Shelf-life studies in cheese with silver-MMT composites promoted an increase in the shelf-life of cheese when incorporated in agar, but were found ineffective when incorporated into zein or PCL (Costa et al., 2011; Gammariello et al., 2011; Incoronato et al., 2011). The inability to reduce bacterial counts in complex food matrices has been attributed to silver being inactivated by proteins (Ilg and Kreyenschmidt, 2011). To our acknowledgement, only one study has applied silver films on real food samples with a significant reduction in viable counts; Martínez-Abad et al. (2012) have incorporated silver on ethylene-vinyl alcohol copolymer (EVOH) films and evaluated its antibacterial efficacy on *Listeria monocytogenes*. However, release capacities of silver from EVOH films were found to be more rapid than from PLA films and challenge tests were only prolonged for 48 h at only 12 ºC. In spite of the good results from PLA films loaded with silver ions, the restriction limits of the EFSA must be considered for their application in the food sector. As explained above, release capacities from films with low concentrations of silver should be optimized in the future, as to

reduce the burst release on contact with humidity and comply with current legislation. This aspect is dealt with in the next chapter.

4. Concluding Remarks

For the first time, we have evaluated active renewable packaging materials for virus control in food samples. Our results show excellent potential for PLA-silver films for food contact applications as well in active packaging technologies to maintain or extend food quality and safety.

5. Acknowledgements

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6. References

Anonymous, 2000. JIS Z 2801:2000 Antimicrobial products-test for antimicrobial activity and efficacy. Japanish Standards Association.

Anonymous, 2006. Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request related to a 12th list of substances for food contact materials. EFSA Journal, 395–401.

Appendini, Hotchkiss, P.J.H., 2002. Review of antimicrobial food packaging. Innovative food science and emerging technologies 3, 113-126.

Auras, R., Harte, B., Selke, S., 2004. An Overview of Polylactides as Packaging Materials. Macromolecular Bioscience 4, 835-864.

Bright, K., Sicairos-Ruelas, E., Gundy, P., Gerba, C., 2009. Assessment of the Antiviral Properties of Zeolites Containing Metal Ions. Food and Environmental Virology 1, 37-41.

Busolo, M. A., Fernandez, P., Ocio, M. J., Lagaron, J. M., 2010. Novel silver-based nanoclay as an antimicrobial in polylactic acid food packaging coatings. Food additives and contaminants Part A: 27, 1617-1626.

Costa, C., Conte, A., Buonocore, G. G., Del Nobile, M.A., 2011. Antimicrobial silver-montmorillonite nanoparticles to prolong the shelf life of fresh fruit salad. International Journal of Food Microbiology 148, 164-167.

De Gusseme, B., Sintubin, L., Baert, L., Thibo, E., Hennebel, T., Vermeulen, G., Uyttendaele, M., Verstraete, W., Boon, N., 2010. Biogenic silver for disinfection of water contaminated with viruses. Applied and Environmental Microbiology 76, 1082-1087.

Doyle, M.P., Erickson, M.C., 2008. Summer meeting 2007 - the problems with fresh produce: an overview. Journal of Applied Microbiology 105, 317–330.

Elizaquível, P., Gabaldón, J. A., Aznar, R., 2011. Quantification of *Salmonella* enterica, *Listeria monocytogenes* and *Escherichia coli* O157:H7 in non-spiked food products and evaluation of real-time PCR as a diagnostic tool in routine food analysis. Food Control 22, 158-164.

EPA, 2002. Initial virucidal effectiveness test. *Antrimicrobials division*. http://www.epa.gov/oppad001/pdf_files/initial_virucidal_test.pdf
European Food Safety Authority, 2012. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in the European Union in 2010. EFSA Journal 10, 442.

FDA/CFSAN, 2010. Listing of food additive status: Silver nitrate-172.167, U. FDA/CFSAN, Editor.

Fortunati, E., Armentano, I., Zhou, Q., Iannoni, A., Saino, E., Visai, L., Berglund, L. A., Kenny, J. M., 2012. Multifunctional bionanocomposite films of poly(lactic acid), cellulose nanocrystals and silver nanoparticles. Carbohydrate Polymers 87, 1596-1605.

Gammariello, D., Conte, A., Buonocore, G.G., Del Nobile, M.A., 2011. Bio-based nanocomposite coating to preserve quality of Fior di latte cheese. Journal of Dairy Science 94, 5298-5304.

Heard, G., 1999 Microbial safety of ready-to-eat salads and minimally processed vegetables and fruits. Food Australia 51:414-420.

Ilg, Y., Kreyenschmidt, J., 2011. Effects of food components on the antimicrobial activity of polypropylene surfaces containing silver ions (Ag +). International Journal of Food Science and Technology 46, 1469-1476.

Incoronato, A.L., Conte, A., Buonocore, G.G., Del Nobile, M.A., 2011. Agar hydrogel with silver nanoparticles to prolong the shelf life of Fior di Latte cheese. Journal of Dairy Science 94, 1697-1704.

Kampmann Y., De Clerck E., Kohn S., Patchala D. K., Langerock R., Kreyenschmidt, J. 2008. Study on the antimicrobial effect of silver-containing inner liners in refrigerators. Journal of Applied Microbiology 106, 1808 – 1814.

Kuorwel, K. K., Cran, M. J., Sonneveld, K., Miltz, J., Bigger, S. W., 2011. Essential Oils and Their Principal Constituents as Antimicrobial Agents for Synthetic Packaging Films. Journal of Food Science 76, R164-R177.

Lee, J., Lee,Y.-H., Jones, K., Sharek, E., Pascall, M.A., 2011. Antimicrobial packaging of raw beef, pork and turkey using silver-zeolite incorporated into the material. International Journal of Food Science and Technology 46, 2382-2386.

Lynch, M.F., Tauxe, R.V., Hedberg, C.W., 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. Epidemiology and Infection 137, 307–315.

Martínez-Abad, A., Sanchez, G., Lagaron, J. M., Ocio, M. J., 2012. On the different growth conditions affecting silver antimicrobial efficacy on *Listeria monocytogenes* and *Salmonella enterica.* International Journal of Food Microbiology 158, 147-154.

Martínez-Abad, A., Lagaron, J. M., Ocio, M. J., 2012. Development and Characterization of Silver-Based Antimicrobial Ethylene–Vinyl Alcohol Copolymer (EVOH) Films for Food-Packaging Applications. Journal of Agricultural and Food Chemistry 60, 5350-5359.

Mattison, K., Harlow, J., Morton, V., Cook, A., Pollari, F., Bidawid, S., Farber, J. M., 2010. Enteric viruses in ready-to-eat packaged leafy greens. Emerging and Infectious Diseases 16, 1815-1817.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M., Tauxe, R. V., 1999. Food-related illness and death in the United States. Emerging and Infectious Diseases 5, 607-625.

Quintavalla, S., Vicini, L., 2002. Antimicrobial food packaging in meat industry. Meat Science 62, 373–380.

Rai, M., Yadav, A., Gade, A., 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances 27, 76-83.

Russell, A. D. (2003). Similarities and differences in the responses of microorganisms to biocides. Journal of Antimicrobial Chemotherapy 52, 750-763.

Russell, A. D., Hugo, W. B., 1994. Antimicrobial activity and action of silver. Progress in Medicinal Chemistry 31, 351-370.

Scharff, R. L., 2010. Health-related Costs from foodborne illness in the United States. http://www.publichealth.lacounty.gov/eh/docs/ReportPublication/HlthRelatedCostsFrom FoodborneIllinessUS.pdf

Silvestry-Rodriguez, N., Sicairos-Ruelas, E. E., Gerba, C. P., Bright, K. R., 2007. Silver as a Disinfectant. Reviews of Environmental Contamination and Toxicology. In (Vol. 191, pp. 23-45): Springer New York.

Sivapalasingam, S., Friedman, C. R., Cohen, L., Tauxe, R. V., 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. Journal of Food Protection 67, 2342-2353.

Stals, A., Baert, L., Jasson, V., Van, C. E., Uyttendaele, M., 2011. Screening of fruit products for norovirus and the difficulty of interpreting positive PCR results. Journal of Food Protection 74, 425-431.

Stine, S. W., Song, I., Choi, C. Y., Gerba, C. P., 2005. Effect of Relative Humidity on Preharvest Survival of Bacterial and Viral Pathogens on the Surface of Cantaloupe, Lettuce, and Bell Peppers. Journal of Food Protection 68, 1352-1358.

Williams, R. L., Doherty, P. J., Vince, D. G., Grashoff, G. J., Williams, D. F., 1989. The biocompatibility of silver. Critical Reviews in Biocompatibility 5, 221-243.

Chapter VII

ANTIMICROBIAL BEESWAX COATED POLYLACTIDE FILMS WITH SILVER CONTROL RELEASE CAPACITY

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ABSTRACT

Although application of silver based antimicrobial systems is a widespread technology, its implementation in areas such as food packaging is still challenging. The present paper describes the fabrication of Poly(lactic acid) (PLA) coated with beeswax with controlled release properties for sustained antimicrobial performance. Release of silver ions from the polymers was monitored voltammetrically under various conditions (surface contact, immersion in various liquid media and at different pH) throughout at least 7 days. A higher release was noted with decreasing pH while surface release was much slower than the release when immersed in liquid medium. While uncoated films demonstrated a high burst release which in some instances implied surpassing some current migration restrictions (<0.05mg/Kg food), the addition of a beeswax layer allowed a sustained release of the antimicrobial compound. Increasing the thickness of the beeswax layer resulted in an increase in the water barrier properties of the films while reducing the relatively constant values of sustained release. Antimicrobial performance was correlated with the release of silver ions, indicating threshold concentrations for biocide action of <6 μg/L and 9-14 μg/L for surface contact and in liquid media, respectively. Either by surface contact or by immersion in growth medium or vegetable soup, the coated films displayed a strong bactericidal effect against *Salmonella enterica*. The application of this functional barrier offers thus the possibility of tuning the release profiles of the films to suit a specific application and puts forth the possible suitability of these materials for food packaging or other migration sensitive applications.

Keywords: Silver ions, poly(lactic acid) or poly(L-lactide), controlled release, antimicrobial food packaging, antimicrobial coatings.

1. Introduction

In recent years, the interest in food packaging with antimicrobial properties has increased considerably, due to the fact that these systems are able to control the microbiological decay of perishable food products (Mastromatteo et al., 2010). Many applications, including food production and storage, might benefit from the incorporation of safe and wide spectrum long-lasting biocides into polymers or working surfaces (Appendini and Hotchkiss, 2002). As bacterial contamination occurs primarily on the surface, the incorporation of the biocide in a film or as a coating has many advantages due to the high exposure areas for the biocide and a reduction of the loss of the antimicrobial compound into the bulk of the food (López-Rubio et al., 2004; Ouattar et al., 2000). Therefore, extensive research has been made to develop packaging strategies to retain the active agent in the polymeric network and control its release as to allow the use of less quantity while assuring the desired effect over the shelf-life of the product (Mastromatteo et al., 2010).

Biopolymers obtained from natural resources are a promising alternative to non-biodegradable petroleum-based plastics in food packaging due to their environmentally friendly nature (Fabra et al., 2013). In particular, polylactides, a renewable family of polymers derived from biobased resources such as maize, are becoming increasingly popular owing to their high mechanical strength, transparency, water resistance, melt processability and relatively low prices in the market compared to other biopolymers (Auras et al., 2004). Additionally, PLA and its copolymers are the most widely used plastics for controlled drug delivery systems because of their biodegradability, biocompatibility and ease of processing (Zhang et al., 2013) Therefore, the use of PLA for controlled release of antimicrobials could be an interesting field of research for food packaging applications.

Silver ions are active against a very broad spectrum of bacteria, yeasts, fungi and viruses and are not toxic to human cells (Russell and Hugo, 1994; Williams et al.,1989). Therefore, a wide variety of materials used in daily life are recently

incorporated with silver or silver salts as key component to control microbial proliferation, ranging from textile clothing (Yuranova et al., 2003), stainless steel coatings in home appliances (Kampmann et al., 2008) and food-contact materials (Bouwmeester et al., 2009; Galeano et al., 2003) (see Chen and Schluesener, 2008; Gupta and Silver, 1998; Rai et al., 2009 for review). In the U.S., the Center for Food Safety and Applied Nutrition in the Food and Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled waters and in the EU, silver is accepted under directive 94/36/EC as a colouring agent (E-174) with no restrictions. Additionally, the European Food Safety Authority (EFSA) has provided positive opinions for silver zeolites, silver zirconium phosphates, and silver containing glasses in its provisional list of additives for food contact materials with a general restriction of ≤0.05 mg/kg food for the whole group (EFSA Journal, 2008). Regardless of the stringent regulations, silver still remains the most widely used antimicrobial polymer additive in food applications (Appendini and Hotchkiss, 2002.; Quintavalla and Vicini, 2002).

In the food sector, silver based antimicrobial systems are mostly based on a thin polymeric layer containing silver exchanged minerals (1-5% silver content) and coated on polymeric or stainless steel surfaces of for example water treatment units or other food processing equipment (cutlery, cutting boards, counter tops, containers); (Chen and Schluesener, 2008; Gupta and Silver, 1998; Rai et al., 2009). The limitation of the use of silver to surfaces is probably attributed to the instability of the active silver species, free silver ions. Silver ions are known to be easily inactivated by many different physical or chemical factors (Ilg and Kreyenschmidt, 2011; Martinez-Abad et al., 2013a). Therefore, the potential application of a silver based system in food packaging implies gathering sufficient knowledge as to be able to control migration profiles, which should comply with current legislation, while at the same time assuring antimicrobial effectiveness under each set of specific conditions.

Previous works demonstrated PLA films incorporating silver were efficient in reducing bacterial contamination either in liquid medium or at the surface of foods (Fortunati et al., 2012; Martinez-Abad et al., 2013b). However, the

sorption-induced release profiles in these materials indicated silver ion concentration could exceed restriction limits in some cases, which would limit their application. Lipids, mainly waxes and resins, are known biopolimers able to produce films with very low water vapour permeability (WVP), although they are opaque and brittle. Among lipids, beeswax is one of the most effective materials employed to decrease WVP due to its high hydrophobicity and solid state at room temperature (Fabra et al., 2008). Therefore, its use as a means to reduce WVP and thus delay or reduce release profiles in coated films could be an interesting approach.

In the present paper, PLA films containing 0.1% silver were coated with beeswax layers of different thicknesses to produce films with different silver ion release profiles. Controlled migration was measured over several days under various conditions and the release capacity correlated with the antimicrobial performance under the same or similar conditions. The aim of the study was to evaluate the factors that govern the release of silver ions under different circumstances and assess the suitability of these materials for its potential implementation in food packaging or other bactericidal applications.

2. Materials and Methods

2.1. Materials

PLA as supplied by Natureworks Llc was used for the preparation of the films. The polylactide with a D-isomer content of approximately 2% had a number-average molecular weight (M_n) of ca. 130,000 g/mol, and an average molecular weight (M_w) of ca. 150,000 g/mol as reported by the manufacturer. Silver nitrate (>98% purity; Sigma-Aldrich, Steinheim, Germany) was used as the antimicrobial compound. White beeswax (VWR, Leuven, Belgium) was used as a functional barrier to produce coated films. The solvents tetrahydrofuran (THF) and dimethylformamide (DMF) used for dissolution of the polymer were purchased at Panreac S.A.U. (Barcelona, Spain). M9 minimal salts 5x (Sigma-Aldrich), glucose, tryptic soy agar (TSA; Condalab, Madrid, Spain), magnesium sulfate (Panreac) and natural vegetable soup (Caldos Aneto, Artés, Barcelona, Spain) were used for preparation of the synthetic growth media and antimicrobial testing.

2.2. Film preparation

Polymer pellets were dissolved in a mixture of THF and DMF (3:1 w/w) in the ratio 5:95 (w/w) at 50 ºC under stirring. After dissolution, a suitable amount of silver nitrate was added to the solution as to achieve films with 0.1% silver nitrate weight in dry conditions. The solution was cast onto glass Petri dishes to obtain a ca. 70 μm thick films after solvent evaporation for 3h at 60ºC. Remaining solvent in the films was further allowed to diffuse out in a vacuum oven at 50 ºC for 18 h. The coated films were prepared using a hydraulic press (Carver 4122, USA). Uncoated films (PLA-0) were placed on the press plate at 90ºC and pellets of white beeswax were placed on both sides and allowed to melt on these specimens. Then, 2MPa of pressure was applied during 3 min to allow homogeneous distribution of the beeswax. To obtain coated films with increasing thickness, aluminium foil sheets of different thicknesses were cut in the shape of the films and used as molds on top of the PLA films. All films were

stored at 0% relative humidity (RH) desiccators before undergoing testing and to a maximum of 14 days.

2.3. Morphology

To investigate the morphology of the films, SEM microphotographs (S4100, Hitachi, Osaka, Japan) were taken with an accelerating voltage of 5 keV on the thickness of the films after cryofracture of the samples immersed in liquid nitrogen. The thickness of the beeswax functional barrier was calculated with the ImageJ software taking 100 random measurements from ten different images of each sample.

2.4. Water Vapour Permeability

The WVP of the films was measured according to the ASTM E96 (2011) gravimetric method, using Payne permeability cups (Elcometer, Hermelle Argenteau, Belgium). Distilled water was placed inside the cup to expose the film (the exposed area was 9.6×10^{-4} m^2) to 100% RH on one side. Once the films were secured, each cup was placed in an equilibrated relative humidity dessicator at 24°C. Relative humidity at 0% was held constant using silica gel. The cups were weighed periodically (±0.0001 g), at least twice a day for 7 days. Aluminium foil was used as a control to rule out vapour loss through the sealing. WVP was calculated from the steady-state permeation slopes obtained from the regression analysis of weight loss data over time. The lower limit of WVP detection of the permeation cells was of ~$1\cdot10^{-17}$ kg·m/s m^2 Pa based on the weight loss measurements of the aluminium films. All measurements were performed in triplicate.

2.5. Quantification of Silver Release

A voltammetric method was used to determine the release of free silver ions over time from the films under different conditions. For the determination of the release profiles of the films in liquid medium at different pH, 9cm^2 of the films were cut and immersed in 30 mL of aqueous solutions either from neutral double distilled water or adjusted to pH 2.5 with HNO_3. For determination of the release

profiles from the surface of the films under conditions stated in the Japanese Industrial Standard Z2801, 2.88mL of aqueous solutions adjusted at pH 2.5 or neutral were spread on 100 cm^2 of the films and covered with a piece of low density polyethylene to assure intimate contact over the whole surface area. Samples in both assays were kept at 24ºC for 24h to allow silver release from the films. Before each measurement, the aqueous solutions in contact with the samples were removed and nitric acid was added to stabilize silver in its ionic form. The silver ion content for each measurement was determined by differential pulse anodic stripping voltammetry (ASV) with an Autolab III (EcoChemie) potentiostat setup under conditions stated in Metrohm application bulletin nº 207/2e "Analysis of silver by stripping voltammetry". Sample volume or/and deposition time was increased to enhance sensitivity. The silver ion working range was 1 – 100 µg/L. After each measurement, film samples were dried with pressurized air, and used again under the same conditions for subsequent release measurements in both assays. This procedure was correlatively repeated every day for at least 7 days or until silver release was below the detection limit (0.5 µg/L). All experiments were carried out in duplicate.

2.6. Bacterial strains and growth conditions

Salmonella enterica CECT 554 strain was obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain) and stored in Phosphate Buffer Saline (PBS) with 10% Tryptone Soy Broth (TSB, Conda Laboratories, Madrid, Spain) and 10% glycerol at -80 ºC until needed. For experimental use, the stock culture was maintained by regular subculture to Tryptone Soy Agar (TSA) slants at 4 ºC and transferred monthly.

Previous to each study, a loopful of bacteria was transferred to 10 mL of TSB and incubated at 37 ºC overnight. A 100 µL aliquot from the overnight culture was again transferred to TSB and grown at 37 ºC to the mid-exponential phase of growth. CFU counts were accurately and reproducibly obtained by inoculation into 10 mL growth medium of 0.1 mL of a culture having an absorbance value of 0.20 as determined by optical density at 600 nm by

ultraviolet visible (UV) spectroscopy (SP-2000 UV, Spectrum Instruments, Shanghai, China).

*2.7. Antimicrobial performance of silver as released from the film*s

The effectiveness of silver ions when released from the films was assessed employing the macro-dilution method M26-A described by the Clinical and Laboratory Standards Institute (CLSI) with modification. 30 cm^2 of the films were cut and immersed in 30 mL of acidic and neutral aqueous solutions. Firstly, samples were incubated at 24ºC for 24h to allow silver release from the films. After incubation, the solution was sterilized by filtration and used for both voltammetric analysis and antimicrobial testing. 8 mL of the solution were added into 2mL 5x M9 medium. A bacterial suspension in mid-log phase was then inoculated in each test tube to achieve an initial inoculum size of approximately 5 x 10^5 CFU/mL and incubated at 37ºC for 24 h. Then, 0.1 mL of each sample was sub-cultivated on TSA plates for viable count after incubation at 37ºC for 24 h. The rest of the aqueous solution containing the silver released over 1 day was analyzed voltammetrically to determine silver ion concentration as described above. After each analysis, film samples were dried with pressurized air, and used again under the same conditions for subsequent analysis. This procedure was correlatively repeated every day for at least 7 days or until silver release was below the detection limit (0.5 µg/L). Samples treated analogously but without silver were used as controls. Each of these experiments was performed in duplicate.

Secondly, the efficacy of the films when tested in liquid environments was assessed by immersing 3cm^2 of the films in 10 mL of M9 minimal medium or natural vegetable soup, in regard to the same ratio as in the release measurements. Then, a bacterial suspension was inoculated as to achieve approximately 5 x 10^5 CFU/mL. The tubes were incubated in static conditions at 12ºC for 7 days and samples were taken every 24h for bacterial enumeration by conventional plate count on TSA, as described above.

2.8. Antimicrobial performance of the films by surface contact

To evaluate the antimicrobial efficacy on bacteria on the surface of the films, the Japanese Industrial Standard Z2801 "Antimicrobial products-Test for antimicrobial activity and efficacy" (JIS) was followed. Briefly, a suspension containing 5 x 10^5 CFU/mL was inoculated and spread onto the surface of the tested films with a square size of 5x5 cm and covered with an inert polyethylene film of 4x4 cm. Then the samples were introduced into Petri dishes and incubated at a temperature of 24ºC and a relative humidity at least 95 % for 24 h. After the incubation period, the surviving cells were collected from the test film sample by using a stomacher and then enumerated by conventional plate count.

3. Results and Discussion

3.1. Morphology

Microstructural analysis of the beeswax coated films provides information about the arrangement of the different film components and may help to understand the mechanisms of water and sorption induced transport through the films. Figure 1 shows images of the cross-sections from the cryo-fractured coated films. The beeswax layer can easily be discerned from the PLA matrix and a good adhesion is observed between the two layers. The average coating thickness for PLA-1, PLA-2 and PLA-3 films was calculated to be 4.56±1.98 μm, 9.00±2.72 μm and 20.32±4.38 μm, respectively.

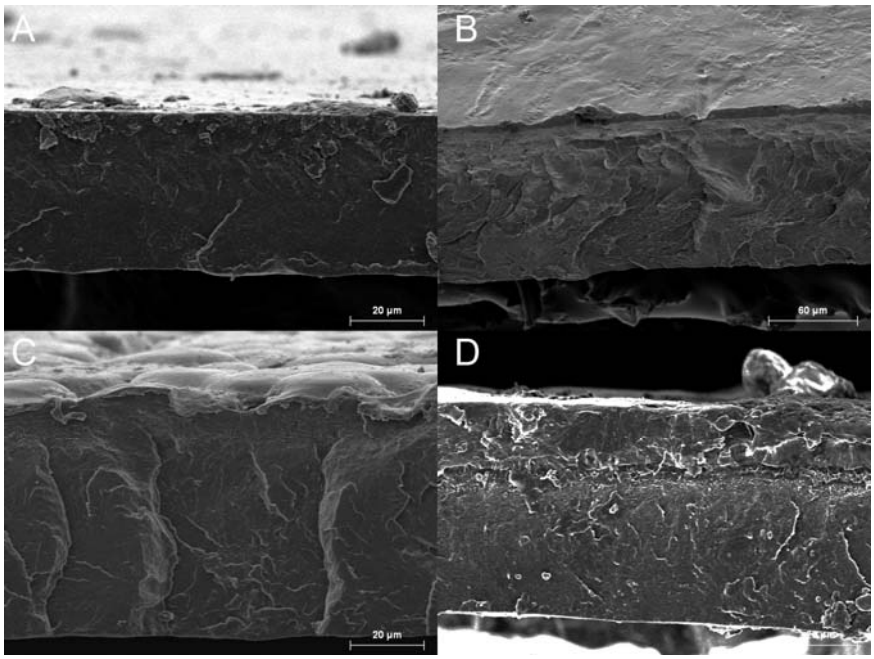

Figure 1. SEM micrographs of the cryofractured cross-sections of PLA-0 (a), PLA-1 (b), PLA-2 (c) and PLA-3 (d) films.

3.2. Water Vapour Permeability

Water vapour permeability (WVP) of the biopolymer films is a very extensively studied parameter since it is directly related with food deteriorative reactions. Additionally, it is well known that most biobased polymers demonstrate higher WVP than their fossil-fuel derived counterparts. The effect of the addition of a hydrophobic functional barrier to the PLA films may improve this important feature, while at the same time serve as a barrier to sustain the release of the antimicrobial compound. Figure 2 gathers the direct water vapour permeability coefficients of the cast PLA films with and without a beeswax coating of different thickness.

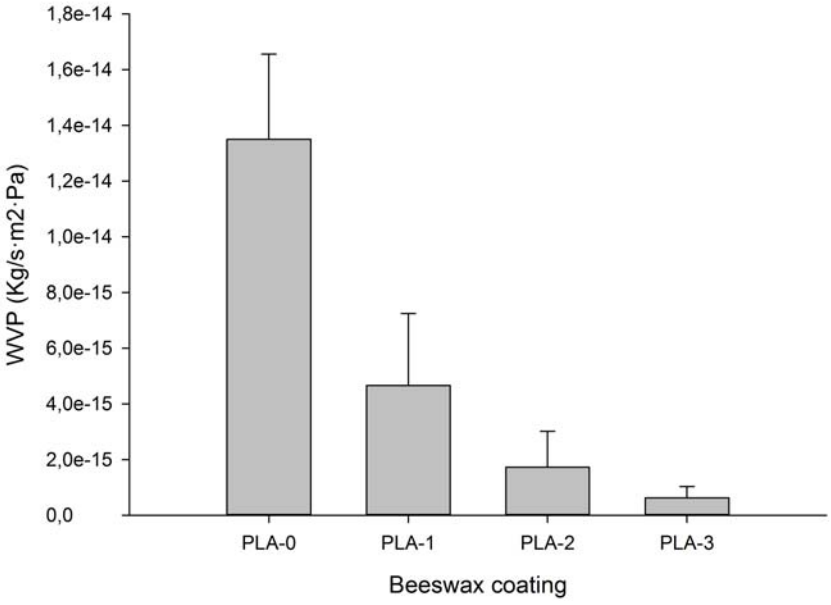

Figure 2. Water vapour permeability of the tested films according to the ASTM E96.

The addition of an increasingly thick beeswax coating results in substantial consecutive reductions in their WVP (Guilbert et al., 1995; Khwaldia, 2010; Zhong et al., 2011). Uncoated films show an average WVP of $1.35 \cdot 10^{-14}$ ($Kg \cdot m/s \cdot m^2 \cdot Pa$). These values are in accordance with those previously reported for PLA cast films (Fortunati et al., 2012; Sanchez-Garcia et al., 2008). When a

thin beeswax layer of about 5 μm is coated on both sides of the films, there is about a 65% reduction in their WVP. PLA-2 films consisting of a beeswax layer of 7-11 μm demonstrate a reduction of about 87% as compared with uncoated PLA films, representing a 63% reduction compared to PLA-1 films. PLA-3 films having a beeswax layer twice as thick as PLA-2 films (about 20 μm) showed another 63% relative reduction in their WVP. In this case, WVP values are not significantly different from those reported for beeswax alone (Donhowe et al., 1993; Shellhammer and Krochta, 1997). The results demonstrate that the addition of a thin beeswax layer can improve the barrier properties of PLA films as to obtain WVP values similar to benchmark non-biodegradable polymers like polyethylene terephthalate (PET) (Staff, 1995). A further increase in the thickness of the functional barrier to 20 μm resulted in WVP values similar to high water barrier polymers, like polypropylene.

3.3. Release study in liquid media

A fundamental issue when evaluating the possible application of silver based antimicrobial system is the correct assessment of silver specific migration. The European Food Safety Authority has for instance restricted migration of silver to foodstuffs to 0.05mg/kg food (EFSA), a very low threshold which could severely limit the use of silver as an antimicrobial in the food sector. Additionally, the release of antimicrobial silver ions can highly vary depending on the moisture content or the pH of the surrounding environment in contact with the films. Silver nitrate is, for instance, known to show higher solubility in water than in organic solvents, and its release from polymer matrices has been reported to be triggered by food moisture (Martinez-Abad et al., 2012a). Considering these issues, the release capacities of the films were evaluated both at the surface of the produced films and when immersed in water. Moreover, each condition was tested at acidic and neutral pH to further assess how the pH may affect release either at the surface of the films or when immersed in aqueous solutions. Table 1 shows the results of the specific migration of silver measured under these conditions. At pH 2.5, uncoated films allow an initial burst release of 122 ng/cm^2. The daily release is approximately halved each day

until day 4 or day 5, when it stabilizes to a relatively constant value of about 10 ng/cm^2. Due to their opposite polar character, silver ions may not be easily dispersed in a PLA matrix. This phenomenon could be at the origin of the release behaviour exhibited in uncoated films (Martinez-Abad et al., 2013b). Considering restriction limits in European legislation (0.05mg/kg) and a hypothetical packaging surface of 6dm^2/kg food, a release of maximum 83 ng/cm^2 would be permitted. Therefore, under these conditions, silver release from uncoated films may be not sustained enough as to comply with current restriction limits in some legislation frames. Films coated with an increasingly thick beeswax layer show a more sustained release throughout the tested time.

Table 1. Silver release from the tested films immersed in aqueous solutions at different pH.

Silver release at acidic pH (ng/cm^2)							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
PLA-0	122±35	61.4±9.5	37.2±4.2	15.7±2.8	11.1±4.3	9.5±1.1	12.3±2.2
PLA-1	24.8±7.2	20.3±3.9	11.0±2.9	10.6±1.4	9.7±0.5	11.5±4.6	7.7±2.3
PLA-2	10.0±1.0	8.9±1.5	9.5±0.2	16.1±3.5	14.2±2.1	12.9±3.6	7.4±1.9
PLA-3	2.5±0.6	1.5±0.3	1.8±0.2	1.0±0.3	0.9±0.2	0.9±0.1	0.9±0.1
Silver release at neutral pH (ng/cm^2)							
PLA-0	110±16	49.4±3.5	22.7±2.6	4.8±0.9	2.1±0.3	1.5±0.1	1.0±0.1
PLA-1	2.8±0.3	4.0±1.3	2.1±0.9	1.6±0.6	1.4±0.1	1.0±0.3	0.8±0.3
PLA-2	1.0±0.3	1.1±0.4	0.7±0.2	0.7±0.1	0.8±0.2	<0.5	<0.5
PLA-3	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

These relatively constant values oscillate between 10ng/cm^2 and 2ng/cm^2 for PLA-3 and PLA-4 films, respectively, while PLA-1 films show a higher release the first two days (about 20 ng/cm^2), which stabilizes to about 10 ng/cm^2 in subsequent days. When the same assay is performed at neutral pH, uncoated films show a burst release very similar to that at pH 2.5 the first three days, but it stabilizes at a much lower value of 1-2 ng/cm^2 from day 5 on. Coated films show again a capacity to prevent a burst release and sustainably deliver silver ions from the first contact, although release values are considerably lower at pH 7

than at pH 2.5. PLA is known to be a relatively hydrophobic polymer. This can slow down plasticization in the presence of moisture. A lower pH in the liquid medium might induce partial hydrolysis of the PLA structure (Burkersroda et al., 2002; Ivanova et al., 1997). This could allow further penetration of water along the polymer matrix, promoting the sorption induced sustained release of silver ions. These results demonstrate that either immersed in an acidic or a neutral aqueous environment, the application of a beeswax coating can serve as a tool to tune the release of silver ions and suit the best application.Additional research on the behaviour of these films in other food simulants or food matrices according to current legislation is required to ascertain its ultimate implementation in active food packaging.

3.4. Surface release study

Current application of silver based technologies is mostly devoted to the preparation of antimicrobial surfaces. In the food sector, these materials are mostly based on a thin polymeric layer containing silver exchanged minerals (1-5% silver content) and coated on polymeric or stainless steel surfaces of for example water treatment units or other food processing equipment (cutlery, cutting boards, counter tops, containers). In these cases, lower migration rates are expected as compared to applications where the films are immersed in an aqueous environment because of the lower amount of moisture in contact with the films. Therefore, a similar setup as in the JIS for testing surface antimicrobial efficacy was performed for the assessment of surface migration. Results in Table 2 display the daily release of silver over consecutive days at acidic and neutral pH. Uncoated films at pH 2.5 present a much lower surface burst release than when immersed, but the same amount of silver ions are delivered once a sustained release is achieved (about 10 ng/cm^2). Nevertheless, accumulated release throughout the tested time would still surpass the hypothetical threshold of 83 ng/cm^2. In coated films, the burst release is prevented and, as before, a more sustained release is attained with a tendency to lower values as the thickness of the functional barrier increases. At neutral pH, silver ion release is in most cases very near to the detection limit of the

experiment in all films. Under these conditions, the silver ion release in any of the films is not expected to surpass restriction limits even after a very prolonged contact exposure.

Table 2. Silver release from the tested films to aqueous solutions at different pH as measured following the JIS Z2801.

Silver release at acidic pH (ng/cm^2)								
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
PLA-0	28±6	21±8	11±5	12±4	13±3	9±3	10±4	9±3
PLA-1	12±3	17±5	7±3	13±5	16±5	12±2	11±5	6±2
PLA-2	5±1	3±1	7±1	4±2	5±1	3±1	4±5	2±0
PLA-3	1±1	<1	<1	<1	<1	<1	<1	<1
Silver release at neutral pH (ng/cm^2)								
PLA-0	18±3.2	0.3±0.2	0.2±0.1	0.1±0.0	<0.1	<0.1	<0.1	<0.1
PLA-1	1.2±0.1	0.1±0.1	0.2±0.1	0.1±0.0	<0.1	<0.1	<0.1	<0.1
PLA-2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
PLA-3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

*3.5. Antimicrobial performance of silver ions as released from the film*s

As current legislation has set stringent limits to silver in foodstuffs, it is crucial to elucidate the threshold of antibacterial effectiveness for silver and correlate silver ion concentrations with their respective effectiveness against foodborne pathogens. *S. enterica* was chosen as model pathogen in this study, considering it is the most common pathogenic bacterium involved in foodborne outbreaks in developed countries (EFSA Journal, 2011). To correlate silver ion concentration with its corresponding antibacterial efficacy, a sample from each release measurement was introduced in M9 medium containing *S. enterica.* The addition of the samples at low pH did not alter the pH in the tubes due to the efficient buffering in M9 (data not shown). The results in Table 3 and Table 4 present the final silver ion concentration in the test tubes and the corresponding *Salmonella* counts after 24h incubation at 37ºC. Uncoated films, PLA-1 and PLA-2 films are able to sustainably release silver ions at pH 2.5 as to inhibit growth or reduce bacterial counts throughout at least 7 days immersion in water. In PLA-3 films,

however, a sustained release of <5 μg/L was not enough to produce any antimicrobial effect. When the release environment is at neutral pH, the released silver ions from coated films are insufficient as to be active against *Salmonella* under optimum growth conditions.

Table 3. Antibacterial performance of silver released to aqueous solutions at acidic pH from the tested films.

	Silver concentration in the test tube (μg/L)								
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
PLA-0	315±45	148±53	69±18	35±13	18±3	18±4	10±3	14±4	8±3
PLA-1	34±10	27±5	14±4	18±6	13±3	13±6	9±3	6±3	9±1
PLA-2	13±2	13±8	13±5	23±3	17±4	15±4	9±1	6±2	6±3
PLA-3	3±1	3±1	3±1	3±2	2±1	2±1	2±0	2±1	2±0
	Bacterial counts (Log UFC/mL)								
PLA-0	<1	<1	<1	<1	1.3±0.2	1.9±0.8	5.3±0.6	1.7±0.4	8.9±0.1
PLA-1	<1	<1	<1	<1	1.4±0.4	2.3±0.3	3.6±0.8	8.9±0.2	5.3±0.5
PLA-2	4.5±0.2	3.8±0.3	4.6±0.3	<1	1.5±0.2	2.3±0.4	5.6±0.3	8.2±0.7	8.9±0.1
PLA-3	8.7±0.1	8.9±0.2	8.8±0.1	8.9±0.2	9.0±0.1	9.0±0.1	8.8±0.1	8.7±0.2	9.0±0.1

Only uncoated films are able to deliver enough silver ions as to produce an antibacterial effect for 4 consecutive days. When the silver ion concentration in each tube is compared with the corresponding bacterial counts, it is revealed that a concentration of < 8 μg/L did not alter bacterial growth, evidencing that this low silver ion concentration is not enough to significantly damage the tested pathogen (Tables 3-4). When more than 14 μg/L are present, however, a reduction of at least 3 log units (or 99.9% of the initial population) is achieved in all cases. Therefore, the threshold concentration of antimicrobial action under the tested conditions might be between 9-14 μg/L. This concentration is below the current restriction limits of 0.05 mg/kg food. Silver is mainly considered to exert a high antimicrobial efficacy by surface contact. Therefore, current application of silver as antimicrobial has mostly been focused on these silver containing surfaces (Martinez-Abad, 2010). The decrease in antimicrobial efficacy can be attributed to the instability of silver ions in solution, which

readily reduce or can be inactivated by a great variety of chemical environments (Ilg and Kreyenschmidt., 2011; Martinez-Abad et al., 2012b) The results in the present paper evidence a high antimicrobial effect can be achieved by the presence of a very small silver ion concentration. While uncoated films show an initial burst release that may surpass restriction limits the first days, PLA-2 films at pH 2.5 are able to produce a sustained release of enough silver ions to produce an antimicrobial effect over time while at the same time complying with relatively stringent legislations. This effect can be only due to silver ions present in solution and is achieved even at optimum incubation temperatures of 37ºC.

Table 4. Antibacterial performance of silver released to aqueous solutions at neutral pH from the tested films.

Silver concentration in the test tube (μg/L)							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
PLA-0	184±33	83±2	38±5	8±3	3±2	2±1	2±0
PLA-1	5±2	7±3	3±2	2±1	2±1	1±0	2±1
PLA-2	3±1	2±1	1±1	2±0	1±1	1±0	<1
PLA-3	1±1	<1	<1	<1	<1	<1	<1
Bacterial counts (Log UFC/mL)							
PLA-0	<1	<1	<1	4.1±0.7	8.79±0.05	8.70±0.03	9.08±0.07
PLA-1	8.88±0.07	9.00±0.09	8.98±0.09	8.86±0.06	9.06±0.10	8.93±0.08	9.13±0.05
PLA-2	9.09±0.02	9.18±0.08	8.62±0.01	8.87±0.06	9.14±0.12	8.75±0.04	8.83±0.06
PLA-3	8.73±0.04	9.09±0.11	8.84±0.06	8.78±0.05	8.98±0.09	8.64±0.02	8.75±0.04

*3.6 Antimicrobial performance of the film*s *immersed in liquid medium*

For the evaluation of the antimicrobial capacity of the films incorporating silver in a liquid medium, a commercial natural vegetable soup and M9 medium were used. The soup has a pH value of 5.8-6.1, contains about 1.7% dry residue and 0.3% protein content. M9 medium is a minimal growth medium, buffered at pH 7.2, without any proteins and glucose as a sole carbon source and was used as to compare the results in the soup with a more restrictive medium void of proteinic possible ligands for silver. Incubation temperature was set to 12ºC as to reflect

temperature abuse in refrigerated samples (EU regulation 2073/2005). Figure 3 and Figure 4 present the bacterial counts over time for both liquid media, respectively. Controls without silver in soup are able to increase their bacterial count number up to maximum growth values of approximately 10^8 CFU/mL. In M9, however, *S. enterica* was not able to proliferate to a great extent throughout 7 days incubation. This contrasts with results in Tables 3 and 4 showing maximum growth values reaching 10^8-10^9 CFU/mL after 24h at 37ºC, indicating incubation temperature might play a relevant role in this nutrient restrictive medium. All films containing silver exert a very high antibacterial effect on *S. enterica*. Uncoated films with silver produce in soup a decrease of about

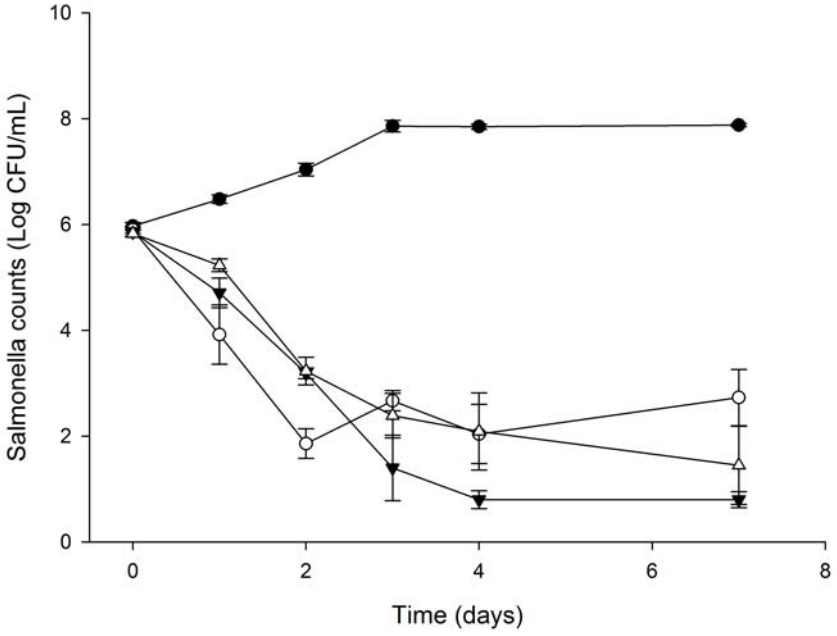

Figure 3. Bacterial viability of *S. enterica* in natural vegetable soup incubated at 12ºC without (●) and with PLA-0 (○), PLA-1 (▼) and PLA-2 (Δ) films for 7 days. The beeswax coating in PLA-1 and PLA-2 had an average thickness of 4.6 and 9.0µm, respectively). Experiments were performed in triplicate..

2 log units both on the first and second day. After that, bacterial counts do not further decrease or even increase throughout the rest of the experiment. Samples with coated films show, in contrast, a gradual decrease in bacterial counts of 4-5 log units after 7 days. In M9 medium, bacterial counts in uncoated film samples decrease 3 log units the first day. After that, the remaining viable bacterial population is able to resist until about day 4 and then decreases again until day 7.

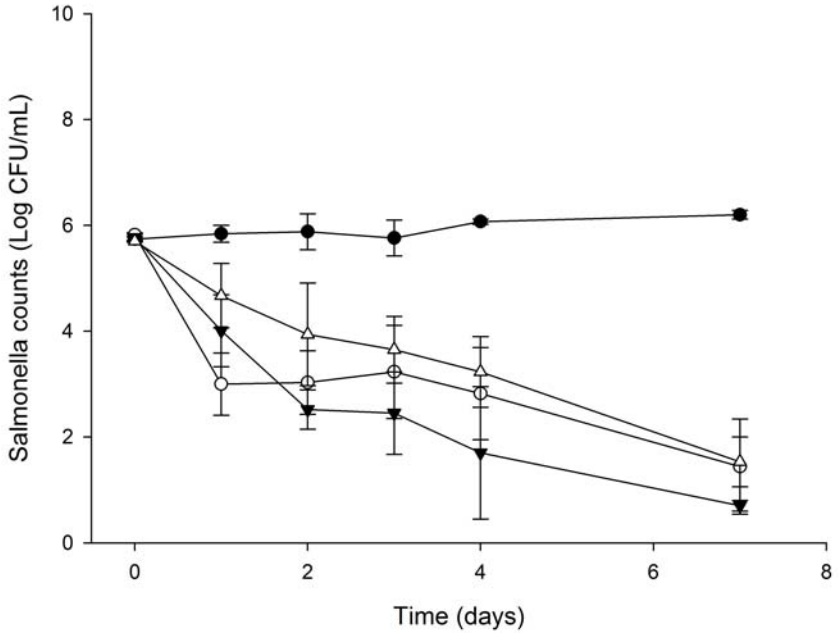

Figure 4. Bacterial viability of *S. enterica* in synthetic M9 medium incubated at 12ºC without (●) and with PLA-0 (○), PLA-1 (▼) and PLA-2 (Δ) films for 7 days. The beeswax coating in PLA-1 and PLA-2 had an average thickness of 4.6 and 9.0µm, respectively. Experiments were performed in triplicate. .

Samples with coated films again show a more gradual decrease in bacterial viability reaching bacterial counts of 10-100 CFU/mL. In general, a more sustained release in coated films is reflected in a more gradual decrease in bacterial viability throughout the 7 days. PLA-1 films were slightly more

effective than PLA-2 films, probably due to the higher silver ion release of these films under these conditions (Table 1). Considering release patterns determined in aqueous solution at similar pH, coated films are not expected to deliver more than 5 ng/cm^2 on a daily basis. This could imply not exceeding European restriction limits even after a very prolonged exposure of the film in contact with the food sample. In uncoated films, however, limitations stated by the EFSA would be probably surpassed from the very first day. Additionally, bacterial population in uncoated film samples showed a tendency to recovery in the nutrient rich environment of the soup, which could indicate a lack of sufficient available silver ions. These results point out the importance of developing a sustained release technology which assures a constant delivery of silver ions to the environment of action, and the possible suitability of the produced coated films for this kind of applications.

3.7. Antimicrobial performance of the films by surface contact

The antimicrobial capacity of the films was further tested according to the JIS Z2801 with modification. An incubation temperature of 24ºC was chosen as to mimic room temperature conditions. Instead of a diluted (500-fold) growth medium, M9 medium was used to allow proliferation of bacteria in case of absence of antibacterial activity and better visualize possible changes as compared to the controls. Table 5 shows bacterial viability after 24h incubation and the silver ion concentration that might have been released in each case calculated from the release study. It must be noted that the conditions in the release assay were not exactly equal to the assays testing antimicrobial efficacy. Although the pH 7 and the area of release (100cm^2) was changed to increase sensitivity, the ratio of 312dm^2/L was maintained. In PLA samples without silver the bacterial population is able to increase its numbers to 10^9-10^{10} CFU/mL in 24h under the tested conditions. In all samples incorporating silver except for PLA-3 films, the bacterial population is reduced to <100 CFU/mL, indicating a high antimicrobial capacity of the films. This effect is reduced in PLA-3 films, probably due to the lower release capacities of these films as shown previously.

Table 5. Antimicrobial performance of the tested films according the the Japanese Induatrial Standard Z2801 and corresponding silver ion concentration after 24h incubation.

	Bacterial viability (log CFU/mL)	Silver concentration (μg/L)
PLA without silver	9.30±0.21	-
PLA-0	<2	95.3±9.7
PLA-1	<2	7.2±1.0
PLA-2	<2	<6
PLA-3	7.41±0.30	<6

Differences between coated PLA films were not found, as silver ion concentration was in these cases near or below the threshold of detection under these testing conditions. Nevertheless, the release concentrations in coated films were far below the restriction limits (50μg/L). Furthermore, considering the much lower surface release compared to samples immersed in liquid medium, exceeding the limits is not expected even on longer terms. It is interesting to remark that even though the surface to liquid volume ratio is greatly increased in this approach, 312 dm^2/L, as compared to migration tests in liquid media (6 dm^2/L); the final concentration of silver in the moisture in intimate contact with the films was not greatly increased. Additionally, concentrations lower than 6 μg/L are still able to produce a bactericidal effect on the bacterial population in contact with the films. This value is below the threshold concentration of 9 μg/L suggested as breakpoint under conditions established in the migration tests in liquid media. These differences could be attributed to inactivation of free silver ions in solution as well as to a more instant and intimate contact of the bacterial population with the ions leaching from the surface of the films.

4. Conclusions

Although application of silver based antimicrobial systems is a widespread phenomenon since recent years, apprehension of the full potential of silver as antimicrobial and its possible implementation in food packaging technologies is still a challenging task. The present paper evidences the possibility of tuning the release profiles of PLA films with silver by the addition of a beeswax layer, evaluates the different parameters affecting their release profiles and correlates silver ion concentration under the various conditions with its corresponding antimicrobial efficacy. This could allow the fabrication of taylor-made antimicrobial coated films as to suit a specific application and puts forth the possible suitability of these materials for food packaging or other "migration sensitive" applications. Future work before its implementation in the food industry should include the tensile test analysis of the materials and their antimicrobial performance in durability studies.

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6. References

Appendini, P., Hotchkiss, J.H. 2002. Review of antimicrobial food packaging. Innovative Food Science and Emerging Technologies 3, 113-126.

Auras, R., Harte, B., Selke, S. 2004. An overview of polylactides as packaging materials. Macromolecular Bioscience 4, 835-864.

Bouwmeester, H., Dekkers, S., Noordam, M.Y., Hagens, W.I., Bulder, A.S., de Heer, C., ten Voorde, S.E.C.G., Wijnhoven, S.W.P., Marvin, H.J.P., Sips, A.J.A.M. 2009. Review of health safety aspects of nanotechnologies in food production. Regulatory Toxicology and Pharmacology 53, 52-62.

Burkersroda, F.v., Schedl, L., Göpferich, A. 2002. Why degradable polymers undergo surface erosion or bulk erosion. Biomaterials 23, 4221-4231.

Chen, X., Schluesener, H.J. 2008. Nanosilver: A nanoproduct in medical application. Toxicology Letters 176, 1-12.

Donhowe, G., Fennema, O. 1993. Water vapor and oxygen permeability of wax films. Journal of the American Oil Chemists' Society 70, 867-873.

EFSA. 2006. 1-21 Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC)
on a request related to a 12th list of substances for food contact materials. The EFSA Journal. 395-401.

EFSA. 2008. Scientific Statement of the Panel on Food Additives and Nutrient Sources added to Food (ANS): Question nº EFSA-Q-2005-169. The EFSA Journal.

EFSA. 2011. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in the European Union in 2010. EFSA Journal 10, 442.

European Comission. 2011. COMMISSION REGULATION (EU) Nº 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food. Official Journal of the European Union L12, 1-89.

Fabra, M.J., Busolo, M.A., Lopez-Rubio, A., Lagaron, J.M. 2013. Nanostructured biolayers in food packaging. Trends in Food Science and Technology 31, 79-87.

Fabra, M.J., Talens, P., Chiralt, A. 2008. Tensile properties and water vapor permeability of sodium caseinate films containing oleic acid-beeswax mixtures. Journal of Food Engineering 85, 393-400.

Fortunati, E., Armentano, I., Zhou, Q., Iannoni, A., Saino, E., Visai, L., Berglund, L.A., Kenny, J.M. 2012. Multifunctional bionanocomposite films of poly(lactic acid), cellulose nanocrystals and silver nanoparticles. Carbohydrate Polymers 87, 1596-1605.

Galeano, B., Korff, E., Nicholson, W.L. 2003. Inactivation of vegetative cells, but not spores, of Bacillus anthracis, B. cereus, and B. subtilis on Stainless steel surfaces coated with an antimicrobial silver- and zinc-containing zeolite formulation. Applied and Environmental Microbiology 69, 4329-4331.

Guilbert, S., Gontard, N., Cuq, B. 1995. Technology and applications of edible protective films. Packaging Technology and Science 8, 339-346.

Gupta, A., Silver, S. 1998. Silver as a biocide: Will resistance become a problem? Nature Biotechnology 16, 888.

Ilg, Y., Kreyenschmidt, J. 2011. Effects of food components on the antimicrobial activity of polypropylene surfaces containing silver ions (Ag +). International Journal of Food Science and Technology 46, 1469-1476.

Ivanova, T., Panaiotov, I., Boury, F., Proust, J.E., Benoit, J.P., Verger, R. 1997. Hydrolysis kinetics of poly(d,l-lactide) monolayers spread on basic or acidic aqueous subphases. Colloids and Surfaces B: Biointerfaces 8, 217-225.

Kampmann, Y., De Clerck, E., Kohn, S., Patchala, D.K., Langerock, R., Kreyenschmidt, J. 2008. Study on the antimicrobial effect of silver-containing inner liners in refrigerators. Journal of Applied Microbiology 104, 1808-1814.

Khwaldia, K. 2010. Water Vapor Barrier And Mechanical Properties Of Paper-Sodium Caseinate And Paper-Sodium Caseinate-Paraffin Wax Films. Journal of Food Biochemistry 34, 998-1013.

López-Rubio, A., Almenar, E., Hernandez-Muñoz, P., Lagarón, J.M., Catalá, R., Gavara, R. 2004. Overview of active polymer-based packaging technologies for food applications. Food Reviews International 20, 357-387.

Martinez-Abad, A. 2010. Silver- and nanosilver-based plastic technologies In: Lagarón, J., Ocio, M.J., Lopez-Rubio, A., (Eds.), Antimicrobial Polymers. John Wiley and sons inc., Hoboken, New jersey. 287-316.

Martinez-Abad, A., Lagaron, J.M., Ocio, M.J. 2012a. Development and characterization of silver-based antimicrobial ethylene-vinyl alcohol copolymer (EVOH) films for food-packaging applications. Journal of Agricultural and Food Chemistry 60, 5350-5359.

Martinez-Abad, A., Sánchez, G., Lagaron, J.M., Ocio, M.J. 2012b. On the different growth conditions affecting silver antimicrobial efficacy on Listeria monocytogenes and *Salmonella enterica*. International Journal of Food Microbiology 158, 147-154.

Martinez-Abad, A., Sánchez, G., Lagaron, J.M., Ocio, M.J. 2013a. Ligands affecting silver antimicrobial efficacy on Listeria monocytogenes and *Salmonella enterica*. Food Chemistry 139, 281-288.

Martinez-Abad, A., Sánchez, G., Lagaron, J.M., Ocio, M.J. 2013b. Evaluation of silver-infused polylactide films for inactivation of *Salmonella* and feline calicivirus *in vitro* and on fresh-cut vegetables. International Journal of Food Microbiology 1, 89-94.

Mastromatteo, M., Conte, A., Del Nobile, M.A. 2010. Advances in controlled release devices for food packaging applications. Trends in Food Science and Technology 21, 591-598.

Maynard, A.D. 2007. Nanotechnology – toxicological issues and environmental safety., Project on Emerging Nanotechnologies, vol. 1-14, Washington, DC.

Ouattar, B., Simard, R.E., Piett, G., Bégin, A., Holley, R.A. 2000. Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. International Journal of Food Microbiology 62, 139-148.

Quintavalla, S., Vicini, L. 2002. Antimicrobial food packaging in meat industry. Meat Science 62, 373-380.

Rai, M., Yadav, A., Gade, A. 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances 27, 76-83.

Russell, A.D., Hugo, W.B. 1994. Antimicrobial activity and action of silver. Progress in medicinal chemistry 31, 351-370.

Sanchez-Garcia, M.D., Gimenez, E., Lagaron, J.M. 2008. Morphology and barrier properties of solvent cast composites of thermoplastic biopolymers and purified cellulose fibers. Carbohydrate Polymers 71, 235-244.

Shellhammer, T.H., Krochta, J.M. 1997. Whey protein emulsion film performance as affected by lipid type and amount. Journal of Food Science 62, 390-394.

Staff, P.D.L., (Ed.) 1995. Permeability and Other Film Properties of Plastics and Elastomers. William Andrew Publishing.

Williams, R.L., Doherty, P.J., Vince, D.G., Grashoff, G.J., Williams, D.F. 1989. The biocompatibility of silver. Critical Reviews in Biocompatibility 5, 205+221-243.

Yuranova, T., Rincon, A.G., Bozzi, A., Parra, S., Pulgarin, C., Albers, P., Kiwi, J. 2003. Antibacterial textiles prepared by RF-plasma and vacuum-UV mediated deposition of silver. Journal of Photochemistry and Photobiology A: Chemistry 161, 27-34.

Zhang, Y., Chan, H.F., Leong, K.W. 2013. Advanced materials and processing for drug delivery: The past and the future. Advanced Drug Delivery Reviews 65, 104-120.

Zhong, J., Li, H., Yu, J., Tan, T. 2011. Effects of Natural Fiber Surface Modification on Mechanical Properties of Poly(lactic acid) (PLA)/Sweet Sorghum Fiber Composites. Polymer - Plastics Technology and Engineering 50, 1583-1589.

Chapter VIII

CHARACTERIZATION OF TRANSPARENT SILVER LOADED POLY(L-LACTIDE) FILMS PRODUCED BY MELT-COMPOUNDING FOR THE SUSTAINED RELEASE OF ANTIMICROBIAL SILVER IONS IN FOOD APPLICATIONS

Food Control (Under Review)

Antonio Martínez Abad, José M. Lagaron, Maria J. Ocio

ABSTRACT

In the present study, thermally stable silver complexes were produced and incorporated into a poly-(L-lactide) (PLA) matrix by melt-compounding. The effect of the different formulations on the mechanical and color properties were first evaluated. Additionally, the release of silver ions to an aqueous environment was also monitored over time by anodic stripping voltammetry and correlated with the antimicrobial performance against *S. enterica*. The incorporation of some silver compounds with contents of 1wt% did not affect the mechanical or optical properties of the films. The films showed a good potential for application in surface treatment and demonstrated a sustainable, antibacterial effectiveness in liquid and solid food environments. These results give new unreported insight into the production of transparent thermally stable and antimicrobial polymers based on the release of ionic silver and about their potential application in antimicrobial food packaging.

Keywords: Silver ions, PLA, poly(lactic acid) or poly(L-lactide), antimicrobial food packaging, melt-compounding.

1. Introduction

Active packaging seeks to improve food preservation extending the shelf-life and/or improving the sensory or nutritional properties while maintaining product quality. In particular, antimicrobial packaging has recently gained much interest, as a means to extend the shelf-life and reduce the risk of contamination by pathogens (Mastromatteo, Conte & Del Nobile, 2010). Many applications, including food production and storage, might benefit from the incorporation of safe and wide spectrum long-lasting biocides into polymers or working surfaces (Appendini & Hotchkiss, 2002). As bacterial contamination occurs primarily on the surface, the incorporation of the biocide in a film or as a coating has many advantages due to the high exposure areas for the biocide and a reduction of the loss of the antimicrobial compound into the bulk of the food (López-Rubio, Almenar, Hernandez-Muñoz, Lagarón, Catalá & Gavara, 2004; Ouattar, Simard, Piett, Bégin & Holley, 2000). The incorporation of antimicrobials into polymers constitutes a solution, as it allows the biocide substance to be released from the package during an extended period, prolonging its effect into the transport and storage phase of food distribution (Quintavalla & Vicini, 2002).

Silver ions are active antimicrobials against a very broad spectrum of bacteria, yeasts, fungi and viruses while not being toxic to human cells (Russell & Hugo, 1994; Williams, Doherty, Vince, Grashoff & Williams, 1989). Therefore, a wide variety of materials used in daily life are recently incorporated with silver or silver salts as key component to control microbial proliferation, ranging from textile clothing (Yuranova et al., 2003), stainless steel coatings in home appliances (Kampmann, De Clerck, Kohn, Patchala, Langerock & Kreyenschmidt, 2008) and food-contact materials (Bouwmeester et al., 2009; Galeano, Korff & Nicholson, 2003) (see Chen & Schluesener, 2008; Gupta & Silver, 1998 and Rai, Yadav & Gade, 2009 for review). In the EU, silver is historically accepted under directive 94/36/EC as a colouring agent (E-174) with no restrictions. More recently, the European Food Safety Authority (EFSA) has provided positive opinions for silver zeolites, silver zirconium phosphates, and

silver containing glasses in its provisional list of additives for food contact materials with a general restriction of ≤0.05 mg/kg food for the whole group (EFSA, 2006). In the U.S., the Center for Food Safety and Applied Nutrition in the Food and Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled waters (FDA, 2012).

However, the outstanding potential of silver for its use as antimicrobial is still severely limited by the instability of the active silver species, the silver ions in its free ionic form. Silver ions are known to be easily inactivated by many different physical or chemical factors (Ilg & Kreyenschmidt, 2011; Martínez-Abad, Sánchez, Lagaron & Ocio, 2013a). Relatively soft thermal treatments or exposure to light or UV can prompt the formation of sulphides or other stable silver complexes void of any biocide properties and usually producing a strong discoloration of the materials. Kasuga et al. solved these problems by forming a transparent light stable, water soluble compound of silver with acetomethionine. Although methionine forms stable complexes with silver, it was found that silver methionine complexes could still exert a high antimicrobial performance (Kasuga, Yoshikawa, Sakai & Nomiya, 2012; Martínez-Abad, Sánchez, Lagaron & Ocio, 2013a). Additionally, the thermal or chemical reduction of silver can lead to formation of silver nanoparticles, which possess a high antimicrobial potential but are only beginning to be considered in many legislation frames and can also present problems of discoloration. Hence, the use of silver in plastics could be severely limited as melt-compounding at high temperature is the most widely manufacturing practice for plastics. Until recent, this problem has been surmounted by incorporating silver exchanged minerals and clays, where the silver ions are absorbed and stabilized within the porous structure (Bedi et al., 2012; Coleman, 2009; Cowan, Abshire, Houk & Evans, 2003; Dogan, Koral & Inan, 2009; Martinez-Abad, 2010). Regardless of the stringent regulations, thermal and chemical instability, and the lack of alternative technologies for silver ion delivery, silver still remains the most widely used antimicrobial polymer additive in food applications (Appendini et al., 2002; Quintavalla et al., 2000). In the food sector, silver based antimicrobial systems are mostly based on a thin polymeric layer containing silver exchanged minerals (1-5% silver content)

and coated on polymeric or stainless steel surfaces of for example water treatment units or other food processing equipment (cutlery, cutting boards, counter tops, containers) (Chen & Schlussener, 2008; Gupta & Silver, 1998.; Rai, Yadav & Gade, 2009.). Expanding the potential applications of a silver based system, for example in food packaging, implies gathering sufficient knowledge as to be able to produce cost-effective silver compounds capable of withstanding thermal processing with adequate optical and physicochemical properties, while at the same time assuring antimicrobial effectiveness according to controlled migration profiles, which should comply with current legislation.

In the present paper, different silver salts and compounds were incorporated in PLA by melt compounding. The materials were characterized on their mechanical and optical properties. The release of silver ions from the materials was monitored over time and their antibacterial effectiveness against the foodborne pathogen *S. enterica* was evaluated in liquid synthetic and food environments as well as for surface treatment and food packaging.

2. Materials and Methods

2.1. Materials

PLA as supplied by Natureworks Llc was used for the preparation of the films. The polylactide with a D-isomer content of approximately 2% had a number-average molecular weight (M_n) of ca. 130,000 g/mol, and an average molecular weight (M_w) of ca. 150,000 g/mol as reported by the manufacturer. Silver nitrate (>98% purity; Sigma-Aldrich, Steinheim, Germany) was used for preparation of the different antimicrobial silver salts. Sodium carbonate, sodium bicarbonate, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium phosphate, sodium triphosphate pentabasic, ethylenediaminetetraacetic acid, polyethylene glycol 900 (PEG) and sodium dodecylsulphate (SDS) were all purchased by Sigma-Aldrich and were also used to prepare the silver antimicrobial compounds. M9 minimal salts 5x (Sigma-Aldrich), glucose, buffered peptone water (BPW), tryptic soy agar (TSA; Condalab, Madrid, Spain), magnesium sulphate (Panreac) and natural vegetable soup (Caldos Aneto, Artés, Barcelona, Spain) were used for preparation of the synthetic growth media and antimicrobial testing.

2.2. Silver compounds and film preparation

The different silver salts were prepared by dropwise adding a 1M silver nitrate solution to a 1M solution of the different sodium salts according to the suitable stoichoimetry (Table 1). The precipitate was filtered and allowed to dry overnight in a vacuum oven at 70ºC with the PLA pellets to eliminate moisture. The PLA blends with 1wt. % of each silver compound were prepared in a Brabender Plastograph mixer (Brabender, Germany) during 3 min at 30 rpm and at 135°C. The batches were subsequently compression molded into films using a hot-plate hydraulic press (Carver 4122, USA) at 155ºC, 2 MPa and 4 min to produce PLA films with a thickness of ~100 μm.

Table 1. Silver compounds and additives incorporated into PLA.

Sample name	Silver Compound	Stechoimetry
PLA-1	$AgHCO_3$	1:1
PLA-2	Ag_2CO_3	2:1
PLA-3	AgH_2PO_4	1:1
PLA-4	Ag_2HPO_4	2:1
PLA-5	Ag_3PO_4	3:1
PLA-6	$AgH_4P_3O_{10}$	1:1
PLA-7	$Ag_2H_3P_3O_{10}$	2:1
PLA-8	$Ag_3H_2P_3O_{10}$	3:1
PLA-9	$Ag_4HP_3O_{10}$	4:1
PLA-10	$Ag_5P_3O_{10}$	5:1
PLA-11	Ag-EDTA	1:1
PLA-12	Ag-EDTA + PEG	1:1
PLA-13	Ag_2-EDTA + PEG	2:1
PLA-14	Ag_3SO_4	3:1
PLA-15	Ag_3SO_4 + SDS	3:1

2.3. Mechanical properties

Tensile tests were performed according to ASTM Standard D 638 in stamped dumbbell-shaped specimens of the samples. An Instron Testing Machine (Model 4469; Instron Corp., Canton, MA, USA) was used, with a crosshead speed of 10 mm/min, at ambient conditions of typically 23ºC and 60%RH. At least, four specimens of each film were tensile tested as to obtain statistically meaningful results. The thickness of all specimens was approximately 100 μm.

2.4. Colour analysis

The transparency and colour of the films was determined using a handheld Minolta Chromameter CR300 (Minolta Camera Co., Ltd., Osaka, Japan). Film specimens were placed on a white standard plate, and the CIELAB colour space was used to determine the parameters L*, a*, and b*. L* value ranges from 0 (black) to 100 (white); a* value ranges from -80 (green) to 100 (red); and b* value ranges from -80 (blue) to 70 (yellow). Samples were evaluated per

triplicate and three measurements were taken at random locations on each of the studied films. ΔE* was calculated as a global parameter (eq. 1) using neat PLA films as the reference samples.

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{(Eq. 1)}$$

2.5. Silver release study

A voltammetric method was used to determine the release of free silver ions over time from the films. 9 cm^2 of the films were cut and immersed in 30 mL of aqueous solutions adjusted to pH 6. Samples were kept at 24ºC for 24h to allow silver release from the films. Before each measurement, the aqueous solutions in the test tubes were removed and nitric acid was added to stabilize silver in its ionic form. The silver ion content for each measurement was determined by differential pulse anodic stripping voltammetry (ASV) with an Autolab III (EcoChemie) potentiostat setup under conditions stated in Metrohm application bulletin nº 207/2e "Analysis of silver by stripping voltammetry". Sample volume or/and deposition time was increased to enhance sensitivity. The silver ion working range was 1 – 100 µg/L. After each measurement, film samples were dried with pressurized air, and used again under the same conditions for subsequent release measurements. This procedure was correlatively repeated every day for at least 7 days to monitor the release of silver from the films over time. All experiments were carried out in duplicate.

2.6. Bacterial strains and growth conditions

Salmonella enterica CECT 554 strain was obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain) and stored in Phosphate Buffer Saline (PBS) with 10% Tryptone Soy Broth (TSB, Conda Laboratories, Madrid, Spain) and 10% glycerol at -80 ºC until needed. For experimental use, the stock culture was maintained by regular subculture to Tryptone Soy Agar (TSA) slants at 4 ºC and transferred monthly.

Previous to each study, a loopful of bacteria was transferred to 10 mL of TSB and incubated at 37 ºC overnight. A 100 µL aliquot from the overnight culture

was again transferred to TSB and grown at 37 ºC to the mid-exponential phase of growth. CFU counts in the inoculum were accurately and reproducibly obtained by inoculation into 10 mL growth medium of 0.1 mL of a culture having an absorbance value of 0.20 as determined by optical density at 600 nm by ultraviolet visible (UV) spectroscopy (SP-2000 UV, Spectrum Instruments, Shanghai, China).

2.7. Antimicrobial testing

For antimicrobial testing in liquid medium, a synthetic growth medium, M9, and a natural vegetable soup were used. $3cm^2$ of the films were immersed in 10 mL of each of the environments, respecting the same ratio as in the release measurements. Then, a bacterial suspension was inoculated as to achieve approximately 5×10^5 CFU/mL. The tubes were incubated in static conditions at 12ºC for 1 to 5 days and samples were then enumerated by conventional plate count on TSA.

To evaluate the antimicrobial efficacy on bacteria on the surface of the films, either for the treatment of surfaces intended to come in contact with bacterial contamination or for assessment of their efficacy in direct contact with food, both the Japanese Industrial Standard Z2801 "Antimicrobial products-Test for antimicrobial activity and efficacy" (JIS) and a challenge test with local lettuces were followed. For the JIS, a suspension containing $5x10^5$ CFU/mL was inoculated and spread onto the surface of the tested films with a square size of 5x5 cm and covered with an inert polyethylene film of 4x4 cm. Then the samples were introduced into Petri dishes and incubated at a temperature of 24ºC and a relative humidity at least 95 % for 24 h. After the incubation period, samples were removed and homogenized with buffered peptone water (BPW) in a sterile plastic bag with lateral filter using a Pulsifier (Microgen Bioproducts, UK) for 2 min. Then, serial dilutions in 0.1% BPW were made in order to quantify the number of viable bacteria by conventional plate count. For the challenge test, locally purchased fresh lettuce was cut in pieces of 3x3 cm and sterilized with UV light in a safety cabinet under laminar flow for 15 min prior to inoculation of the test microorganisms. A 100 µL aliquot of *S. enterica* was

inoculated on the food sample as to achieve concentrations of about 5×10^5 CFU/cm^2. After inoculation, samples were held for 10 min to allow sorption of the tested microorganisms. Then, samples were covered with pieces of 2.5 x 2.5 cm of the different PLA films with silver and incubated at 12 °C for 24h. After incubation, surviving cells were enumerated by plate count after homogenization in stomacher bags as described above. Each experimental condition was analyzed in triplicate.

3. Results and Discussion

3.1. Mechanical properties

PLA is known to be inherently brittle, which has prompted much research on improving this by for example incorporating different additives (Nordqvist, Sanchez-García, Hedenqvist & Lagaron, 2010). Mechanical properties of the films were analyzed as to evaluate if processing of the melts at lower temperatures or the addition of all tested silver salts may alter the mechanical properties of the produced films (Table 2).

Table 2. Mechanical properties of the tested films.

Sample	Modulus (Mpa)	Elongation at break (%)	Max. Tensile Strength (MPa)
PLA	1701 ± 85 A[a]	5.87 ± 0.16 A	47.1 ± 1.2 A
PLA-1	1668 ± 46 A	4.43 ± 0.70 B	44.9 ± 2.0 AB
PLA-2	1665 ± 89 A	4.65 ± 0.85 B	45.9 ± 5.7 AB
PLA-3	1513 ± 90 AB	5.03 ± 0.50 AB	48.6 ± 6.0 AB
PLA-4	1571 ±109 AB	5.09 ± 0.87 AB	47.9 ± 2.8 AB
PLA-5	1697 ± 108 A	5.53 ± 0.94 A	46.7 ± 2.4 AB
PLA-6	1532 ± 75 AB	5.32 ± 0.32 AB	48.9 ± 3.8 A
PLA-7	1565 ± 88 AB	5.64 ± 0.89 AB	46.9 ± 4.5 AB
PLA-8	1555 ± 38 AB	5.45 ± 0.93 AB	47.5 ± 4.9 AB
PLA-9	1585 ± 45 AB	5.74 ± 0.25 AB	48.2 ± 2.3 AB
PLA-10	1544 ± 7 AB	4.20 ± 1.11 B	43.5 ± 3.5 AB
PLA-11	1476 ± 16 B	5.15 ± 0.61 AB	40.8 ± 3.7 BC
PLA-12	1371 ± 20 C	3.88 ± 0.30 C	35.7 ± 3.8 C
PLA-13	1498 ± 41 B	4.45 ± 0.45 B	37.1 ± 1.7 C
PLA-14	1545 ± 38 AB	5.70 ± 0.69 A	46.7 ± 2.9 AB
PLA-15	1528 ± 22 AB	4.53 ± 0.47 B	46.8 ± 0.8 AB

[a]Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. (n=4)

Elastic modulus, maximum tensile strength or elongation at brake of neat PLA films was found to be in agreement with previously reported values for similar materials processed at higher temperatures (Auras, Lim, Selke & Tsuji, 2010;

Sanchez-Garcia, Nordqvist, Hedenqvist & Lagaron, 2011). Elastic modulus is slightly reduced for all films incorporated with silver salts, although this reduction was only found significant for PLA films with silver-EDTA complexes. These films also demonstrated a weakening in the films tenacity and brittleness as reflected in significantly lower maximum tensile strengths and lower elongation at break, respectively. In the rest of the samples, elongation at break is also somewhat reduced, although differences were not found to be significant and are in line with previously reported values (Auras et al., 2010; Lin, Liu, Han, Wang, Bian & Dong, 2013), while maximum tensile strength is not significantly affected by the addition of all tested silver salts except for silver-EDTA salts.

3.2. Colour analysis

Transparency is highly desirable in many applications and PLA is a highly transparent polymer. Surface plasmon phenomena of silver particles and compounds may produce a discoloration in silver based antimicrobial materials during processing and storage. These changes, although useful when evaluating the presence of silver compounds and the size or shape of silver nanoparticles, may represent an important drawback for consumer acceptance. Colour measurements contribute to objectively differentiate and evaluate changes in the colour of the films. Table 3 shows the colour parameters L*, a*, b* and ΔE for all tested films after 4 weeks storage. ΔE as a global parameter of colour alteration is significantly different from neat PLA in PLA-1, PLA-2, PLA-4, PLA-5 and all films produced with silver-EDTA complexes (PLA-11, PLA-12, PLA-13), indicating rather deficient optical properties. PLA-6, PLA-7, PLA-9, PLA-10, PLA-14 and PLA-15 displayed no significant differences from neat PLA in any of the tested parameters, indicating these films possess the best optical attributes. Films incorporating silver phosphate salts (PLA-3, PLA-4 and PLA-5) show a notable and significant increase in greenness and yellowness, while PLA-1, PLA-2, PLA-6 and PLA-8 show a significant decrease in transparency and/or an increase in yellowness. Figure 1 displays a digital photograph for visual orientation. These results evidence that silver may be

efficiently incorporated into PLA films by melt-compounding without compromising their optical properties.

Table 3. Colour measurements of the films.

Sample	L*	a*	b*	ΔE
Standard plate	94.91 A[a]	0.26 A	2.26 A	-
PLA	94.88 A	0.24 A	2.30 A	0.14 A
PLA-1	85.87 BC	0.40 A	7.97 BCDE	10.70 BC
PLA-2	79.67 C	0.17 A	8.39 CDE	16.43 C
PLA-3	93.25 AB	-1.75 B	7.28 BCDE	5.79 AB
PLA-4	87.12 BC	-2.85 B	9.24 DE	10.92 BC
PLA-5	88.56 BC	-3.19 B	11.27 E	11.55 BC
PLA-6	91.06 B	1.20 A	5.63 ABCD	4.88 AB
PLA-7	92.53 AB	0.44 A	4.75 ABC	3.45 AB
PLA-8	90.78 AB	0.53 A	8.54 CDE	7.53 ABC
PLA-9	91.65 AB	0.81 A	5.91 ABCD	4.93 AB
PLA-10	91.76 AB	0.79 A	6.07 ABCD	5.02 AB
PLA-11	87.29 BC	0.11 A	10.91 E	11.53 BC
PLA-12	85.08 BC	0.82 A	3.52 A	10.27 BC
PLA-13	54.01 D	0.90 A	3.83 A	40.94 D
PLA-14	94.42 A	0.31 A	2.33 A	17.53 AB
PLA-15	94.11 A	0.27 A	2.60 A	0.49 A

[a]Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. (n=3)

3.3. Silver release study

A fundamental issue when evaluating the possible application of silver based antimicrobial systems in active packaging is the correct assessment of specific migration. The European Food Safety Authority, for instance, has restricted migration of silver to foodstuffs to 0.05mg/kg food [27], a very low threshold which could severely limit the use of silver as antimicrobial in the food sector. Therefore a sustained release of only the necessary antibacterial concentrations of the biocide is desired. Many vegetables as well as the tested vegetable soup have a pH of around 6. Therefore, the pH of these aqueous solutions was

adjusted to 6 as to mimic the conditions in the antimicrobial testing and correlate release with antimicrobial performance. Table 4 shows the release of silver ions results of the specific migration of silver measured under these conditions. In general, all tested films show significantly higher release values the first 1-3 days after which a lower sustained release is noted. However, the release in these films is generally more sustained as compared to films produced by solvent evaporation (casting) technique (Martínez-Abad, Ocio, Lagarón & Sánchez, 2013b). This is reflected in lower differences between the initial migration values and the sustained release values over subsequent days, which constitutes a significant improvement as to previous works (Martinez-Abad, Lagarón & Ocio, 2013c; Martínez-Abad, Ocio, Lagarón & Sánchez, 2013b).

Table 4. Release profiles of PLA films with 1% of different silver salts to aqueous solutions at pH 6.

	Daily release of silver (ng/cm^2)						
Sample	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
PLA-1	162±29^{a}	269±79^{a}	61±26^{b}	73±15^{b}	84±31^{b}	53±13^{b}	61±9^{b}
PLA-2	107±41^{a}	84±35^{a}	9±4^{b}	10±3^{b}	3±2^{b}	4±2^{b}	5±1^{b}
PLA-3	74±19^{a}	100±36^{a}	49±19^{ab}	22±6^{b}	20±5^{b}	15±8^{b}	27±10^{b}
PLA-4	81±17^{a}	64±13^{a}	19±3^{b}	22±7^{b}	12±5^{b}	16±2^{b}	14±5^{b}
PLA-5	228±75^{a}	323±85^{a}	67±21^{b}	70±23^{b}	82±36^{b}	70±15^{b}	49±21^{b}
PLA-6	512±98^{a}	182±14^{b}	25±0^{c}	20±7^{c}	12±3^{c}	18±1^{c}	19±4^{c}
PLA-7	154±16^{a}	74±5^{b}	11±1^{c}	10±3^{c}	6±3^{c}	5±3^{c}	8±2^{c}
PLA-8	97±12^{a}	129±44^{a}	32±8^{b}	54±10^{b}	33±8^{b}	38±5^{b}	25±0^{b}
PLA-9	274±5^{a}	248±5^{a}	59±9^{b}	41±20^{b}	21±0^{b}	26±5^{b}	25±3^{b}
PLA-10	168±9^{a}	61±6^{b}	22±2^{c}	12±0^{c}	29±6^{c}	16±5^{c}	20±2^{c}
PLA-11	17±5^{a}	10±3^{a}	13±6^{a}	7±1^{a}	<1^{b}	<1^{b}	<1^{b}
PLA-12	16±7^{a}	10±4^{a}	7±2^{a}	7±3^{a}	<1^{b}	<1^{b}	<1^{b}
PLA-13	26±8^{a}	10±3^{ab}	2±0^{b}	<1^{b}	<1^{b}	<1^{b}	<1^{b}
PLA-14	2072±306^{a}	1195±162^{ab}	535±173^{b}	475±272^{b}	183±56^{b}	95±24^{b}	55±11^{c}
PLA-15	6508±1050^{a}	2094±184^{b}	629±85^{c}	323±42^{d}	201±3^{d}	185±15^{d}	152±36^{d}

^{a}Mean values with different letters in the same line represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. (n=2)

However, strong differences are still noted depending on the silver salt added to the PLA melt. Films containing silver sulfate release much higher quantities of silver than the rest. The addition of SDS further enhances this release, probably because the surfactant allows a better distribution of the silver salt. Films with silver-EDTA complexes, on the contrary, produce a very low migration profile throughout a week exposure. PLA-1 and PLA-5 are able to produce a sustained release of silver of >50 ng/cm^2 through the 7 days. PLA-3, PLA-4, PLA-8, PLA-9 and PLA-10 films display release values of 10-50 ng/cm^2 between day 3 and day 7, although initial values are somewhat higher, especially in PLA-6 and PLA-9. PLA-2 and PLA-7 show a similar burst release as before but their release is strongly reduced after the second day. The different release profiles may be useful for implementation of these technologies in the food or in other application areas. For example in food products there may be a need for an initial relatively higher biocide capacity followed by a slower sustained release to prevent recontamination. Although the release values are relatively low (except for PLA-14 and PLA-15), the stringent migration limits appointed by some legislation bodies, such as the EFSA, impose the need to carefully understanding the final application of the silver based technology. For instance, this limit may be surpassed if the contact surface to volume ratio is high enough as to allow more than 50μg/Kg food to be released to the food sample. Additionally, it is expected that release rates vary considerably depending on the pH or moisture content of the sample in contact with the films (Cushen, Kerry, Morris, Cruz-Romero & Cummins, 2013; Echegoyen & Nerín, 2013; Fortunati et al., 2012; Martinez-Abad, Lagarón & Ocio, 2013c). These issues further stress the need for a profound assessment of silver release and point out the complexity of designing silver based antimicrobial systems.

3.4. Antimicrobial performance of the films immersed in liquid medium

For the evaluation of the antimicrobial capacity of the films incorporating silver in a liquid medium, both a commercial natural vegetable soup and the synthetic medium M9 were used. The soup has a pH value of 5.8-6.1, contains about 1.7% dry residue and 0.3% protein content. M9 medium is a minimal growth medium,

buffered at pH 7.2, without any proteins and glucose as a sole carbon source and was used as to compare the results in the soup with a more restrictive medium void of proteinic possible ligands for silver. Incubation temperature was set to 12ºC as to reflect temperature abuse in refrigerated samples (EU regulation 2073/2005). *S. enterica* was chosen as model pathogen in this study, considering it is the most common pathogenic bacteria involved in foodborne outbreaks in developed countries (EFSA, 2013}. Table 5 reflects the viability of the tested bacteria when incubated in M9 with all tested PLA films. While control samples without silver allow an increase of about 0.5 log units of the bacterial population after 24h, all films with silver produce a reduction in viable counts except for samples with silver-EDTA complexes. In these cases, bacterial numbers do not decrease or even increase as compared to the initial inoculum size (5.7 log units), evidencing a lower antimicrobial efficacy. Highest antimicrobial performance in M9 medium is noted for PLA-1, PLA-10 and PLA-9, respectively, with more than a 99% reduction (2 log units). More than 1 log reduction is achieved with PLA-2, PLA-3, PLA-4, PLA-6, PLA-8, PLA-14 and PLA-15.

Table 5. Bacterial viability of *S. enterica* after incubation with PLA films with different silver salts in synthetic M9 medium.

Sample	Bacterial Counts Log (CFU/mL)	Sample	Bacterial Counts Log (CFU/mL)
PLA	6.15 (0.04)[a]	PLA-8	4.37 (0.11)
PLA-1	3.58 (0.34)	PLA-9	3.63 (0.30))
PLA-2	4.23 (0.21)	PLA-10	3.46 (0.11)
PLA-3	3.90 (0.43)	PLA-11	6.05 (0.12)
PLA-4	4.23 (1.00)	PLA-12	6.12 (0.05)
PLA-5	5.00 (0.77)	PLA-13	5.87 (0.07)
PLA-6	4.57 (0.20)	PLA-14	4.05 (0.69)
PLA-7	4.91 (0.39)	PLA-15	4.51 (0.12)

[a] Standard deviation

When the assay is performed analogously in a commercial vegetable soup, viability of *S. enterica* is shown to be higher than in the synthetic medium (Fig.

2). This could be related to inactivation of the active silver species by various components in the soup as suggested before (Ilg & Kreyenschmidt, 2001; Martínez-Abad, Sánchez, Lagaron & Ocio, 2013a). Additionally, the nutrient rich soup may allow better effectiveness of the bacterial repair mechanisms activated with the unspecific damage produced by silver ions. In this assay and for further antimicrobial testing, only a selection of 9 films was used. PLA films with silver-EDTA complexes (PLA-11, PLA-12, PLA-13) were cast aside because of their deficient optical, mechanical and antimicrobial properties. PLA-4 and PLA-5 were rejected because of significantly detrimental optical properties. PLA-15 was also cast aside from the experiment because the additive SDS did not enhance antimicrobial efficacy even though it boosted release from the films as compared to films with silver sulfate alone (PLA-14). Incubation in this assay was prolonged for 5 days to assess if the antimicrobial effect was sustained over time even in the presence of a chemically complex environment of the soup (Fig. 2).

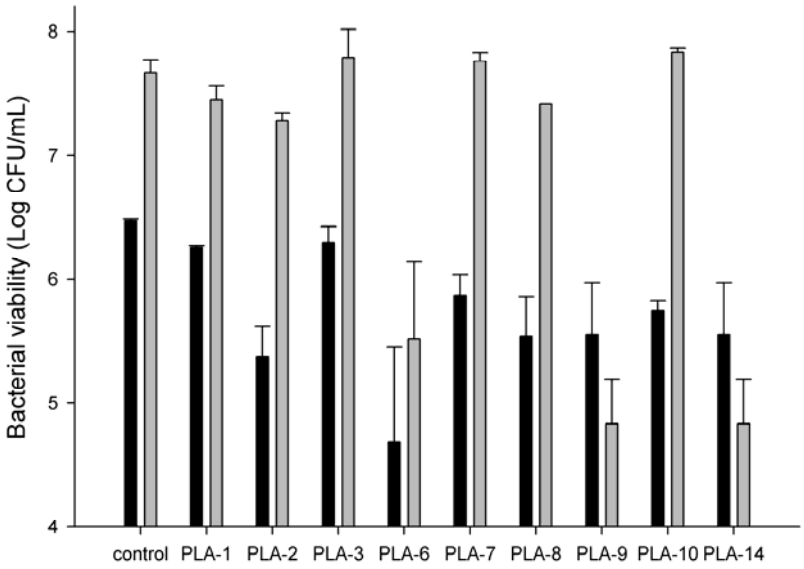

Figure 2. Bacterial viability of *S. enterica* after 1 ■ and 5 days ▭ incubation with PLA films with different silver salts in natural vegetable soup.

After 24h incubation, controls without silver allowed the increase of the bacterial population of about 1 log. Except for PLA-1 and PLA-3, in all samples with silver bacteria were unable to grow or even decreased in number. After 5 days incubation, however, most samples were able to recover and proliferate to a certain extent, except for PLA-6, PLA-9 and PLA-14. The recovery of bacterial cultures treated with silver in rich media has already been reported (Hwang, Katayama & Ohgaki, 2006; Woo, Hye, Ki, Shin, So & Yong, 2008; Martínez-Abad, Sánchez, Lagaron & Ocio, 2012) and would imply an important drawback for the possible use of these antimicrobial materials. Therefore the ability of the aforementioned samples to exert a high and sustained antimicrobial effectiveness over time even after immersion in a liquid food may be a good prospect for the future application of silver based technologies in food applications other than surface finishing.

3.5. Antimicrobial performance of the films on the surface

When evaluating the antimicrobial activity of films on the surface, two methodologies were performed. First, to assess the possible application of these plastics in food contact or other antimicrobial surfaces, the JIS was followed with modification. Secondly, a challenge test was performed with fresh lettuces to investigate the suitability of the plastics for food packaging. In the JIS, an incubation temperature of 24ºC was chosen as to mimic room temperature conditions. Instead of a diluted (500-fold) growth medium, undiluted M9 medium was used to allow proliferation of bacteria in case of absence of antibacterial activity and better visualize possible changes as compared to the controls. After 24h incubation with the tested PLA samples, all films with silver produced a reduction of viable bacteria below the detection limit (100 CFU/mL; Table 6). In PLA samples without silver, the bacterial population was allowed to proliferate to 8-9 log units. The surface assay indicates that the antimicrobial efficacy of the films is much higher when surface contamination occurs than when immersed in a contaminated liquid medium. The results are in line with previous works based on surface contact of PLA films with 0.01wt % and 0.1wt % silver produced by casting (Martinez-Abad, Sanchez, Lagaron & Ocio, 2013b;

Martínez-Abad, Lagaron & Ocio, 2013c). Although lower migration rates were detected as compared to applications where the films are immersed in an aqueous environment, much less quantities of silver ions were needed to exert an antimicrobial effect. This is probably due to inactivation of free silver ions in solution as well as to a more instant and intimate contact of the bacterial population with the ions leaching from the surface of the films. The astounding effectiveness of silver by surface contact has probably been the motive force for its current application, mostly limited to the preparation of antimicrobial surfaces (Simpson, 2003; Gupta & Silver, 1998; Martinez-Abad, 2010). To investigate the suitability of the plastics for food packaging, and how the continuous contact of a food matrix may affect their efficacy; a challenge test was performed with artificially inoculated lettuce. Results in Table 7 show a reduction of at least 3 log units (99.9%) of the bacterial population in all samples containing silver except for PLA-1 and PLA-14, which display a decrease of 2 log units (99%) compared with the controls.

Table 6. Surface antibacterial performance of the tested PLA films incubated with *S. enterica* on fresh cut lettuces and according to the Japanese Industrial Standard Z2801.

Sample	Bacterial Counts on lettuce (log CFU/cm^2)	Bacterial Counts JIS Z2801 (log CFU/mL)
PLA	5.32 (0.33)	6.08 (0.10)
PLA-1	3.39 (0.22)	<1
PLA-2	2.18 (0.82)	<1
PLA-3	1.60 (0.43)	<1
PLA-6	1.65 (0.49)	<1
PLA-7	2.11 (1.15)	<1
PLA-8	2.61 (0.53)	<1
PLA-9	2.99 (1.00)	<1
PLA-10	1.45 (0.21)	<1
PLA-14	3.53 (0.23)	<1

Controls without silver are not able to proliferate beyond the initial inoculum size probably due to lack of sufficient nutrients on the surface of the vegetable.

The lower effectiveness of the plastic in the challenge test as compared to results for the JIS may be attributed to the roughness of the food sample, which does not allow such a homogeneous and intimate contact or to partial inactivation of the silver ions by food constituents. This indicates that surface assays testing antimicrobial efficacy, such as in the JIS, the ISO 22196 or the ASTM 2180 may overestimate the antibacterial performance of the plastic if this is intended to be in prolonged contact with a food matrix. The reduction of silver efficacy by nutrient rich environments, mainly proteinic, can be observed by browsing the different efficacy results in scientific literature and has been reported previously (Ilg & Kreyenschmidt, 2011; Lee, Lee, Jones, Sharek & Pascall, 2011; Martinez-Abad, Sanchez, Lagaron & Ocio, 2012). The present results evinced a high antibacterial effect of these relatively transparent melt-compounded plastics on a food matrix, which represents a step forward in the implementation of silver in active packaging or other technologies.

4. Conclusions

Although application of silver based antimicrobial systems is a widespread phenomenon since recent years, their possible implementation in food packaging technologies is still a challenging task, due to instability of silver ions for thermal processing as well as in other chemical environments. In this work, a number of different silver salts were found to be directly incorporated into the PLA melt without compromising the mechanical or optical properties. The materials could sustainably release silver ions as to efficiently reduce bacterial contamination under four different experimental conditions: in liquid synthetic media as well as in a commercial vegetable soup, for surface treatment and in direct contact with a solid food. These materials could pose an alternative to other silver based plastics either produced by casting or incorporated into carriers. Further studies need to be performed to ascertain if the materials are also suitable for packaging of other more complex food products and to more

accurately tailor the release kinetics and delimit the best combination of silver complexes and concentrations.

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6. References

Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Science and Emerging Technologies*, *3*(2), 113-126.

Auras, R., Lim, L. T., Selke, S., & Tsuji, H., eds (2010). *Poly(lactic acid): Synthesis, Structures, Properties, Processing, and Applications*. Hoboken, New Jersey: Wiley and Sons.

Bedi, R. S., Cai, R., O'Neill, C., Beving, D. E., Foster, S., Guthrie, S., Chen, W., & Yan, Y. (2012). Hydrophilic and antimicrobial Ag-exchanged zeolite a coatings: A year-long durability study and preliminary evidence for their general microbiocidal efficacy to bacteria, fungus and yeast. *Microporous and Mesoporous Materials*, *151*, 352-357.

Bouwmeester, H., Dekkers, S., Noordam, M. Y., Hagens, W. I., Bulder, A. S., de Heer, C., ten Voorde, S. E. C. G., Wijnhoven, S. W. P., Marvin, H. J. P., & Sips, A. J. A. M. (2009). Review of health safety aspects of nanotechnologies in food production. *Regulatory Toxicology and Pharmacology*, *53*(1), 52-62.

Coleman, N. J. (2009). Aspects of the in vitro bioactivity and antimicrobial properties of Ag +- and Zn2+-exchanged 11 Å tobermorites. *Journal of Materials Science: Materials in Medicine*, *20*(6), 1347-1355.

Cowan, M. M., Abshire, K. Z., Houk, S. L., & Evans, S. M. (2003). Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel. *Journal of Industrial Microbiology and Biotechnology*, *30*(2), 102-106.

Cushen, M., Kerry, J., Morris, M., Cruz-Romero, M., & Cummins, E. (2013). Migration and exposure assessment of silver from a PVC nanocomposite. *Food Chemistry*, *139*(1-4), 389-397.

Chen, X., & Schluesener, H. J. (2008). Nanosilver: A nanoproduct in medical application. *Toxicology Letters*, *176*(1), 1-12.

Dogan, H., Koral, M., & Inan, T. Y. (2009). Ag/Zn zeolite containing antibacterial coating for food-packaging substrates. *Journal of Plastic Film and Sheeting*, *25*(3-4), 207-220.
Echegoyen, Y., & Nerín, C. (2013). Nanoparticle release from nano-silver antimicrobial food containers. *Food and Chemical Toxicology*, *62*, 16-22.

EFSA (2006). 1-21 Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC)
on a request related to a 12th list of substances for food contact materials. (pp. 395-401): The EFSA Journal.

EFSA (2008). Scientific Statement of the Panel on Food Additives and Nutrient Sources added to Food (ANS): Question nº EFSA-Q-2005-169. *The EFSA Journal*(884), 1-3.

EFSA (2013). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011 *The EFSA Journal*, *11*(4), 250.

FDA/CFSAN. Listing of Food Addittives status. Silver nitrate-172.167

Fortunati, E., Armentano, I., Zhou, Q., Iannoni, A., Saino, E., Visai, L., Berglund, L. A., & Kenny, J. M. (2012). Multifunctional bionanocomposite films of poly(lactic acid), cellulose nanocrystals and silver nanoparticles. *Carbohydrate Polymers*, *87*(2), 1596-1605.

Galeano, B., Korff, E., & Nicholson, W. L. (2003). Inactivation of vegetative cells, but not spores, of Bacillus anthracis, B. cereus, and B. subtilis on Stainless steel surfaces coated with an antimicrobial silver- and zinc-containing zeolite formulation. *Applied and Environmental Microbiology*, *69*(7), 4329-4331.

Gupta, A., & Silver, S. (1998). Silver as a biocide: Will resistance become a problem? *Nature Biotechnology*, *16*(10), 888.

Hwang, M. G., Katayama, H., & Ohgaki, S. (2006). Effect of intracellular resuscitation of Legionella pneumophila in Acanthamoeba polyphage cells on the antimicrobial properties of silver and copper. *Environmental Science and Technology*, *40*(23), 7434-7439.

Ilg, Y., & Kreyenschmidt, J. (2011). Effects of food components on the antimicrobial activity of polypropylene surfaces containing silver ions (Ag +). *International Journal of Food Science and Technology*, *46*(7), 1469-1476.

Kampmann, Y., De Clerck, E., Kohn, S., Patchala, D. K., Langerock, R., & Kreyenschmidt, J. (2008). Study on the antimicrobial effect of silver-containing inner liners in refrigerators. *Journal of Applied Microbiology*, *104*(6), 1808-1814.

Kasuga, N. C., Yoshikawa, R., Sakai, Y., & Nomiya, K. (2012). Syntheses, structures, and antimicrobial activities of remarkably light-stable and water-soluble silver complexes with amino acid derivatives, silver(I) N-acetylmethioninates. *Inorganic Chemistry*, *51*(3), 1640-1647.

Lee, J., Lee, Y. H., Jones, K., Sharek, E., & Pascall, M. A. (2011). Antimicrobial packaging of raw beef, pork and turkey using silver-zeolite incorporated into the material. *International Journal of Food Science and Technology*, *46*(11), 2382-2386.

Lin, H.-J., Liu, S.-R., Han, L.-J., Wang, X.-M., Bian, Y.-J., & Dong, L.-S. (2013). Effect of a phosphorus-containing oligomer on flame-retardant, rheological and mechanical properties of poly (lactic acid). *Polymer Degradation and Stability*, *98*(7), 1389-1396.

López-Rubio, A., Almenar, E., Hernandez-Muñoz, P., Lagarón, J. M., Catalá, R., & Gavara, R. (2004). Overview of active polymer-based packaging technologies for food applications. *Food Reviews International*, *20*(4), 357-387.

Martinez-Abad, A. (2010). Silver- and nanosilver-based plastic technologies In: J. Lagarón, M. J. Ocio, & A. Lopez-Rubio, *Antimicrobial Polymers* (pp. 287-316). Hoboken, New jersey: John Wiley and sons inc.

Martínez-Abad, A., Sánchez, G., Lagaron, J. M., & Ocio, M. J. (2012). On the different growth conditions affecting silver antimicrobial efficacy on Listeria monocytogenes and *Salmonella enterica*. *International Journal of Food Microbiology*, *158*(2), 147-154.

Martínez-Abad, A., Sánchez, G., Lagaron, J. M., & Ocio, M. J. (2013a). Ligands affecting silver antimicrobial efficacy on Listeria monocytogenes and *Salmonella enterica*. *Food Chemistry*, *139*(1-4), 281-288.

Martínez-Abad, A., Ocio, M. J., Lagarón, J. M., & Sánchez, G. (2013b). Evaluation of silver-infused polylactide films for inactivation of *Salmonella* and feline calicivirus in vitro and on fresh-cut vegetables. *International Journal of Food Microbiology*, *162*(1), 89-94.

Martinez-Abad, A., Lagarón, J., & Ocio, M. J. (2013c). Active packaging strategies based on silver ion releasing biopolymers. In: L. Cabedo, *Polymar 2013*.
Mastromatteo, M., Conte, A., & Del Nobile, M. A. (2010). Advances in controlled release devices for food packaging applications. *Trends in Food Science and Technology*, *21*(12), 591-598.

Nordqvist, D., Sanchez-García, M. D., Hedenqvist, M. S., & Lagaron, J. M. (2010). Incorporating amylopectin in poly(lactic Acid) by melt blending using poly(ethylene-co-vinyl Alcohol) as a thermoplastic carrier. (I) morphological characterization. *Journal of Applied Polymer Science*, *115*(3), 1315-1324.

Ouattar, B., Simard, R. E., Piett, G., Bégin, A., & Holley, R. A. (2000). Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. *International Journal of Food Microbiology*, *62*(1-2), 139-148.

Quintavalla, S., & Vicini, L. (2002). Antimicrobial food packaging in meat industry. *Meat Science*, *62*(3), 373-380.

Rai, M., Yadav, A., & Gade, A. (2009). Silver nanoparticles as a new generation of antimicrobials. *Biotechnology Advances*, *27*(1), 76-83.
Russell, A. D., & Hugo, W. B. (1994). Antimicrobial activity and action of silver. *Progress in medicinal chemistry*, *31*, 351-370.

Sanchez-Garcia, M. D., Nordqvist, D., Hedenqvist, M., & Lagaron, J. M. (2011). Incorporating amylopectin in poly(lactic acid) by melt blending using poly(ethylene-co-vinyl alcohol) as a thermoplastic carrier. II. Physical

General Discussion

Research on silver as antimicrobial has gained much interest in recent years in many sectors, most predominantly the healthcare, medical and food area, in the pursuit for the development of antimicrobial materials with long lasting effectiveness and suiting diverse applications. But even though publications related to antimicrobial silver can be found by the thousands in any search engine or database, knowledge about the intricate mechanisms of effectiveness and inactivation of the different silver species is still incomplete. Additionally, the enormous potential scope of application of silver is such that has allowed research to greatly expand in terms of the development of antimicrobial materials with antimicrobial efficacy, without exhausting the infinite possibilities these technologies seem to offer. Nanotechnology being a very hot and trendy topic nowadays, a great extent of this research is focused on the production of silver nanoparticles, and their incorporation in all kinds of materials. However, the toxicity of silver nanoparticles to humans and to the environment is still being questioned and its use for food contact materials remains out of legislation in most countries. Therefore, the development of silver ion releasing technologies continues to be a field of research with great potential for application in the food or other sectors.
For the efficient design of a silver based antimicrobial material, a correct assessment of the reactivity of silver and its effectiveness under different conditions is mandatory. This issue is dealt with in the first chapter of this thesis.

Silver was found to be strongly antibacterial even at the nanomolar level. On the other hand, silver efficacy was found to be completely inactivated by thiol groups, which may impose some limitations as to their use in food contact materials, considering the chemical complexity of food matrices [135-138]. Methylsulphur groups, such as in methionine, allowed a sufficient fraction of silver ions to be free in solution, and thus retained antimicrobial effectiveness. In line with these findings, recent studies have used sulphur compounds as a means to deliver silver ions while at the same time increasing their half-life in the presence of bacteria or during thermal treatment [132, 135, 139].

As commented in the introduction, the elucidated mechanism of action of silver implies several unspecific pathways. This raised the question as if bacteria may be able to resuscitate and regrow after or during exposure to silver and how this damage may affect the correct assessment of bacterial viability by enumeration methods. In the second chapter, *L. Monocytogenes and S. enterica* were incubated in the presence of silver and different growth media. Viability was evaluated by traditional plate counting and flow cytometry (FC) analysis with a Live/Dead® staining kit. The results showed that bacteria could be able to recover and proliferate even in the presence of silver. Resuscitation took place after a prolonged lag time where a small fraction of bacteria were not cultivable but remained viable according to FC. FC also revealed most dead bacteria were lysed by the action of silver [140]. The existence of a resilient fraction of bacteria is in agreement with other recently published works. [141-142]. The differences in efficacy and resuscitation were found to be dependent on chemical interactions taking place in nutrient rich environments, which again stressed the importance of investigating these issues before designing a silver based antimicrobial material.

Considering the instability of silver ions, a sustained release is crucial as to assure antimicrobial efficacy over the shelf-life of the products. As an alternative to inorganic mineral clays with very low release rates, an EVOH copolymer was used to directly incorporate silver ions without further fillers by casting technique (Chapter III). As release is sorption induced and EVOH is a relatively hygroscopic polymer and sensitive to moisture, immersion in aqueous solutions produced a release of most part of the silver within less than half an hour. This produced a remarkable antibacterial performance in synthetic minimal medium even when films loaded with only 0.0001% silver were tested. Furthermore, the effectiveness of low concentrations of silver as released from the films was higher than when silver nitrate alone was tested. This was ascribed to higher stability of the silver ions in the polymer matrix than in solution. Films were able to efficiently kill 99% of the bacterial load in artificially inoculated samples, such as apples, eggshells or lettuce. However, the antimicrobial

performance and transparency of the films when assayed on food samples with high proteinic content, such as chicken, cheese or pork, was drastically decreased. These results are in agreement with other recent publications, studying the matrix effects of food samples on silver efficacy [136, 143]. To the best of our knowledge, successful application of silver releasing films on proteinic matrices has not yet been reported, at least not producing drastic bactericidal effects. In these cases, decreases of less than 1 log unit were found [116, 144]. Studies incorporating silver in inorganic fillers, as in montmorillonites, could not drastically reduce bacterial counts but still produced an increase in the shelf-life of food products with high proteinic content, as tested in durability studies [116-118, 145]. Therefore, even though not being so extraordinary effective, silver based materials may still be suitable for food packaging of proteinic products.

The excellent antibacterial performance of the produced EVOH coatings and their ability to yield 100% of its silver content in form of silver ions was further exploited by generating EVOH fibres with silver ions by electrospinning (Chapter IV). The solvents used and the absence of any thermal treatments allowed 100% of the silver content to instantly delivered in its ionic form, unlike previously reported [146-150]. This may be important if concerns about nanoparticle migration are to be avoided. This release mechanism may be useful in a number of applications, such as tissue engineering, food packaging or other targeted delivery systems where release is to be instant upon contact with moisture. Additionally, a new insight on these materials was given by analyzing silver speciation before and after thermal treatments. These treatments prompted the formation of silver nanoparticles, which were mostly retained within the polymer and produced much slower release kinetics with increasing thermal treatment. The antibacterial efficacy may be attributed to the free ions rather than to nanoparticles, which again correlated with previous results.

PLA and its copolymers are the most widely used plastics for controlled drug delivery systems because of their biodegradability, biocompatibility and ease of

processing [151] Additionally, it is one of the most widely used biopolymers in food packaging. Therefore, PLA was chosen for incorporation of silver within the next sections (Chapters V-VIII), as a means to obtain materials with a prolonged antimicrobial efficacy based on the sustained release of silver ions.

Chapter V dealt with the incorporation of silver into PLA by casting using different solvent mixtures. The materials were characterized on their morphology, silver distribution, thermal, mechanical, optical and release properties over weeks to months and after successive washings. Alterations were only found for silver filler contents of >1wt.%. The use of different solvent mixtures affected the morphology of the films and the distribution of silver, which in turn had a strong influence in the release profiles. The antibacterial performance of the released silver ions was tested in vitro and breakpoints were established at optimum conditions in synthetic medium. Silver ion concentrations necessary to exert a strong bactericidal effect were found to be in the range of 10-20 μg/L. These values are well below the stringent migration limits stated by the EFSA, which demonstrated that silver ion technology may be applied to foods within the European legislation frame. A sustained release of sufficient silver ions was achieved even after successive washings during days to months depending on the preparation method and the silver contents. The possibility of developing PLA films with a sustained antimicrobial efficacy over relatively long time without the need of further inorganic fillers was evinced.

If the films were intended to be used as food contact antimicrobial materials, either as coatings in food contact surfaces or food packaging, their effectiveness against foodborne pathogens was to be evaluated under more realistic conditions. In chapter VI, the films were tested according to the Japanese Industrial standard (JIS) and on food samples (challenge tests), such as lettuce and paprika. Films were artificially inoculated with *Salmonella* and feline calicivirus, a human norovirus surrogate, as they are the most common food-borne pathogens in developed countries [6]. Plate counting and the 50% tissue culture infectious dose, respectively, were used to enumerate the pathogens after

prolonged exposure to food samples and surfaces in contact with the films. The results revealed great antiviral and antibacterial effectiveness according to the JIS, while effectiveness in food samples somewhat decreased and was highest at refrigeration temperatures. This was ascribed to a more intimate contact of the pathogens under the JIS. In lettuce samples incubated at 4°C during 6 days, 4 log reductions of *Salmonella* were reported for films with 0.1 wt.-% and 1 wt.-% and no infectious FCV were reported. Literature dealing with materials with antiviral properties is scarce [73]. Although viruses were, in general, significantly less susceptible than bacteria, this study showed great antiviral activity at relatively low silver concentrations, as compared to previous studies {Bright, 2009}. However, the release profiles from the films still exhibited an initial burst release which under certain conditions may surpass restriction limits in European legislation.

In order to produce a more sustained release over time and assure restriction limits would not be surpassed even after prolonged exposure while at the same time assuring antimicrobial performance, a beeswax coating of different thickness was applied as a functional barrier (Chapter VII). This not only served as to eliminate the burst release and tune the release profiles, but also enhanced the water vapour barrier properties of the films. The release profiles and antibacterial performance were studied at the surface of the films and immersed in liquid media both at acidic and neutral pH. The release profiles varied substantially, higher at acidic pH and when immersed, than at neutral pH or at the surface. The application of a beeswax barrier of different thickness allowed tailoring the release profiles to suit the conditions for a specific application. Additionally, the PLA/beeswax films were effective in reducing *Salmonella* counts in liquid synthetic medium, liquid food products and by surface contact (JIS).

It was thus demonstrated that these films may be useful for the preparation of antimicrobial coatings in food packaging or food contact materials with long lasting antibacterial activity and complying with current legislation. However,

food packaging materials are commonly and most cost-effectively fabricated by melt compounding. The challenge of stabilizing silver ions in its ionic form as to be able to melt-compound the materials at high temperatures without compromising the sustained release capacities and subsequent prolonged efficacy was dealt with in Chapter VIII.

Different silver salts and compounds were formed and incorporated into PLA melts to form 15 different PLA films which were evaluated on their mechanical and optical properties as well as on their silver ion release capacities. The release of silver ions from melt-compounded films was found to be somewhat more sustained as compared with cast films although a higher release at the first 1-2 days was noted in most samples. The antimicrobial efficacy of the films revealed a lower efficacy of the silver as compared to cast films, which could be attributed to lower release capacities, lower silver content in the salts with heavier anions, lower reactivity of the silver compounds against the pathogen, or a combination of these. Nevertheless, all tested films showed a remarkable antibacterial effect, efficiently killing more than 99% of bacteria both on a solid food substrate and according to the JIS. Many of the tested films were also able to produce a substantial decrease in bacterial counts after prolonged incubation with liquid food samples. The search for light and thermally stable complexes with antibacterial efficacy has been also confronted in other studies. Silver cyanoxymates were found to be light and thermally stable but lacked transparency [152]. Stable complexes with methionine were light stable and could still exert a high antimicrobial performance but thermal stability or possible incorporation into polymers was not evaluated [132]. Naftoquinone-oxime ligands were thermally stable to up to 700ºC and showed antibacterial efficacy against bacteria and fungi, although quantification of ion release was not evaluated and their incorporation into polymers was not considered [153]. Min et al. were able to absorb silver nitrate onto porous silica and produce heat stable colourless silver chloride nanoparticles whose blends with polypropylene showed a high antibacterial effect according to the JIS [154]. This, however, implies the need of a porous inorganic carrier for the silver compound, as is the

case in commercially available silver based materials [97, 155]. In this study, silver salts were directly incorporated in the PLA melt to produce transparent films which could sustainably release silver ions as to reduce bacterial contamination in a number of different applications. The release of silver ions from the films may, however, surpass restriction limits under certain conditions, which evinces the need for further studies tailoring the release kinetics and delimiting the best combination of silver complexes and concentrations.

Conclusions

1. Silver is only active in form of free silver ions. The activity of other silver compounds is due to the presence of small quantities of ions at equilibrium or leaching from the compounds under certain conditions.
2. Sublethal damage to bacteria and parallel inactivation of the active silver species may cause resuscitation of bacteria after prolonged exposure as evaluated by flow cytometry.
3. When incorporated into EVOH copolymer, silver ions were found to be homogeneously distributed within the polymer film and did not affect molecular or water uptake profiles of the material even at high concentrations. The materials were able to release 20-100% of the silver ions when in contact with a food sample or immersed in aqueous solutions, respectively. The sorption induced release followed in a relatively short period depending on the moisture level in the environment of action.
4. EVOH copolymers with 0.0001% silver ions were able to exert a higher antibacterial effect than the bulk material under laboratory conditions. However, the effectiveness of the films drastically decreased in contact with food samples of high proteinic content. In low proteinic content food, films were found to reduce 99% of the bacterial load over 48h contact.
5. Silver ions incorporated into EVOH fibres by electrospinning were found to instantly deliver the antimicrobial and exerted a high antibacterial effect. Thermal treatment of the fibres produced the reduction of silver and much slower release kinetics from the fibres.
6. When incorporated into a PLA matrix by casting, different morphology, distribution and release profiles were found depending on the solvent mixture used. The incorporation of silver contents of up to 1wt.% did not affect morphology, thermal or mechanical properties of the films. Sustainable, antibacterial effectiveness over days to months was found in liquid medium for films containing silver loading between 0.01wt.% and 1wt.%. In all cases, an initial burst release was observed which arrested with time.

7. These films also showed a remarkable efficiency according to the JIS and to challenge tests performed on real food samples against human noroviruses and *Salmonella*.
8. The application of a functional barrier with beeswax improved the release patterns of the films by eliminating the initial burst release and allowing a more sustained release over at least 7 days.
9. Release profiles were found to be dependent on the pH and the moisture in contact with the film, being more rapid when at acidic pH and completely immersed in aqueous solutions. Increasing the thickness of a functional barrier, such as a beeswax layer, may allow tailoring the release kinetics of the films to suit a specific set of conditions without surpassing restriction limits.
10. Silver ions could be stabilized by the formation of various compounds which were subsequently incorporated to PLA by melt-compounding. The transparent, thermally stable PLA-silver compounds showed a prolonged antibacterial performance in liquid and solid food samples and on surfaces, which represents a step forward in the implementation of silver in active packaging or other technologies.

References

1. Environmental Protecttion Agency, *Municipal Solid Waste (MSW) in the United States: 2011 Facts and Figures (full report)*. http://www.epa.gov/osw/nonhaz/municipal/pubs/MSWcharacterization_fnl_060713_2_rpt.pdf.

2. Eurostat, E.C.-. *Packaging waste statistics*.; Statistics explained:: http://epp.eurostat.ec.europa.eu/statistics_explained/index.php

3. Lagarón, J.M., et al., *Improving packaged food quality and safety. Part 2: Nanocomposites.* Food Additives and Contaminants, 2005. **22**(10): p. 994-998.

4. Havelaar, A.H., et al., *Future challenges to microbial food safety.* International Journal of Food Microbiology, 2010. **139**(SUPPL. 1): p. S79-S94.

5. Newell, D.G., et al., *Food-borne diseases - The challenges of 20years ago still persist while new ones continue to emerge.* International Journal of Food Microbiology, 2010. **139**(SUPPL. 1): p. S3-S15.

6. EFSA, *The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011* The EFSA Journal, 2013. **11**(4): p. 250.

7. Hoffmann, S., M.B. Batz, and J.G. Morris Jr, *Annual cost of illness and quality-adjusted life year losses in the united states due to 14 foodborne pathogens.* Journal of Food Protection, 2012. **75**(7): p. 1292-1302.

8. Valero, M. and E. Francés, *Synergistic bactericidal effect of carvacrol, cinnamaldehyde or thymol and refrigeration to inhibit Bacillus cereus in carrot broth.* Food Microbiology, 2006. **23**(1): p. 68-73.

9. Quintavalla, S. and L. Vicini, *Antimicrobial food packaging in meat industry.* Meat Science, 2002. **62**(3): p. 373-380.

10. Appendini, P. and J.H. Hotchkiss, *Review of antimicrobial food packaging.* Innovative Food Science and Emerging Technologies, 2002. **3**(2): p. 113-126.

11. López-Rubio, A., et al., *Overview of active polymer-based packaging technologies for food applications.* Food Reviews International, 2004. **20**(4): p. 357-387.

12. Ouattar, B., et al., *Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan.* International Journal of Food Microbiology, 2000. **62**(1-2): p. 139-148.

13. Mastroeni, P., et al., *Antimicrobial actions of the nadph phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo.* Journal of Experimental Medicine, 2000. **192**(2): p. 237-247.

14. Coma, V., *Bioactive packaging technologies for extended shelf life of meat-based products.* Meat Science, 2008. **78**(1-2): p. 90-103.

15. Cruz-Romero, M.C., et al., *Antimicrobial activity of chitosan, organic acids and nano-sized solubilisates for potential use in smart antimicrobially-active packaging for potential food applications.* Food Control, 2013. **34**(2): p. 393-397.

16. Schirmer, B.C., et al., *A novel packaging method with a dissolving CO 2 headspace combined with organic acids prolongs the shelf life of fresh salmon.* International Journal of Food Microbiology, 2009. **133**(1-2): p. 154-160.

17. Wanda, S., et al., *Incorporation of lactic acid as an antimicrobial agent in polyamide food-packaging films.* Archiv fur Lebensmittelhygiene, 2013. **64**(1): p. 8-14.

18. Campos, C.A., L.N. Gerschenson, and S.K. Flores, *Development of Edible Films and Coatings with Antimicrobial Activity.* Food and Bioprocess Technology, 2011. **4**(6): p. 849-875.

19. Brody, A.L., *What's active in active packaging.* Food Technology, 2001. **55**(9): p. 104.

20. Mecitoğlu, C., et al., *Incorporation of partially purified hen egg white lysozyme into zein films for antimicrobial food packaging.* Food Research International, 2006. **39**(1): p. 12-21.

21. Gemili, S., A. Yemenicioğlu, and S.A. Altinkaya, *Development of cellulose acetate based antimicrobial food packaging materials for controlled release of lysozyme.* Journal of Food Engineering, 2009. **90**(4): p. 453-462.

22. Oram, J.D. and B. Reiter, *Inhibition of bacteria by lactoferrin and other iron-chelating agents.* BBA - General Subjects, 1968. **170**(2): p. 351-365.

23. Guerra, N.P., et al., *Antimicrobial activity of nisin adsorbed to surfaces commonly used in the food industry.* Journal of Food Protection, 2005. **68**(5): p. 1012-1019.

24. Mauriello, G., et al., *Development of polythene films for food packaging activated with an antilisterial bacteriocin from Lactobacillus curvatus 32Y.* Journal of Applied Microbiology, 2004. **97**(2): p. 314-322.

25. Scaffaro, R., et al., *Incorporation of nisin in poly (ethylene-co-vinyl acetate) films by melt processing: A study on the antimicrobial properties.* Journal of Food Protection, 2011. **74**(7): p. 1137-1143.

26. Iseppi, R., et al., *Anti-listerial activity of a polymeric film coated with hybrid coatings doped with Enterocin 416K1 for use as bioactive food packaging.* International Journal of Food Microbiology, 2008. **123**(3): p. 281-287.

27. Cutter, C.N., J.L. Willett, and Grsiragusa, *Improved antimicrobial activity of nisin-incorporated polymer films by formulation change and addition of food grade chelator.* Letters in Applied Microbiology, 2001. **33**(4): p. 325-328.

28. Dutta, P.K., et al., *Perspectives for chitosan based antimicrobial films in food applications.* Food Chemistry, 2009. **114**(4): p. 1173-1182.
29. Aider, M., *Chitosan application for active bio-based films production and potential in the food industry: Review.* LWT - Food Science and Technology, 2010. **43**(6): p. 837-842.
30. Fernandez-Saiz, P., M.J. Ocio, and J.M. Lagaron, *Antibacterial chitosan-based blends with ethylene-vinyl alcohol copolymer.* Carbohydrate Polymers, 2010. **80**(3): p. 874-884.
31. Burt, S., *Essential oils: Their antibacterial properties and potential applications in foods - A review.* International Journal of Food Microbiology, 2004. **94**(3): p. 223-253.
32. Frydrych, A., J. Arct, and K. Kasiura, *Zinc: A critical importance element in cosmetology.* Journal of Applied Cosmetology, 2004. **22**(1): p. 1-13.
33. Lansdown, A.B.G., et al., *Zinc in wound healing: Theoretical, experimental, and clinical aspects.* Wound Repair and Regeneration, 2007. **15**(1): p. 2-16.
34. Espitia, P.J.P., et al., *Zinc Oxide Nanoparticles: Synthesis, Antimicrobial Activity and Food Packaging Applications.* Food and Bioprocess Technology, 2012. **5**(5): p. 1447-1464.
35. Noyce, J.O., H. Michels, and C.W. Keevil, *Use of copper cast alloys to control Escherichia coli O157 cross-contamination during food processing.* Applied and Environmental Microbiology, 2006. **72**(6): p. 4239-4244.
36. Al-Holy, M.A., L.F. Castro, and H.M. Al-Qadiri, *Inactivation of cronobacter enterica (Enterobacter sakazakii) in infant formula using lactic acid, copper sulfate and monolaurin.* Letters in Applied Microbiology, 2010. **50**(3): p. 246-251.
37. Ibrahim, S.A., H. Yang, and C.W. Seo, *Antimicrobial activity of lactic acid and copper on growth of Salmonella and Escherichia coli O157:H7 in laboratory medium and carrot juice.* Food Chemistry, 2008. **109**(1): p. 137-143.
38. Maness, P.C., et al., *Bactericidal activity of photocatalytic TiO2 reaction: Toward an understanding of its killing mechanism.* Applied and Environmental Microbiology, 1999. **65**(9): p. 4094-4098.
39. Nel, A.E., et al., *Understanding biophysicochemical interactions at the nano-bio interface.* Nature Materials, 2009. **8**(7): p. 543-557.
40. Chorianopoulos, N.G., et al., *Use of titanium dioxide (TiO2) photocatalysts as alternative means for Listeria monocytogenes biofilm disinfection in food processing.* Food Microbiology, 2011. **28**(1): p. 164-170.
41. Cerrada, M.L., et al., *Self-sterilized EVOH-TiO2 nanocomposites: Interface effects on biocidal properties.* Advanced Functional Materials, 2008. **18**(13): p. 1949-1960.

42. Kim, Y., et al., *Disinfection of iceberg lettuce by titanium dioxide-UV photocatalytic reaction.* Journal of Food Protection, 2009. **72**(9): p. 1916-1922.

43. Chawengkijwanich, C. and Y. Hayata, *Development of TiO2 powder-coated food packaging film and its ability to inactivate Escherichia coli in vitro and in actual tests.* International Journal of Food Microbiology, 2008. **123**(3): p. 288-292.

44. Vaidyanathan, R., et al., *Nanosilver-The burgeoning therapeutic molecule and its green synthesis.* Biotechnology Advances, 2009. **27**(6): p. 924-937.

45. Chen, X. and H.J. Schluesener, *Nanosilver: A nanoproduct in medical application.* Toxicology Letters, 2008. **176**(1): p. 1-12.

46. Bhattacharya, R. and P. Mukherjee, *Biological properties of "naked" metal nanoparticles.* Advanced Drug Delivery Reviews, 2008. **60**(11): p. 1289-1306.

47. Klasen, H.J., *Historical review of the use of silver in the treatment of burns. I. Early uses.* Burns, 2000. **26**(2): p. 117-130.

48. Hetrick, E.M. and M.H. Schoenfisch, *Reducing implant-related infections: Active release strategies.* Chemical Society Reviews, 2006. **35**(9): p. 780-789.

49. Liau, S.Y., et al., *Interaction of silver nitrate with readily identifiable groups: Relationship to the antibacterial action of silver ions.* Letters in Applied Microbiology, 1997. **25**(4): p. 279-283.

50. Choi, O., et al., *Role of sulfide and ligand strength in controlling nanosilver toxicity.* Water Research, 2009. **43**(7): p. 1879-1886.

51. Russell, A.D. and W.B. Hugo, *Antimicrobial activity and action of silver.* Progress in medicinal chemistry, 1994. **31**: p. 351-370.

52. Feng, Q.L., et al., *A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus.* Journal of Biomedical Materials Research, 2000. **52**(4): p. 662-668.

53. Guggenbichler, J.P., et al., *A new technology of microdispersed silver in polyurethane induces antimicrobial activity in central venous catheters.* Infection, 1999. **27**(SUPPL. 1).

54. Lok, C.N., et al., *Proteomic analysis of the mode of antibacterial action of silver nanoparticles.* Journal of Proteome Research, 2006. **5**(4): p. 916-924.

55. Semeykina, A.L. and V.P. Skulachev, *Submicromolar Ag⁺ increases passive Na⁺ permeability and inhibits the respiration-supported formation of Na⁺ gradient in Bacillus FTU vesicles.* FEBS Letters, 1990. **269**(1): p. 69-72.

56. Jung, R., et al., *Antimicrobial properties of hydrated cellulose membranes with silver nanoparticles.* Journal of Biomaterials Science, Polymer Edition, 2009. **20**(3): p. 311-324.

57. Sondi, I. and B. Salopek-Sondi, *Silver nanoparticles as antimicrobial agent: A case study on E. coli as a model for Gram-negative bacteria.* Journal of Colloid and Interface Science, 2004. **275**(1): p. 177-182.
58. Park, H.J., et al., *Silver-ion-mediated reactive oxygen species generation affecting bactericidal activity.* Water Research, 2009. **43**(4): p. 1027-1032.
59. Mijnendonckx, K., et al., *Antimicrobial silver: Uses, toxicity and potential for resistance.* BioMetals, 2013. **26**(4): p. 609-621.
60. Silver, S., *Bacterial silver resistance: Molecular biology and uses and misuses of silver compounds.* FEMS Microbiology Reviews, 2003. **27**(2-3): p. 341-353.
61. Chopra, I., *The increasing use of silver-based products as antimicrobial agents: A useful development or a cause for concern?* Journal of Antimicrobial Chemotherapy, 2007. **59**(4): p. 587-590.
62. Ruparelia, J.P., et al., *Strain specificity in antimicrobial activity of silver and copper nanoparticles.* Acta Biomaterialia, 2008. **4**(3): p. 707-716.
63. Hwang, M.G., H. Katayama, and S. Ohgaki, *Inactivation of Legionella pneumophila and Pseudomonas aeruginosa: Evaluation of the bactericidal ability of silver cations.* Water Research, 2007. **41**(18): p. 4097-4104.
64. Li, W.R., et al., *Antibacterial activity and mechanism of silver nanoparticles on Escherichia coli.* Applied Microbiology and Biotechnology, 2010. **85**(4): p. 1115-1122.
65. Nair, L.S. and C.T. Laurencin, *Silver nanoparticles: Synthesis and therapeutic applications.* Journal of Biomedical Nanotechnology, 2007. **3**(4): p. 301-316.
66. Sharma, V.K., R.A. Yngard, and Y. Lin, *Silver nanoparticles: Green synthesis and their antimicrobial activities.* Advances in Colloid and Interface Science, 2009. **145**(1-2): p. 83-96.
67. Li, Q., et al., *Antimicrobial nanomaterials for water disinfection and microbial control: Potential applications and implications.* Water Research, 2008. **42**(18): p. 4591-4602.
68. Liu, J. and R.H. Hurt, *Ion release kinetics and particle persistence in aqueous nano-silver colloids.* Environmental Science and Technology, 2010. **44**(6): p. 2169-2175.
69. Sotiriou, G.A., et al., *Quantifying the origin of released Ag+ ions from nanosilver.* Langmuir, 2012. **28**(45): p. 15929-15936.
70. Kim, J.S., et al., *Antimicrobial effects of silver nanoparticles.* Nanomedicine: Nanotechnology, Biology, and Medicine, 2007. **3**(1): p. 95-101.
71. Sun, L., et al., *Silver nanoparticles inhibit replication of respiratory syncytial virus.* Journal of Biomedical Nanotechnology, 2008. **4**(2): p. 149-158.

72. Kim, J.Y., et al., *Enhanced inactivation of E. coli and MS-2 phage by silver ions combined with UV-A and visible light irradiation.* Water Research, 2008. **42**(1-2): p. 356-362.

73. De Gusseme, B., et al., *Biogenic silver for disinfection of water contaminated with viruses.* Applied and Environmental Microbiology, 2010. **76**(4): p. 1082-1087.

74. Lara, H.H., et al., *Mode of antiviral action of silver nanoparticles against HIV-1.* Journal of Nanobiotechnology, 2010. **8**.

75. Elechiguerra, J.L., et al., *Interaction of silver nanoparticles with HIV-1.* Journal of Nanobiotechnology, 2005. **3**.

76. Takenaka, S., et al., *Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats.* Environmental Health Perspectives, 2001. **109**(SUPPL. 4): p. 547-551.

77. Tian, J., et al., *Topical delivery of silver nanoparticles promotes wound healing.* ChemMedChem, 2007. **2**(1): p. 129-136.

78. Peng, H., et al., *Response of Ceriodaphnia dubia to ionic silver: Discrepancies among model predictions, measured concentrations and mortality.* Chemosphere, 2002. **46**(7): p. 1141-1146.

79. Reinfelder, J.R. and S.I. Chang, *Speciation and microalgal bioavailability of inorganic silver.* Environmental Science and Technology, 1999. **33**(11): p. 1860-1863.

80. Bielmyer, G.K., K.V. Brix, and M. Grosell, *Is Cl⁻ protection against silver toxicity due to chemical speciation?* Aquatic Toxicology, 2008. **87**(2): p. 81-87.

81. Glover, C.N., S.K. Sharma, and C.M. Wood, *Heterogeneity in physicochemical properties explains differences in silver toxicity amelioration by natural organic matter to Daphnia magna.* Environmental Toxicology and Chemistry, 2005. **24**(11): p. 2941-2947.

82. Kolts, J.M., C.J. Boese, and J.S. Meyer, *Acute toxicity of copper and silver to Ceriodaphnia dubia in the presence of food.* Environmental Toxicology and Chemistry, 2006. **25**(7): p. 1831-1835.

83. Nichols, J.W., et al., *Influence of salinity and organic matter on silver accumulation in Gulf toadfish (Opsanus beta).* Aquatic Toxicology, 2006. **78**(3): p. 253-261.

84. Lee, D.Y., C. Fortin, and P.G.C. Campbell, *Contrasting effects of chloride on the toxicity of silver to two green algae, Pseudokirchneriella subcapitata and Chlamydomonas reinhardtii.* Aquatic Toxicology, 2005. **75**(2): p. 127-135.

85. Choi, O., et al., *The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth.* Water Research, 2008. **42**(12): p. 3066-3074.

86. Bartłomiejczyk, T., et al., *Silver nanoparticles - Allies or adversaries?* Annals of Agricultural and Environmental Medicine, 2013. **20**(1): p. 48-54.

87. Behra, R., et al., *Bioavailability of silver nanoparticles and ions: From a chemical and biochemical perspective.* Journal of the Royal Society Interface, 2013. **10**(87).

88. Durán, N., et al., *Potential use of silver nanoparticles on pathogenic bacteria, their toxicity and possible mechanisms of action.* Journal of the Brazilian Chemical Society, 2010. **21**(6): p. 949-959.

89. Levard, C., et al., *Environmental transformations of silver nanoparticles: Impact on stability and toxicity.* Environmental Science and Technology, 2012. **46**(13): p. 6900-6914.

90. Panyala, N.R., E.M. Peña-Méndez, and J. Havel, *Silver or silver nanoparticles: A hazardous threat to the environment and human health?* Journal of Applied Biomedicine, 2008. **6**(3): p. 117-129.

91. Yu, S.J., Y.G. Yin, and J.F. Liu, *Silver nanoparticles in the environment.* Environmental Sciences: Processes and Impacts, 2013. **15**(1): p. 78-92.

92. Ip, M., et al., *Antimicrobial activities of silver dressings: An in vitro comparison.* Journal of Medical Microbiology, 2006. **55**(1): p. 59-63.

93. Lo, S.F., et al., *A systematic review of silver-releasing dressings in the management of infected chronic wounds.* Journal of Clinical Nursing, 2008. **17**(15): p. 1973-1985.

94. Rai, M., A. Yadav, and A. Gade, *Silver nanoparticles as a new generation of antimicrobials.* Biotechnology Advances, 2009. **27**(1): p. 76-83.

95. Simpson, K., *Using silver to fight microbial attack.* Plastics, Additives and Compounding, 2003. **5**(5): p. 32-35.

96. Gupta, A. and S. Silver, *Silver as a biocide: Will resistance become a problem?* Nature Biotechnology, 1998. **16**(10): p. 888.

97. Martinez-Abad, A., *Silver- and nanosilver-based plastic technologies* in *Antimicrobial Polymers*, J. Lagarón, M.J. Ocio, and A. Lopez-Rubio, Editors. 2010, John Wiley and sons inc.: Hoboken, New jersey. p. 287-316.

98. Kim, B.R., et al., *Literature review - Efficacy of various disinfectants against Legionella in water systems.* Water Research, 2002. **36**(18): p. 4433-4444.

99. Han, D.W., et al., *The use of silver-coated ceramic beads for sterilization of Sphingomonas sp. in drinking mineral water.* World Journal of Microbiology and Biotechnology, 2005. **21**(6-7): p. 921-924.

100. Lv, Y., et al., *Silver nanoparticle-decorated porous ceramic composite for water treatment.* Journal of Membrane Science, 2009. **331**(1-2): p. 50-56.

101. Busolo, M.A., et al., *Novel silver-based nanoclay as an antimicrobial in polylactic acid food packaging coatings.* Food Additives and Contaminants -

Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 2010. **27**(11): p. 1617-1626.

102. EFSA, *Scientific Statement of the Panel on Food Additives and Nutrient Sources added to Food (ANS): Question nº EFSA-Q-2005-169.* The EFSA Journal, 2008(884): p. 1-3.

103. Biswal, N., et al., *Incorporation of silver ions into zirconium titanium phosphate: A novel approach toward antibacterial activity.* Industrial and Engineering Chemistry Research, 2011. **50**(16): p. 9479-9486.

104. Miola, M., et al., *Surface silver-doping of biocompatible glasses to induce antibacterial properties. Part II: Plasma sprayed glass-coatings.* Journal of Materials Science: Materials in Medicine, 2009. **20**(3): p. 741-749.

105. Fiedler, J., et al., *Copper and silver ion implantation of aluminium oxide-blasted titanium surfaces: Proliferative response of osteoblasts and antibacterial effects.* International Journal of Artificial Organs, 2011. **34**(9): p. 882-888.

106. Zhao, L., et al., *Antibacterial coatings on titanium implants.* Journal of Biomedical Materials Research - Part B Applied Biomaterials, 2009. **91**(1): p. 470-480.

107. Bai, X., et al., *Functionally graded hydroxyapatite coatings doped with antibacterial components.* Acta Biomaterialia, 2010. **6**(6): p. 2264-2273.

108. Ewald, A., et al., *Silver-doped calcium phosphate cements with antimicrobial activity.* Acta Biomaterialia, 2011. **7**(11): p. 4064-4070.

109. Francolini, I., et al., *Polyurethane anionomers containing metal ions with antimicrobial properties: Thermal, mechanical and biological characterization.* Acta Biomaterialia, 2010. **6**(9): p. 3482-3490.

110. Bajpai, M., P. Gupta, and S.K. Bajpai, *Silver(I) ions loaded cyclodextrin-grafted-cotton fabric with excellent antimicrobial property.* Fibers and Polymers, 2010. **11**(1): p. 8-13.

111. Dogan, H., M. Koral, and T.Y. Inan, *Ag/Zn zeolite containing antibacterial coating for food-packaging substrates.* Journal of Plastic Film and Sheeting, 2009. **25**(3-4): p. 207-220.

112. Zehui, Z., et al., *Migration model of silver-loaded zeolite in antibacterial kraft paper*. 2012, 2012 International Conference on Packaging Technology and Science, ICPTS 2012. p. 271-277.

113. Fernández, A., et al., *Migration of antimicrobial silver from composites of polylactide with silver zeolites.* Journal of Food Science, 2010. **75**(3): p. E186-E193.

114. Incoronato, A.L., et al., *Active systems based on silver-montmorillonite nanoparticles embedded into bio-based polymer matrices for packaging applications.* Journal of Food Protection, 2010. **73**(12): p. 2256-2262.

115. Bo, L., et al., *A simple and 'green' synthesis of polymer-based silver colloids and their antibacterial properties.* Chemistry and Biodiversity, 2009. **6**(1): p. 111-116.

116. Lee, J., et al., *Antimicrobial packaging of raw beef, pork and turkey using silver-zeolite incorporated into the material.* International Journal of Food Science and Technology, 2011. **46**(11): p. 2382-2386.

117. Incoronato, A.L., et al., *Agar hydrogel with silver nanoparticles to prolong the shelf life of Fior di Latte cheese.* Journal of Dairy Science, 2011. **94**(4): p. 1697-1704.

118. Gammariello, D., et al., *Bio-based nanocomposite coating to preserve quality of Fior di latte cheese.* Journal of Dairy Science, 2011. **94**(11): p. 5298-5304.

119. Møretrø, T., et al., *Antibacterial activity of cutting boards containing silver.* Food Control, 2012. **28**(1): p. 118-121.

120. Johnston, J.H., et al., *Nano-structured composite calcium silicate and some novel applications.* Current Applied Physics, 2008. **8**(3-4): p. 504-507.

121. Fernández, A., P. Picouet, and E. Lloret, *Cellulose-silver nanoparticle hybrid materials to control spoilage-related microflora in absorbent pads located in trays of fresh-cut melon.* International Journal of Food Microbiology, 2010. **142**(1-2): p. 222-228.

122. Kumar, R. and H. Münstedt, *Silver ion release from antimicrobial polyamide/silver composites.* Biomaterials, 2005. **26**(14): p. 2081-2088.

123. Kumar, R. and H. Münstedt, *Polyamide/silver animicrobials: Effect of crystallinity on the silver ion release.* Polymer International, 2005. **54**(8): p. 1180-1186.

124. Kumar, R., S. Howdle, and H. Münstedt, *Polyamide/silver antimicrobials: Effect of filler types on the silver ion release.* Journal of Biomedical Materials Research - Part B Applied Biomaterials, 2005. **75**(2): p. 311-319.

125. Damm, C. and H. Münstedt, *Kinetic aspects of the silver ion release from antimicrobial polyamide/silver nanocomposites.* Applied Physics A: Materials Science and Processing, 2008. **91**(3): p. 479-486.

126. Dowling, D.P., et al., *Anti-bacterial silver coatings exhibiting enhanced activity through the addition of platinum.* Surface and Coatings Technology, 2003. **163-164**: p. 637-640.

127. Ward, T.J. and J.R. Kramer, *Silver speciation during chronic toxicity tests with the mysid, Americamysis bahia.* Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, 2002. **133**(1-2): p. 75-86.

128. Hu, W., et al., *In situ synthesis of silver chloride nanoparticles into bacterial cellulose membranes.* Materials Science and Engineering C, 2009. **29**(4): p. 1216-1219.

129. Kim, T.N., et al., *Antimicrobial effects of metal ions (Ag⁺, Cu²⁺, Zn²⁺) in hydroxyapatite.* Journal of Materials Science: Materials in Medicine, 1998. **9**(3): p. 129-134.

130. Bjarnsholt, T., et al., *Silver against Pseudomonas aeruginosa biofilms.* APMIS, 2007. **115**(8): p. 921-928.

131. Thomas, V., et al., *A versatile strategy to fabricate hydrogel-silver nanocomposites and investigation of their antimicrobial activity.* Journal of Colloid and Interface Science, 2007. **315**(1): p. 389-395.

132. Kasuga, N.C., et al., *Syntheses, structures, and antimicrobial activities of remarkably light-stable and water-soluble silver complexes with amino acid derivatives, silver(I) N-acetylmethioninates.* Inorganic Chemistry, 2012. **51**(3): p. 1640-1647.

133. Martínez-Abad, A., et al., *Ligands affecting silver antimicrobial efficacy on Listeria monocytogenes and Salmonella enterica.* Food Chemistry, 2013. **139**(1-4): p. 281-288.

134. Mastromatteo, M., A. Conte, and M.A. Del Nobile, *Advances in controlled release devices for food packaging applications.* Trends in Food Science and Technology, 2010. **21**(12): p. 591-598.

135. Zhou, Y.T., et al., *Effect of silver nanomaterials on the activity of thiol-containing antioxidants.* Journal of Agricultural and Food Chemistry, 2013. **61**(32): p. 7855-7862.

136. Ilg, Y. and J. Kreyenschmidt, *Effects of food components on the antimicrobial activity of polypropylene surfaces containing silver ions (Ag +).* International Journal of Food Science and Technology, 2011. **46**(7): p. 1469-1476.

137. Lloret, E., P. Picouet, and A. Fernández, *Matrix effects on the antimicrobial capacity of silver based nanocomposite absorbing materials.* LWT - Food Science and Technology.

138. Jin, X., et al., *High-throughput screening of silver nanoparticle stability and bacterial inactivation in aquatic media: Influence of specific ions.* Environmental Science and Technology, 2010. **44**(19): p. 7321-7328.

139. Singh, S.P., M.K. Bajpai, and B. Razdan, *Synthesis and study of anti-bacterial activity of complexes of diallyldisulphide from garlic.* International Journal of Green Pharmacy, 2012. **6**(3): p. 180-183.

140. Grigor'Eva, A., et al., *Fine mechanisms of the interaction of silver nanoparticles with the cells of Salmonella typhimurium and Staphylococcus aureus.* BioMetals, 2013. **26**(3): p. 479-488.

141. Percival, S.L., et al., *Use of flow cytometry to compare the antimicrobial efficacy of silver-containing wound dressings against planktonic Staphylococcus aureusand Pseudomonas aeruginosa.* Wound Repair and Regeneration, 2011. **19**(3): p. 436-441.

142. Irwin, P., et al., *Antimicrobial activity of spherical silver nanoparticles prepared using a biocompatible macromolecular capping agent: Evidence for induction of a greatly prolonged bacterial lag phase.* Journal of Nanobiotechnology, 2010. **8**.

143. Llorens, A., et al., *Metallic-based micro and nanocomposites in food contact materials and active food packaging.* Trends in Food Science and Technology, 2012. **24**(1): p. 19-29.

144. Fernández, A., P. Picouet, and E. Lloret, *Reduction of the spoilage-related microflora in absorbent pads by silver nanotechnology during modified atmosphere packaging of beef meat.* Journal of Food Protection, 2010. **73**(12): p. 2263-2269.

145. Costa, C., et al., *Antimicrobial silver-montmorillonite nanoparticles to prolong the shelf life of fresh fruit salad.* International Journal of Food Microbiology, 2011. **148**(3): p. 164-167.

146. Dong, G., et al., *Functional Ag porous films prepared by electrospinning.* Applied Surface Science, 2009. **255**(17): p. 7623-7626.

147. Rujitanaroj, P.O., N. Pimpha, and P. Supaphol, *Preparation, characterization, and antibacterial properties of electrospun polyacrylonitrile fibrous membranes containing silver nanoparticles.* Journal of Applied Polymer Science, 2010. **116**(4): p. 1967-1976.

148. Jeon, H.J., et al., *Preparation of poly(ε-caprolactone)-based polyurethane nanofibers containing silver nanoparticles.* Applied Surface Science, 2008. **254**(18): p. 5886-5890.

149. Sheikh, F.A., et al., *Electrospun antimicrobial polyurethane nanofibers containing silver nanoparticles for biotechnological applications.* Macromolecular Research, 2009. **17**(9): p. 688-696.

150. Lee, Y.J. and W.S. Lyoo, *Preparation of Atactic poly(vinyl alcohol)/silver composite nanofibers by electrospinning and their characterization.* Journal of Applied Polymer Science, 2010. **115**(5): p. 2883-2891.

151. Zhang, Y., H.F. Chan, and K.W. Leong, *Advanced materials and processing for drug delivery: The past and the future.* Advanced Drug Delivery Reviews, 2013. **65**(1): p. 104-120.

152. Gerasimchuk, N., et al., *Light insensitive silver(I) cyanoximates as antimicrobial agents for indwelling medical devices.* Inorganic Chemistry, 2010. **49**(21): p. 9863-9874.

153. Gonewar, N.R., et al., *Synthesis, characterisation and antimicrobial activities of 1, 2 naphthoquinone-1-oxime ligand and its metal chelates of Hg (II), Pb (II), Ag (I), Zn (II) and Cd (II).* International Journal of Chemical Sciences, 2012. **10**(3): p. 1493-1505.

154. Min, S.H., et al., *Development of white antibacterial pigment based on silver chloride nanoparticles and mesoporous silica and its polymer composite.* Microporous and Mesoporous Materials, 2010. **128**(1-3): p. 19-25.
155. Wright, T., *Alphasan: A thermally stable silver based inorganic antimicrobial technology.* Chemical Fibers International, 2002. **52**(2): p. 125.

Annexes

Annex A. Silver ions and nanoparticle minimal inhibitory/bactericidal concentrations against *Escherichia coli* according to different publications.

Authors, Year	Incubation Time	Growth conditions	MIC/MBC (ppm)	Method used	Ag^{+}/Ag^{0}
Hwang et al. (2007)	3h	Synthetic water	0,01	macrodilution	Ions
Kim et al. (1998)	20h	PBS	0,02	microdilution	NP
Lok et al. (2007)	16h	M9	0,3	macrodilution	Ions
Xu et al. (2008)	8h	MHB 1:100	0,39	Two-fold dilution	NP
Panacek et al. (2006)	24h	MHB	3,38	microdilution	NP
Luo et al. (2008)	18h	TSB	16	macrodilution	Ions
Hamilton-Miller et al. (1996)	24h	Isosensitest agar	32	Agar dilution	Ions
Ruparelia et al. (2007)	24h	Beef extract,yeast extract, peptone, NaCl	50	Macrodilution	NP
Lee et al. (2005)	20h	LB	>100	Agar dilution	NP
Pal et al. (2007)	24h	NB (beef extract, peptone)	>100 20/100	Agar dilution	Ions NP
Sanpui et al. (2008)	18-24h	LB	>100	macrodilution	NP
Sondi and Salopek-Sondi (2004)	24h	LB	>100	Agar dilution	NP

Annex B. Overview of this doctoral thesis: objectives, brief description of each chapter and conclusions linking chapters with each other..

Objectives	Chapters	Description	Conclusions/Questions
1. Gathering of a deep understanding of the interactions of silver ions with bacteria, the chemical environment, and how these may affect bacterial viability.	Chapter I	Ligands affecting silver efficacy: Influence of chlorides, sulphur aminoacids, complex synthetic growth media. Bonding and antimicrobial performance	Cysteine and complex growth media produce drastic decrease. Ag^+ attaches to bacteria. Type of Ag^+-TSB interactions unclear Bacteria really dead? (Chapter II)
	Chapter II	Viability (Flow cytometry and live/dead staining) vs cultivability	Increase in lag phase and resuscitation of bacteria under different growth conditions in the presence of silver.
2. Incorporation of silver into polar matrices, such as EVOH, to promote the sorption induced release, and evaluation of the release of ions and the efficacy of the films under food contact conditions.	Chapter III	EVOH-Ag^+ films generated by casting. Release and antimicrobial patterns under laboratory conditions and with food samples of low or high protein content.	<1 log reduction in high protein foods. Good effect with low protein foods. Active in M9 at the nanomolar level. Release is very fast in EVOH. Application? (Chapter IV)
	Chapter IV	EVOH-Ag^+ films generated by electrospinning. Release patterns and antimicrobial performance before and after thermal annealing of the fibres	Instant release of 100% silver content in form of ions. Thermal annealing produces nanoparticles and slows down release. Good antimicrobial properties. Sustained release needed for food packaging applications (Chapter V)

3. Evaluation of different methods for incorporation of silver ions into PLA as to their suitability as long lasting antimicrobial and antiviral materials for food applications.	Chapter V Chapter VI	$PLA\text{-}Ag^{+}$ films generated by casting. Characterization, long-term release and antimicrobial patterns under laboratory conditions after successive washings. Breakpoints established for S. enterica minimal antibacterial concentration. Same materials tested after successive washings for surface treatment and on food samples against *S. enterica* and feline Calicivirus	Physico-chemical properties not affected by low Ag^{+} contents. Release and antibacterial effect prolonged for days to months. Initial burst release doesn't comply with EU regulation (Chapter VII). Effective against the most common foodborne pathogens in foods and on surfaces (JISZ2801)
4. Application of a functional barrier to the PLA-Ag+ films as to tailor the release capacities of the films and achieve a prolonged antimicrobial performance without surpassing restriction limits.	Chapter VII	Beeswax layers of different thickness applied to $PLA\text{-}Ag^{+}$ films. Characterization, long-term release profiles and antimicrobial performance in liquid and solid foods and according to the JIS Z2801.	Long-term effectiveness under all tested conditions. Release kinetics can be tailored as to comply with migration restriction limits. Melt-compoundable films would be preferred for industrial applications (Chapter VIII)
5. Fabrication of PLA films incorporating silver compounds, capable of withstanding thermal plastic processing while maintaining a prolonged antimicrobial effectiveness over time.	Chapter VIII	Melt-compounded PLA films with 15 different silver compounds. Characterization on the optical, mechanical, thermal properties, release patterns and antimicrobial performance.	Some films were transparent and showed good thermal, mechanical and long-term antibacterial effects.

Annex C. Composition of the synthetic media TSB and M9.

Tryptic Soy Broth

Pancreatic Digest of Casein	17g/L
Sodium Chloride	5g/L
Papaic Digest of Soy Bean	3g/L
Glucose Monohydrate	2.5g/L
Dipotassium Phosphate	2.5g/L

M9 minimal medium

Disodium phosphate heptahydrate	6.8 g/L
Monopotassium phosphate	3g/L
Ammonium Chloride	1g/L
Sodium chloride	0.5g/L
Glucose	3.6g/L
Magnesium sulphate	0.24g/L

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Ligands affecting silver antimicrobial efficacy on *Listeria monocytogenes* and *Salmonella enterica*

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ABSTRACT

Although silver is being extensively used in food or other applications as the key component to control microbial proliferation, many factors affecting its real potential are still unknown. In the present work, the presence of specific ligands or the contents in organic matter was correlated with silver speciation and its antibacterial performance. Silver was found to be only active in form of free silver ions (FSI). The presence of chloride ions produced an equilibrium of stable silver chloride complexes which were void of antimicrobial efficacy. However, even at relatively high concentrations of chlorides, a small fraction of FSI may still be present, producing a bactericidal effect with concentrations at the nanomolar level under optimum conditions. Low concentrations of thiol groups completely inactivated silver, while methylsulphur groups only affected its efficacy at very high concentrations. Antibacterial performance revealed differences of about 1000-fold between results for environments with high organic matter content and results for aqueous salt buffers. Thiol groups were nonetheless not found directly associated with the decrease in antimicrobial performance in a nutrient rich environment. These results point out the complexity of the antimicrobial systems based on silver and can have relevance in food or other applications of silver as an antimicrobial.

1. Introduction

In the last decade, the demand for minimally processed, easily prepared and ready-to-eat 'fresh' food products has globally increased which has encouraged the industry to develop new technologies as an alternative to food-thermal technologies. These new alternative technologies such as lower thermal or high pressure treatments may in some instances allow pathogenic bacterial growth (Valero & Francés, 2006). However, even if foodborne pathogens are totally eliminated by efficient thermal treatments, microbial recontamination of the food surface could take place during the post-processing steps, when the risk of cross-contamination is elevated. As a result, a reduction of food shelf-life is observed and the risk of foodborne illnesses is greatly increased. Therefore, new preservation techniques, such as incorporation of antibacterial substances to the food products in order to extend its preservation is currently being investigated and applied.

The use of silver as antimicrobial for food-related applications has been recognised since silver pottery and cutlery where used in antiquity (Klasen, 2000). Although the mechanism remains disputed (Dibrov, Dzioba, Gosink, & Hase, 2002; Texter, Ziemer, Rhoades, & Clemans, 2007), it is generally accepted that free silver ions (FSI) bind to membrane constituents, destabilising the membrane potential and causing proton leakage (Liau, Read, Pugh, Furr, & Russell, 1997; Matsumura, Yoshikata, Kunisaki, & Tsuchido, 2003) and it also interferes with DNA replication and ion transport across the respiratory chain (Feng et al., 2000; Semeykina & Skulachev, 1990), all of which eventually lead to cell death. Due to this combination of unspecific mechanisms, silver ions are not likely to develop any resistances and are active against a very broad spectrum of bacteria, yeasts, fungi and even viruses in tiny concentrations (Thomas & McCubbin, 2003), remaining nontoxic to human cells (Russell & Hugo, 1994; Williams, Doherty, Vince, Grashoff, & Williams, 1989).

Therefore, its use has become more and more popular in the past few years. Apart from the medical field, silver is nowadays incorporated as the key component to control microbial proliferation in a wide variety of materials used in our daily life like textile clothing, coatings in home appliances and food related applications like water treatment units or a great variety of food-contact materials (see Bosetti, Massè, Tobin, & Cannas, 2002; Chen & Schluesener, 2008; Gupta & Silver, 1998; Li et al., 2008; Rai, Yadav, & Gade, 2009 for review). In most of these materials, the antimicrobial effect relies on the leaking of silver ions based on ion-exchange from mineral carriers, like montmorillonites (Busolo, Fernandez, Ocio, & Lagaron, 2010; Malachová, Praus, Pavlíčková, & Turicová, 2009), tobermorites (Coleman, 2009) and most predominantly

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On the different growth conditions affecting silver antimicrobial efficacy on *Listeria monocytogenes* and *Salmonella enterica*

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ABSTRACT

Silver is known to inhibit microorganisms and therefore it is an ideal candidate for its incorporation in a wide variety of materials for food applications. However, there is still a need for understanding how silver prolonged exposure to bacterial contamination affects the bioavailability of the active silver species. In the present study, growth curves of *Listeria monocytogenes* and *Salmonella enterica* were performed for 3–5 days in Tryptic Soy Broth (TSB) and M9 minimal medium (M9) in the presence of silver ions and silver solutions previously in contact with the growth media. The cultivability of the bacteria under these conditions was correlated with the viability of the bacterial populations as measured by flow cytometry analysis (FC) using a LIVE/DEAD BacLight kit. It was found that, after a period where viable counts were not detected, bacterial populations recovered and were able to proliferate in most cases. The resuscitation of the cultures was explained by both the existence of a resilient fraction of bacteria in a compromised state and the parallel inactivation of the silver species. This inactivation was found to be highly influenced by time dependant chemical reactions taking place in the environment of exposure, producing differences of at least 3 fold between results for nutrient rich environments and results for limiting environments. This study points out the need for understanding these chemical interactions and bacterial mechanisms of adaptation and may have relevance in the design of silver-based antimicrobial systems for food-related applications.

1. Introduction

In the last decade, the demand for minimally processed, easily prepared and ready-to-eat 'fresh' food products has globally increased which has encouraged the industry to the development of new technologies as an alternative to food-thermal technologies. These new alternative technologies such as lower thermal or high pressure treatments may in some instances allow pathogenic bacterial growth (Valero and Francés, 2006). However, even if foodborne pathogens are totally eliminated by efficient thermal treatments, microbial recontamination of the food surface could take place during the processing steps, when the risk of cross-contamination is elevated. As a result, the risk of foodborne illnesses is increased. Therefore, new preservation techniques, such as incorporation of antibacterial substances into materials intended to come in contact with food products, are currently being investigated and applied.

The use of silver as antimicrobial for food-related applications has been recognized since silver pottery and cutlery were used in antiquity (Klasen, 2000). Although the mechanism of action still remains disputed, it is generally accepted that free silver ions (FSI), present or leaking from the materials in contact with the food matrix, are able to bind to membrane constituents, destabilizing the membrane potential and causing proton leakage (Liau et al., 1997; Matsumura et al., 2003). They also interfere with DNA replication and ion transport across the respiratory chain (Feng et al., 2000; Semeykina and Skulachev, 1990; Texter et al., 2007), all of which eventually lead to cell death. Due to this combination of unspecific mechanisms, silver ions are not likely to develop any resistances and are active against a very broad spectrum of bacteria, yeasts, fungi and even viruses in tiny concentrations, remaining nontoxic to human cells (Russell and Hugo, 1994).

Therefore, its use has become more and more popular in the past few years. Apart from the medical field, silver is nowadays incorporated as the key component to control microbial proliferation in a wide variety of materials used in our daily life like textile clothing, coatings in home appliances and food related applications like water treatment units (Han et al., 2005) or a great variety of food-contact materials (Appendini and Hotchkiss, 2002; Bouwmeester et al., 2009; Simpson, 2003) (see Gupta and Silver, 1998; Li et al., 2008; Rai et al., 2009 for review). In most of these materials, the antimicrobial effect relies on the leaking of silver ions based on ion-exchange from mineral carriers, like montmorillonites, tobermorites and most predominantly zeolites (Busolo et al., 2010; Cowan et al., 2003; Galeano et al., 2003; Nakane

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Development and Characterization of Silver-Based Antimicrobial Ethylene−Vinyl Alcohol Copolymer (EVOH) Films for Food-Packaging Applications

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ABSTRACT: The use of silver as an antimicrobial in the food area has raised wide interest in recent years. In the present work, 0.001−10 wt % silver ions was satisfactorily incorporated into an ethylene−vinyl alcohol (EVOH) copolymer matrix by a solvent casting technique. The antibacterial efficacy of the composite was evaluated under laboratory conditions and in contact with some foods. The ionic compound did not affect the crystallinity or the water-induced plasticization of the materials and was homogeneously distributed across the surface and thickness of the films. When immersed in water, sorption-induced release of 50−100% of the silver ions took place in <30 min. In the bacterial minimal growth medium M9, the minimal inhibitory concentration (MIC) of the film was in the range of 0.01−0.1 ppm. High protein content food samples displayed low susceptibility to the films (<1 log reduction in any case), whereas low protein content food samples exhibited no detectable bacterial counts for films with 1 and 10 wt % silver and about 2 log reduction for films with 0.1 wt % silver. These results represent a step forward in the understanding of silver antimicrobial efficacy and its possible application in the food-packaging industry, most likely as food coatings.

KEYWORDS: *active packaging, antimicrobial silver, ethylene−vinyl alcohol (EVOH)*

INTRODUCTION

Market trends toward minimally processed, easily prepared, and ready-to-eat "fresh" food products involve the use of alternative technologies such as lower thermal, high-pressure, UV irradiation, or electric pulse treatments, which might allow survival and proliferation of pathogenic bacteria.[1−4] Recent foodborne microbial outbreaks, globalization of food trade, and distribution from centralized processing are driving a search for innovative ways to inhibit microbial growth in the foods while maintaining quality, freshness, and safety.[5] The combination of these emerging technologies with antimicrobial packaging technologies could allow extension of the shelf life of foods and the prevention of recontamination with pathogens.

In antimicrobial packaging, a substance with biocide properties is included in a sachet, coated, adsorbed, or immobilized on the surface or directly incorporated in the polymer during its processing. As microbial contamination occurs primarily at the surface, the incorporation of the biocide in a film or as a coating has many advantages due to the high exposure areas for the biocide.[6,7]

Various antimicrobials have been incorporated in polymers for food-packaging applications such as organic acids[8,9] or triclosan.[10] Recently, natural antimicrobials such as enzymes,[11] bacteriocins,[12−14] essential oils,[15] chitosan,[16] and others have attracted much attention due to consumer demand trends (see refs 5, 17, and 18 for reviews). Antimicrobial silver has emerged as a new effective technology to prevent microbial proliferation on food contact surfaces in the food industry.

The antimicrobial efficacy of silver has been recognized since ancient times.[19,20] In the past few years, the use of silver or silver salts as key components to control microbial proliferation has become more and more popular. Much of the research on this compound is still focused on medical applications, such as wound[21] and burn[22] treatments, dentistry, catheters,[23−25] or orthopedics.[26] However, new applications have emerged, so that silver is currently being incorporated in a wide variety of materials used in daily life, ranging from textile clothing,[27,28] coatings in washing machines, refrigerators, and furniture handles,[29−32] home water treatment units, food-contact materials,[5,33] deodorants,[34] and tooth brushes[35] (see refs 36−38 for reviews).

Due to their unspecific mechanism of action, silver ions are active against a very broad spectrum of bacteria, yeasts, fungi, and viruses[39,40] and are not toxic to human cells.[19,20] In the United States, the Center for Food Safety and Applied Nutrition in the Food and Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled waters, and in the European Union, silver is accepted under directive 94/36/EC as a coloring agent (E-174) with no restrictions. Additionally, the European Food Safety Authority (EFSA) has provided positive opinions for silver zeolites, silver zirconium phosphates, and silver-containing glasses in its provisional list of additives for food contact materials with a general restriction of ≤0.05 mg/kg food for the whole group. Regardless of the stringent regulations, silver remains the most

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ORIGINAL CONTRIBUTION

Influence of speciation in the release profiles and antimicrobial performance of electrospun ethylene vinyl alcohol copolymer (EVOH) fibers containing ionic silver ions and silver nanoparticles

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Abstract In the present study, tailor-made ethylene vinyl alcohol copolymer (EVOH) fibers containing different amounts of antimicrobial silver ions and nanoparticles were developed by electrospinning and subsequent thermal annealing. The morphology of the fibers was examined by scanning and transmission electron microscopy and thermal properties were characterized by differential scanning calorimetry. Speciation and controlled release of silver from the fibers was monitored by anodic stripping voltammetry and energy dispersive X-ray spectroscopy. Before aging, 100 % of the silver recovered from the electrospun structures was in ionic form to be instantly released in contact with moisture with varying temperature-dependent kinetics. Thermal annealing of the fibers at 100 °C for 1, 2, and 4 days prompted the gradual transformation of 70, 93–94, and 98–99 % of the total silver into nanoparticles homogeneously distributed along the fibers, which were mostly retained within them, producing a substantial decrease in their release capacity. Speciation and release profiles from the fibers were correlated with their antibacterial performance against *Listeria monocytogenes* and *Salmonella enteric*. This study is a step forward in the understanding of silver-based electrospun antimicrobial polymers and puts forth the suitability of EVOH for the development of targeted delivery systems in a number of applications.

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Introduction

Electrospinning is a simple technique to continuously generate ultrafine fibrous mats with fiber diameters ranging from tenths of nanometers to several microns. Their distinct characteristics, like a very high specific surface and porosity, and the suitability of the technique for impregnating other materials within the fibers at the nanoscale level have prompted their use in a wide range of applications [1].

Silver is particularly important due to its unique physical and chemical properties at the nanoscale and high antimicrobial effect. The incorporation of silver in electrospun fibers has enabled the development of new materials with useful features. Ultrafine fibers with enhanced photoconductivity [2], tensile strength [3] or photocatalytic properties [4, 5], materials which act as biobatteries [6] or biosensors [7] have been produced, as well as fibrous membranes with antibacterial properties with applications in water filtration [8], protective clothing [9], wound dressings, implant materials, or tissue engineering [10].

The nanoparticles can be either purchased in its reduced form or produced from silver salts by physical, chemical, or biological reduction [11]. This reduction can take place without addition of any further-reducing agents by heat or irradiation treatments after the electrospinning process [12–16]. The annealing of the fibers by heat treatment or ultraviolet visible (UV) irradiation also offers the possibility of tuning the size of the nanoparticles by changing the irradiation time [17]. Although many studies have been devoted to the incorporation of silver and silver nanoparticles into polymeric matrices, its speciation is usually

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Evaluation of silver-infused polylactide films for inactivation of *Salmonella* and feline calicivirus *in vitro* and on fresh-cut vegetables

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ABSTRACT

There is a growing trend to develop packaging materials with an active role in guarantying that the quality and safety characteristics of packaged products will remain or improve from preparation throughout shelf-life. In the present study, 0.001–1.0 wt.% silver ions were satisfactorily incorporated into polylactide (PLA) films by a solvent casting technique. Silver migration from the films was measured by voltamperometry and then correlated with its antimicrobial efficacy against *Salmonella enterica* and feline calicivirus (FCV), a human norovirus surrogate, by using the Japanese industrial standard (JIS Z 2801). The PLA-silver films showed strong antibacterial and antiviral activity *in vitro*, with increasing effects at higher silver concentrations. Moreover, results show that FCV was less susceptible to silver than *Salmonella*. When films were applied on food samples, antibacterial and antiviral activity was reduced as compared to *in vitro*. Antimicrobial activity was very much dependent on the food type and temperature. In lettuce samples incubated at 4 °C during 6 days, 4 log CFU of *Salmonella* was inactivated for films with 1.0 wt.% and no infectious FCV was reported under the same conditions. On paprika samples, no antiviral effect was seen on FCV infectivity whereas films showed less antibacterial activity on *Salmonella*.

1. Introduction

The consumption of fresh-cut vegetables has increased globally as they are generally considered safe and healthy by consumers (Lynch et al., 2009). However, agricultural irrigation with wastewater that may be raw, treated and/or partially diluted, is a common practice worldwide and constitutes the main source of pathogen contamination. Several factors affect microbial quality and shelf-life of vegetables, such as intrinsic properties of the vegetables (e.g. pH, water content), processing factors (e.g. washing, cutting, blanching), extrinsic factors (e.g. storage temperature, packaging) and implicit factors (e.g. microbial characteristics) (Heard, 1999).

The increase in mass production and distribution of food products will lead to an increase in the number of multinational outbreaks. A wide range of pathogens has been associated with outbreaks related to vegetable products. Among them, the most common agents causing fresh produce-related outbreaks are human norovirus (NV) and *Salmonella* (Doyle and Erickson, 2008; EFSA, 2012). In addition, human norovirus and *Salmonella* have been listed in the top 5 highest-ranking pathogens with respect to the total cost of foodborne illness in the United States (Scharff, 2010). As a means to prevent recontamination with pathogens and allow extending shelf-life of foods, antimicrobial packaging is one of the most promising technologies in the food area. However, few studies have confronted the task of fabricating and evaluating materials with antimicrobial properties in real food applications, and very scarce information is available about packaging materials with both antibacterial and virucidal properties.

Among natural antimicrobials, silver has emerged as a very efficient technology to prevent microbial proliferation on food contact surfaces in the food industry. Due to its unspecific mechanism of action, silver ions are active not only against a very broad spectrum of bacteria, but also against yeasts, fungi and even viruses, being nontoxic to human cells (Russell and Hugo, 1994; Williams et al., 1989). In the U.S., the Center for Food Safety and Applied Nutrition in the Food and Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled waters and in the EU, silver is accepted under directive 94/36/EC as a colouring agent (E-174) with no restrictions. Additionally, the European Food Safety Authority (EFSA) has provided positive opinions for silver zeolites, silver zirconium phosphates, and silver containing glasses in its provisional list of additives for food contact materials with a general restriction of ≤0.05 mg/kg food for the whole group.

As a result of its outstanding potential, and given the absence of cost-effective alternatives among other natural antimicrobials, silver is the most widely used polymer additive for food contact applications (Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002).

The approach of the use of silver in the food industry has been mostly bounded to ion exchange from mineral carriers, which are

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Antimicrobial beeswax coated polylactide films with silver control release capacity

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ABSTRACT

Although the application of silver based antimicrobial systems is a widespread technology, its implementation in areas such as food packaging is still challenging. The present paper describes the fabrication of poly(lactic acid) (PLA) coated with beeswax with controlled release properties for sustained antimicrobial performance. Release of silver ions from the polymers was monitored voltammetrically under various conditions (surface contact, immersion in various liquid media and at different pH values) throughout at least 7 days. A higher release was noted with decreasing pH while surface release was much slower than the release when immersed in liquid medium. While uncoated films demonstrated a high burst release which in some instances implied surpassing some current migration restrictions (<0.05 mg/kg food), the addition of a beeswax layer allowed a sustained release of the antimicrobial compound. Increasing the thickness of the beeswax layer resulted in an increase in the water barrier properties of the films while reducing the relatively constant values of sustained release. Antimicrobial performance was correlated with the release of silver ions, indicating threshold concentrations for biocide action of <6 μg/L and 9–14 μg/L for surface contact and in liquid media, respectively. Either by surface contact or by immersion in growth medium or vegetable soup, the coated films displayed a strong bactericidal effect against *Salmonella enterica*. The application of this functional barrier thus offers the possibility of tuning the release profiles of the films to suit a specific application and puts forth the possible suitability of these materials for food packaging or other migration sensitive applications.

1. Introduction

In recent years, the interest in food packaging with antimicrobial properties has increased considerably, due to the fact that these systems are able to control the microbiological decay of perishable food products (Mastromatteo et al., 2010). Many applications, including food production and storage, might benefit from the incorporation of safe and wide spectrum long-lasting biocides into polymers or working surfaces (Appendini and Hotchkiss, 2002). Although contamination depends highly on the characteristics of the food product, microbiological exposure might in many instances take place on the surface of the food. In this sense, the incorporation of the biocide in a film or as a coating has many advantages due to the high exposure areas for the biocide and a reduction of the loss of the antimicrobial compound into the bulk of the food (López-Rubio et al., 2004; Ouattar et al., 2000). Therefore, extensive research has been made to develop packaging strategies to retain the active agent in the polymeric network and control its release

Biopolymers obtained from natural resources are a promising alternative to non-biodegradable petroleum-based plastics in food packaging due to their environmentally friendly nature (Fabra et al., 2013). In particular, polylactides, a renewable family of polymers derived from biobased resources such as maize, are becoming increasingly popular owing to their high mechanical strength, transparency, water resistance, melt processability and relatively low prices in the market compared to other biopolymers (Auras et al., 2004). Additionally, PLA and its copolymers are the most widely used plastics for controlled drug delivery systems because of their biodegradability, biocompatibility and ease of processing (Zhang et al., 2013) Therefore, the use of PLA for controlled release of antimicrobials could be an interesting field of research for food packaging applications.

Silver ions are active against a very broad spectrum of bacteria, yeasts, fungi and viruses and are not toxic to human cells (Russell and Hugo, 1994; Williams et al., 1989). Therefore, a wide variety of materials used in daily life are recently incorporated with silver or silver salts

Additional publications not included in this PhD thesis carried out by the candidate or in which he has collaborated:

A. Martinez-Abad, J.M. Lagaron, M.J. Ocio and G. Sánchez *Antibacterial performance of solvent cast polycaprolactone (PCL) films containing essential oils* (2013) Food Control 34 (1) 214-220

A. Martinez-Abad *Silver- and nanosilver based plastic technologies* (2011) in *Antimicrobial Polymers*, J. Lagarón, M.J. Ocio, and A. Lopez-Rubio, Editors. 2011, John Wiley and sons inc.: Hoboken, New jersey. p. 287-316. ISBN: 978-0-470-59822-1

A. Martinez-Abad. *Silver based nanoreinforced polymers for food packaging* in *Multifunctional and nanoreinforced polymers for food packaging,* J. Lagarón, Editor. 2011 Woodhead Publishing. p. 347-367 ISBN: 1 84569 738 3

A. Martinez-Abad**,** G. Sánchez, M.J. Ocio and J.M. Lagaron *Polymeric Materials Containing Natural Compounds with Antibacterial and Virucide Properties* in *Polymeric Materials with Antimicrobial Activity: From Synthesis to Applications,* M. Fernandez-Garcia, Editor, 2013, RSC Publishing. p. 310-326 ISBN:

S. Torres-Giner, A. Martinez-Abad, M.J. Ocio and J.M. Lagaron *Stabilization of a Nutraceutical Omega-3 Fatty Acid by Encapsulation in Ultrathin Electrosprayed Zein Prolamine* (2010) Journal of Food Science 75 (6) N69-N79

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