Traditional and Bio-based packaging materials with antimicrobial activity: effectiveness in selected food products

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I declare that my PhD thesis has been amended to address all the Referee's comments.

Introduction and aim of the thesis

Packaging has a significant role in the food industry and it is an integral part both of the food processes and the supply chain.
Food packaging has to perform several tasks as well as fulfilling many demands and requirements. It protects food from environmental conditions, such as oxygen, light, microbes, moisture, mechanical stress and dust; at the same time a food package makes distribution easier. Other basic tasks are to ensure adequate labeling for providing information and a proper convenience to the consumer, e.g., easy opening, reclosable lids and suitable dosing mechanisms. Basic requirements for each packaging are good marketing properties, reasonable price, technical feasibility (e.g. suitability for automatic packaging machines, sealability), suitability for food contact, low environmental stress and suitability for recycling or refilling. A package has to satisfy all these various requirements effectively and economically.
Changes in the way food products are produced, distributed, stored and retailed, reflecting the continuing increase in consumer request for improved safety, quality and extended shelf life for packaged foods, are placing greater demands on the performance of food packaging. Consumers want to be assured that the packaging is fulfilling its function of protecting the quality, freshness and safety of foods. The trend to ensure the quality and safety of food without, or at least fewer, additives and preservatives means that packaging has a more significant role in the preservation of food and in guaranteeing the safety of food in order to avoid wastage and food poisoning and to reduce allergies. So the growing consumer demand for safe, fresh, preservative-free and minimally processed foods has led to the development of new processing and preservation techniques.
While traditionally food packaging has been defined as a passive technology, which allows to preserve food product, recently a new concept of packaging has been proposed: active packaging, so called because it doesn't represent only an inert barrier but has an active role and interacts with the food or the surrounding media.
Active packaging can be defined as a system that modifies the environment inside the food package, thereby altering the state of the packaged food system and its headspace to enhance its quality by extension of shelf life, enhancement of sensory qualities, and maintenance of microbial safety (Malhotra *et al.*, 2015). It has been developed as alternative method to control the microbial contamination and extend the shelf-life of food.
Food condition in the definition of active packaging includes various aspects that may play a role in determining the shelf life of packaged food, such as physiological processes (e.g. respiration of fresh fruit and vegetables), microbiological aspect (e.g. spoilage by microorganisms), chemical processes (e.g. lipid oxidation), physical processes (e.g. staling of bread, dehydration)

and infestation (e.g. by insects). Some traditional food preservation techniques like drying, freezing, heating, fermentation, salting can extend the shelf life of food products, but recontamination may occur, that may render the food unpalatable for the consumers. Antimicrobial packaging system is a novel development which incorporates an antimicrobial agent into a polymer film to suppress the activities of targeted microorganisms that are contaminating foods (Sung *et al*., 2013). Through the application of appropriate active packaging systems, these conditions can be regulated in numerous ways depending on the requirements of the packaged food. As reported in Table 1, active packaging techniques for preservation and improving quality and safety of foods can be divided into three categories: absorbers (i.e. scavengers); releasing systems and other systems.

Absorbing (scavenging) systems remove undesired compounds such as oxygen, carbon dioxide, ethylene, excessive water, taints and other specific compounds. Releasing systems actively add or emit compounds to the packaged food or into the headspace of the package such as antioxidants, carbon dioxide and preservatives.

Depending on the physical form of active packaging systems, absorbers and releasers can be a sachet, label or film type. Sachets are placed freely in the head-space of the package while labels are attached into the lid of the package. Direct contact with food should be avoided because it impairs the function of the system and, on the other hand, may cause migration problems.

Table 1. Example of active packaging application for use within the food industry (Kerry *et al.*, 2006).

Absorbing/scavenging properties	Oxygen, carbon dioxide, moisture, ethylene, flavours, taints, UV light
Releasing/emitting properties	Ethanol, carbon dioxide, antioxidants, preservatives, sulphur dioxide, flavours, pesticides
Removing properties	Catalyzing food component removal: lactose, cholesterol
Temperature control	Insulating materials, self-heating and self-cooling packaging, microwave susceptors and modifiers, temperature-sensitive packaging
Microbial and quality control	UV and surface-treated packaging materials

Another category of active packaging includes systems in which an antimicrobial agent is integrated either directly into food particle or to the packaging material, where it is released over a period of time to maintain the

products quality, as well as its safety leading to its extended shelf life. Direct adding of antimicrobial agents onto foods is less efficient in the microorganism inhibition than the antimicrobial packaging itself. This is due to the rapid diffusion of antimicrobial agent into foods and denaturation of the active substances by food constituents which reduce the reactivity of antimicrobial agent. On the other hand, antimicrobial packaging offers slow and continuous migration of the antimicrobial agent from packaging material to food surfaces which enable antimicrobial agent to maintain at high concentration over a long period.

Among the various solutions of active packaging available on the market the attention of this PhD thesis was paid to the antimicrobial ones; at first the work was aimed to verify the antimicrobial effectiveness of a commercial active system called *Food Touch*® by Microbeguard Corp. (USA). *Food Touch*® liners were made with material containing silver zeolite that dispensed silver metal ions in a controlled release over time, resulting in preserving the color and texture, minimizing the odor of food items, and extending shelf life 1 to 3 days longer. This product was provided for lining sheet pans, storage containers, for covering and wrapping food, both at professional and domestic kitchens. The liners were indicated for the preservation of any perishable food items except for the baked products.

In this work *Food Touch*® liner ability to improve the microbiological quality and so extend the shelf life of the food product as well as its reliability and application were investigated using as food matrix a typical Italian soft cheese (*Stracchino* cheese). *Stracchino* is a commercially-available fresh cheese, preferentially consumed by children and the elderly, and was selected as a perishable and high value food suitable for use in these packaging systems. Microbiological, chemical and sensorial tests were carried out at different stages of the food shelf life. The selected microbiological parameters were spoilage-related microorganisms while pathogens were not considered because of their not significant incidence rate in the *Stracchino* cheese.

The *Food Touch*® performance was compared to another innovative packaging (*Ovtene*® by Arcadia Spa, Italy), based on calcium carbonate and titanium oxide, regularly used at that time by the cheese manufacturer, and a traditional passive packaging. *Ovtene*® was claimed by the manufacturer not as an active packaging but as an innovative foodstuff packaging which protects the purity and freshness of foodstuffs kept in the refrigerator for a longer time.

The experimentation on the fresh cheese packed with silver-based liners is described in the second part of this thesis (publication n.1): the findings did not show a marked antibacterial action on the microbial spoilage population neither an improving effect on the organoleptic characteristics of the cheese. Otherwise a silver migration and accumulation were observed in higher

quantity than suggested by the European Food Safety Authority (EFSA) as a precautionary measure, i.e. 0,05 mg/kg of food (EFSA Journal, 2011).
In front of the results obtained from the silver-based film the second part of the research was dedicated to explore the application of active packaging containing natural antimicrobial organic agents with particular regard to consumer expectations and food safety needs.
As recently there has been a very high interest in polymers derived from renewable and bio-compostable sources, an experimental gluten-based edible film has been developed and subsequently implemented by the addition of natural substances with antimicrobial action.
Several studies reported the antimicrobial effect of various essential oils or their constituents. The active substances incorporated in the experimental film were selected among those described in literature having the capacity of reducing or controlling the growth of pathogenic microorganisms such as *Salmonella* spp. and *Listeria monocytogenes.*
Different formulations of gluten-based films were produced in laboratory with the addition of the following substances: cinnamaldehyde, eugenol and carvacrol at different concentrations.
Despite publishing lots of works about the antimicrobial activity on model media, this study evaluated the effect of active packaging not only *in vitro*, but also on real food matrices; in particular the experimental film was tested on fish and meat products artificially contaminated with *Salmonella* spp. and *Listeria monocytogenes* and assessing the antimicrobial effectiveness at different times of the shelf life of the products. The results obtained from the active packed samples were compared to those packed with edible films without antimicrobial substances. The experimental tests are extensively described in the second part of the thesis (publication n.2).
In the last years packaging development has been driven by the continuous request of best performing materials and technologies for food industry.
Active packaging systems using natural organic or inorganic compounds, such as aroma compounds or silver ions, would be a valid alternative to the usage of chemical preservatives, rendering the food safer from a hygienic and health point of view for longer time.
On the other hand further investigations are necessary aiming to improve characterizing the new packaging materials in terms of their potential impacts on human health.

Part I

ANTIMICROBIAL FOOD PACKAGING

1. ANTIMICROBIAL AGENTS

Food products can be subjected to microbial contamination that is mainly caused by bacteria, yeasts and fungi. Many of these microorganisms can cause undesirable reactions that deteriorate the flavor, color, odor, sensory and textual properties of food. Microbial growth in food products is a major concern because some microorganisms can potentially cause food-borne illness. In packaged foods, the growth and survival of common spoilage and pathogenic microorganisms such as *Listeria monocytogenes* , *Salmonella* spp., *Escherichia coli* O157, *Staphylococcus aureus*, *Campylobacter* spp., *Clostridium perfringens*, *Aspergillus niger* and *Saccharomyces cerevisiae* are affected by a variety of intrinsic factors such as pH, water activity and the presence of oxygen or by extrinsic factors associated with storage conditions including temperature, time and relative humidity (Kuorwel *et al.*, 2011).

Many food products including various types of cheeses, meats, poultry and fish are highly susceptible to microbial spoilage. To prevent the growth of spoilage and pathogenic microorganisms on foods, various traditional preservation techniques such as heat treatment, salting, acidification and drying are used in food industry.

As reported in the introduction food packaging films added with antimicrobial agents have been developed as alternative method to control the microbial contamination and extend the shelf-life of food.

Approaches to antimicrobial packaging can be classified as either of two types. The first one consists of binding an agent to the surface of the package and this would require a molecular structure large enough to retain activity on the microbial cell wall even though bound to the plastic. Such agents are likely to be limited to enzymes or other antimicrobial proteins.

The second approach involves the release of active agents onto the surface of the food.

Antimicrobial agents are divided into two groups:

- synthetic;
- natural.

Synthetic antimicrobial agents (Table 2) including various organic acids and salts and have been used for the preservation of food products since they have been approved by regulatory agencies. Synthetic antimicrobial agents that have demonstrated inhibitory activity against different microorganisms include sodium benzoates and propionates, potassium sorbates, sulfites, chlorides, nitrites, triclosan, fungicides (e.g. benomyl, imazalil) and various metal ions including silver zeolites, quaternary ammonium salts and cooper ions. Other antimicrobial agents such as acetic acid from vinegar and benzoic acid from cranberries are found in nature, but are classified as synthetic antimicrobial agents when produced synthetically.

In Japan, silver-substituted zeolite has been developed as the most common antimicrobial agent incorporated into plastic. The zeolite, which has some of its surface atoms replaced by silver, is laminated as a thin layer (3-6 μm) in the surface of the food contact polymer and appears to release silver ions as aqueous solution from the food enters the exposed cavities of the porous structure.

Silver ions, which inhibit a wide range of metabolic enzymes, have strong antimicrobial activity with a broad spectrum; studies reported in the literature have shown that silver ions are capable of inactivating vital enzymes and preventing DNA replication within bacterial cells.

Silver ions have been included in several industry applications such as synthetic fabrics and medical devices as an antimicrobial agent; the antibacterial activity of silver-zeolite has been tested for use in antimicrobial packaging materials such as plastic, stainless steel and fabric materials.

As regard the application in the food industry, little is reported about the antimicrobial activity of silver-zeolite packaging applied to real food items. The positive aspect of the antimicrobial activity of the silver-zeolite in a real food could be impeded by high protein and salt concentration. This is so because silver ions will bind to amino acids, chlorides, phosphate and sulphide ions and thus lose their antimicrobial efficacy (Lee *et al.*, 2011).

On 9 June 2000 the AgION™ Silver Ion Technology received the approval of the Food and Drug Administration for use in all types of food-contact polymers in USA market (Quintavalla *et al.*,2002). In 2011 the Ministère de l'Économie de l'Industrie et de l'Emploi, France asked on behalf of AgION Technologies Ing, to the European Commission a risk assessment of the substance silver zeolite A (silver zinc sodium ammonium alumino silicate), silver content 2-5 % for use to control microorganism growth on the article in polyolefin, poly(ethylene terephthalate) (PET) and polycarbonate (PC) made with up to 3% w/w of silver zeolite A containing around 2.5% silver. Finished articles are intended to be used for single contact with all types of foodstuffs at room temperature for a long period (EFSA Journal 2011;9(2):1999). The EFSA Panel on food contact materials, enzymes, flavorings and processing aids concluded that there was no safety concern for the consumer if migration of silver ion does not exceed the group specific migration limit of 0.05 mg Ag/kg food.

Another example is Triclosan, the antimicrobial substance used by Microban (UK). For many years this chemical compound has been used effectively in personal hygiene products such as toothpaste, deodorant, soap and mouthwash, as well as an antibacterial agent in the hospital environment. This protection is achieved by combining Triclosan with any of the major polymers, e.g. polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC). The Triclosan fits into the empty spaces of the polymer and migrates to the surface to start its work against any developing bacterium. During

washing, the molecules closest to the surface are cleaned away but are immediately replaced by other protective molecules (Coma, 2008; Kerry *et al.* 2006) reported that a 1% triclosan film could have a strong antimicrobial effect on *Listeria monocytogenes* in *in vitro* assays whereas the film did not effectively reduce spoilage bacteria and *Listeria monocytogenes* growth on refrigerated vacuum packaged chicken breasts stored at 7°C.

Table 2. Antimicrobial activity of common synthetic antimicrobial agents.

Antimicrobial agent	**Packaging material[a]**	**Substrate**	**Reference**
Imazalil	LDPE	Culture media	Vartiainen et al. (2003)
Potassium sorbate	Starch polymer	Culture media	Barzegar et al. (2014)
Silver-zeolite	Paper	Beef, pork, turkey	Lee et al. (2011)
Triclosan	PVC	Culture media	Ji et al. (2009)

[a]LDPE, low-density polyethylene; PVC, polyvinyl chloride.

In recent years, natural antimicrobial agents (Table 3) have attracted much attention in the food and packaging industries as a replacement for synthetic ones for food preservation. Natural antimicrobial agents are classified by their sources: antimicrobial agents derived from microbial sources (nisin, natamycin) and naturally polymers (chitosan), animal sources (for example, lysozyme, lactoferrin) and plant essential oils (for example thyme, cinnamon, oregano, clove and rosemary) (Kuorwel *et al.*, 2011).

Bacteriocin are peptides produced by lactic acid bacteria (Coma, 2008), with antibacterial activity against many Gram-positive bacteria (Marcos *et al.*, 2007). These agents are generally heat-stable, apparently hypoallergenic and readily degraded by proteolitic enzymes in the human intestinal tract. Class IIa bacteriocins, described as being active against *Listeria* spp., are one of the most interesting groups of antimicrobial peptides used for food preservation. Among class IIa bacteriocins, enterocins have proved to be controlling *Listeria monocytogenes* growth in meat product (Marcos *et al.*, 2007). Moreover, it is particularly effective against heat-resistant bacterial spores of *Clostridium botulinum* and against food-borne pathogens such as *Listeria monocytogenes, Staphylococcus aureus or Bacillus cereus* (Lucera *et al.*, 2012).

The enzymes represent another group of natural compounds that find application in food as valid preservatives. Lysozyme for example, has been used primarily to prevent late blowing in semi-hard cheeses, caused by *Clostridium tyrobutyricum.* It is well know that lysozyme is bactericidal

against Gram-negative bacteria, whereas it is essentially ineffective against Gram-negative bacteria, owing to the presence of a lipopolysaccharide layer in the outer membrane (Lucera *et al.,* 2012).

Table 3. Antimicrobial activity of natural antimicrobial agents.

Antimicrobial agent	**Packaging material**[a]	**Substrate**	**Reference**
Glucose oxidase	WPI	Culture media	Murillo-Martinez et al. (2013)
Lysozyme	Cellulose based	Meat slices	Barbiroli et al. (2012)
Lysozyme	Corn starch film, pea protein	Culture media	Fabra et al. (2014)
Lysozyme	PET	Culture media	Corradini et al. (2013)
Nisin	Cellulose film	Wurstel	Nguyen et al. (2008)
Nisin	Gelatin or corn zein film	Culture media	Ku et al. (2007)
Pediocin	PLA	Raw sliced pork	Woraprayote et al. (2013)
Enterocin	Alginate, zein and polyvinyl alcohol	Cooked ham	Marcos et al. (2007)
Oregano, Clove	WPI	Culture media	Fernández-Pan et al. (2012)
Cinnamaldehyde, Eugenol	Cellulose-based	Culture media	Sanla-Ead et al. (2011)
Carvacrol	Tomato-based	Culture media	Du W.X. et al. (2008)
Green tea	Chitosan	Pork sausage	Siripatrawan et al. (2012)

[a]WPI, whey protein isolate; PET, polyethylene terephalate; PLA, polylactic acid.

The essential oils extracted from plant sources consist of various mixtures, 20-60 components at quite different concentrations, with some compounds at fairly high concentrations (20-70%), and others in trace amounts (Table 4). The components at high concentrations (terpenes, terpenoids, molecules with an aromatic ring) play a major role in the antimicrobial/biological effect of essential oils (Bakkali *et al.,* 2008).

Some important compounds of essential oils are mono and sesquiterpenes, carbohydrates, phenols, alcohols, ethers, aldehydes and ketones. Phenolic compounds have been recognized as bioactive components (Perricone *et al.*, 2015). These plant essential oils are volatile and generally possess relatively strong odors.

Essential oils are considered to be safe and have "Generally Recognized As Safe" (GRAS) status as designated by the American Food Drug Administration (Rocha *et al.,* 2013; Lucera *et al.,* 2012; Kuorwel *et al.,* 2011).

Antimicrobial agents derived from plant sources are produced as secondary metabolites and are associated generally with the volatile essential oil fraction (Kuorwel *et al.*, 2011).

The antimicrobial activity of plant essential oils is related to their chemical structure, namely, the presence of hydrophilic functional groups such as the hydroxyl groups of phenolic components and/or lipophilicity of the components in the essential oils which depends on their concentration. Essential oils and their principal constituents inhibit microorganisms via a range of mechanisms such as disruption of the cytoplasmic membrane; leakage of intracellular constituents such as metabolites and ions; coagulation of cell content; inhibition of protein synthesis, enzymes associated with cell wall synthesis, DNA/RNA synthesis, general metabolite/pathways; and/or destruction of the osmotic integrity of the cell membrane (Figure 1).

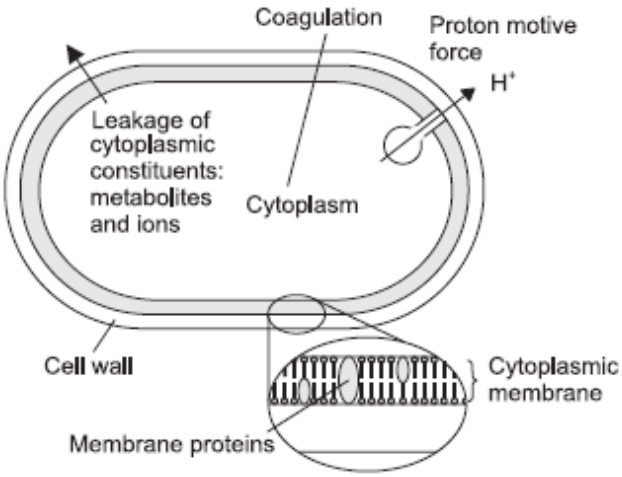

Fig. 1. Mode of antimicrobial action (Burt, 2004).

Extracted derived from various herbs and essential oils contain a range of natural compounds such as thymol, linalool and carvacrol which have a broad antimicrobial spectrum against different pathogenic and spoilage microorganisms including Gram negative species such as *Escherichia coli*,

Yersinia enterocolitica, Pseudomonas aeruginosa and *Salmonella choleraesuis*; Gram positive bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus*; yeasts such as *Saccaromices cerevisiae, Candida albicans*; molds such as *Aspergillus niger*.
The antimicrobial activity of different essential oils is very difficult to compare given the variation of essential oil composition among the plant species, differences in the geographic origin of the plants, harvesting season, extraction methods, and the part of plant that is used (Kuorwel *et al.*, 2011; Faleiro, 2011; Burt, 2004).
There are a number of test methods used to determine the antimicrobial activity of various essential oils and their principal constituents. These include diffusion methods (agar diffusion), dilution methods and micro atmosphere methods.

Table 4. Antimicrobial and aroma characteristics of essential oils (Perricone *et al.*, 2015, modified).

Essential oils	**Major constituents**	**Antimicrobial effect against**
Garlic root (Allium sativum)	Methyl disulfide, allyl sulfide, allyl disulfide, allyl trisufide, allyl tetrasulfide	B. cereur; E. coli; Shigella spp.; V. parahaemolyticus; Y. eneterocolitica; Salmonella enterica serovars Enteritis, Infantis, typhimurium; Bacillus subtilis; E. feacalis; A. alternata.
Cinnamon leaf (Cinnamon zeylanicum)	Cinnamaldehyde, copaene, ß-caryophyllene	E. coli; P. aeruginosa; E. feacalis; S. aureus; S. epidermidis; methicillin-resistant S. aureus; K. pneumonia; Salmonella spp.; V. parahaemolyticus.
Thyme (Thymus vulgaris)	Thymol, *p*-cymene, ɤ-terpenine, linalool	B. cereus; C. botulinum; E. feacalis; E. coli; S.aureus; L. monocytogenes; A. flavus; A. niger; K. pneumoniae; P. aeuroginosa; Salmonella spp.

Oregano (Origanum vulgare)	Sabinyl monoterpenes, terpinen-4-ol, ɤ-terpenine, carvacrol, thymol	B. cereus; B. subtilis; C. botulinum; E. faecalis; E.coli; S. aureus; A. niger; L. monocytogenes; K. pneumonia; P. aeruginosa; Salmonella spp.
Clove (Syzygium aromaticum)	Eugenol, eugenly acetate, caryophllene	B. brevis; B. subtilis; C. botulinum; E. faecalis; Candida spp., A. flavus; A. niger; E.coli; K. pneumonia; P. aeruginosa; S. aureus; Salmonella spp.; L. monocytogenes.
Basil (Ocimum basilicum)	Linalool, methylchalvicol, eugenol, methyl eugenol, methyl cinnamate, 1,8-cineole, caryophyllene	B. brevis, E. coli, A. flavus, A. niger, E. faecalis, E. coli, K. pneumonia, P. aeruginosa, S. aureus.
Coriander (Coriandrum sativum)	2(E)-decanal, 2(E) dodecenal, linalool	E. coli; L. monocytogenes; L. plantarum; S. aureus.
Citrus (Citrus spp.)	Liminene, linalool, citral	A. niger; A. flavus; P. verrucosum; P. chrysogenum.
Laurel (Laurus nobilis)	1,8-cineole, α-terpinyl acetate, linalool, methyl eugenol	S. aureus; B. cereus; E. faecalis
Ginger (Zingiber officinale)	ß-sesquiphellandrene, zingiberene	A. flavus; A. niger; E. faecalis; E. coli; K. pneumonia; P. aeruginosa; S. aureus.
Rosemary (Rosmarinus officinalis)	Borneol, verbenone, camphor, a-pinene, 1,8-cineole	A. flavus; A. niger. E. faecalis; E.coli; K. pneumonia; P. aeruginosa; S. aureus; L. monocytogenes; L. plantarum; Salmonella spp.; B. cereus.
Peppermint (Mentha piperita)	Menthol, menthone, menthyl acetate, menthofurane	B. brevis; S. aureus; V. cholera; E. faecalis; E. coli; K. pneumonia; P. aeruginosa; A. flavus.

Carvacrol is one of the major components of oregano and thyme oils. They have received substantial attention as useful natural antimicrobial agents due to their natural origin and GRAS status, and because they exhibit a broad antimicrobial spectrum against different microorganisms and possess heat stability when incorporated into packaging materials (Kuorwel *et al.,* 2011).

The inhibitory effect of phenols could be explained by interactions with the cell membrane of microorganisms and in often correlated with the hydrophobicity of the compounds. For instance, oregano essential oil was reported to induce permeability alteration in the microorganisms' membranes with a consequent leakage of protons, phosphates and potassium (Ben Arfa *et al.,* 2006).

Cinnamaldehyde is one the major components of cinnamon oils. Although cinnamaldehyde is known to be inhibitive to growth of *E. coli* O157:H7 and *Salmonella* Typhimurium at similar concentration to carvacrol and thymol, it did not disintegrate the outer membrane or deplete the intracellular ATP pool (Burt, 2004).

Eugenol is a major component of clove oil. Sub-lethal concentrations of eugenol have been found to inhibit production of amylase and proteases by *Bacillus cereus*. Cell wall deterioration and a high degree of cell lysis were also noted (Burt, 2004).

Antimicrobial agents have different activities on different pathogenic microorganisms due to their various diverse physiologies. Several *in vitro* studies have demonstrated the antimicrobial properties of natural organic and inorganic compounds against pathogenic microorganisms (Lloret *et al.,* 2012, Gutierrez *et al.,* 2010; Fernandez *et al.,* 2010, Rojas-Grau *et al.,* 2007; Seydim *et al.,* 2006), but more data about their action and effectiveness on real food matrices are necessary (Kuorwel *et al.,* 2011).

2. FOOD PACKAGING MATERIALS

The quality of food products depends on organoleptic, nutritional, and hygienic characteristics, but these evolve during storage and commercialization. Interactions between food and its surrounding media could lead to different phenomena that affect food quality. In particular, mass transfer of various molecules could take place between food and micro/macro environment or among food components (such as for composite products), and cause physicochemical changes and food deterioration. Different phenomena could imply quality loss and alteration, such as chemical reactions (Maillard reaction, oxidation) catalyzed or not (enzyme, microorganisms) and/or physicochemical reactions (e.g. phase transition, loss of crispness, caking of powders,…). Packaging acts as a barrier between food product and surrounding medium preventing from:

- Mechanical shocks and deformation;
- Light;
- Organic compounds vapours, especially aroma compounds;
- Gases, such as oxygen and carbon dioxide, which control vegetable products and microorganisms metabolism as well oxidation reactions;
- Solutes, such as salts, pigments, additives and lipids;
- Water vapour, which causes product desiccation or hydration and the consequent increase of reaction rate.

Nowadays food preservation, quality maintenance, and safety are major growing concerns of the food industry. It is evident an increasing consumers' demand for natural and safe food products with stringent regulation to prevent food-borne infectious diseases. Antimicrobial packaging which is thought to be a subset of active packaging and controlled release packaging is one such promising technology which effectively impregnates the antimicrobial into the food packaging material and subsequently delivers it over the stipulated period of time to kill the pathogenic microorganisms affecting food products thereby increasing the shelf life to severe fold (Malhotra *et al.,* 2015).

Because a packaged food product is a coordinated system including food matrix, packaging film and possibly the head-space, the choice of material with which to make the packaging is fundamental and critical for the achievement of the purposes for which the packaging was designed.

Antimicrobial packaging materials are divided into two major groups called:

- biodegradable packaging;
- non-biodegradable packaging.

Most synthetic polymers are non-biodegradable. These packaging materials with the advantages of low cost, low density, excellent barrier properties,

inert, good mechanical strength, high transparency, ability to be heat-sealed and easy to be printed on are the superior candidates for usage in the food industry. The most widely used plastics in packaging included low density polyethylene (LDPE), linear low density polyethylene (LLDPE), high density polyethylene (HDPE), polypropylene (PP), ethylene vinyl acetate (EVA), polystyrene (PS), polyethylene terephthalate (PET) and polyvinyl chloride (PVC) (Sung *et al.*, 2013).

Petroleum derivate plastics represent a serious environmental problem (Atarés *et al.*, 2016). It causes landfill depletion, environmental pollution and high energy consumption of manufacturing process. Human health can be endangered by the diffusion of additives from polymers into food products. Therefore, the usage of biodegradable materials in recent years has been increasing (Sung *et al.*, 2013). Biodegradable films and coatings represent an interesting alternative to conventional plastic materials, which is why several biopolymers have been exploited to develop material for eco-friendly food packaging (Atarés *et al.*, 2016).

Biodegradable antimicrobial films were produced by natural polymer that possesses inherently antimicrobial reactivity or through addition of antimicrobial agents into natural polymer. Examples of renewable biopolymers are polysaccharides, proteins, gums, lipids and their complexes, derived from animal and plant origin.

Most of the biodegradable-based packaging researches are focused on the blending of thermoplastic starches (TPS) with biodegradable polyesters such as polycaprolactone (PCL), polylactic acid (PLA), polyhydroxybutyrate-co-hydroxyvalerate, polybutylene succinate-adipate, poly(butylenes adipate-co-terephtalate), and poly(hydroxyl ester ether). The PLA is the most widely used polymer among the biodegradable ingredients and it is also biocompatible thermoplastic produced by fermentation from renewable resources.

It can also be synthesized either by condensation polymerization of lactic acid or by ring opening polymerization of lactide in the presence of a catalyst. PLA-based films could perform better than other antimicrobial films because of several advantages, such as competitive price, regulation matter and eco-friendliness.

Apart from the biodegradable materials previously mentioned, edible films made from edible biopolymer (e.g. proteins, lipids, polysaccharides) and food-grade additives (e.g.: plasticizers, antimicrobial agents, colorant, flavors, emulsifiers) are also the good candidates for fabrication of antimicrobial films (Table 5).

Table 5. Components of natural biopolymer-based films (Jong *et al.*, 2007).

Film-forming materials
- Polysaccharides: starch, cellulose derivatives, pectin, alginate, carrageenan, chitosan, pullulan and natural gums
- Protein: casein, whey protein, collagen, gelatin, keratin, fish myofibrillar protein, soy protein, wheat gluten and corn zein
- Lipids: neutral lipids, fatty acids and wax
Additives
- Plasticizers: glycerol, propylene glycol, polyethylene glycol, sorbitol and water
- Functional ingredient: antimicrobial, antioxidant, flavor, colorants, vitamins and other nutraceuticals

Edible films have been defined in different ways. According to Hernandez-Izquierdo *et al.* (2008) an edible film can be defined as a thin layer of edible material, which can be performed in a food as a coating or performed as a film that can be placed between food components, used as a food wrap, or formed into a pouch to contain foods. The advantage of natural biopolymer films over traditional plastic materials are summarized in Table 6. The principal interest in introducing edible packaging is related to the possibility to use renewable sources of ingredients and decrease waste through biological recycling to the system (Balaguer *et al.*, 2011).

Table 6. Benefits of and possible uses for natural biopoymer-based packaging materials (Jong, *et al.*, 2007).

- Edible
- Biodegradable
- Supplement the nutritional value of food
- Enhanced organoleptic characteristic of food, such as appearance odor and flavor
- Reduced packaging volume, weight and waste
- Incorporated antimicrobial agents and antioxidants
- Extended shelf-life and improved quality of usually non packaged items
- Control over intercomponent migration of moisture, gases, lipids and solutes
- Individual packaging of small particulate foods, such as nuts and raisins
- Function as carrier for antimicrobial and antioxidant agents
- Microencapsulation and controlled release of active ingredients

- Possible use in multilayer food packaging materials together with non-edible films
- Low cost and abundant
- Annually renewable resources

Proteins cover a broad range of polymeric compounds that provide structure or biological activity in plants or animals. Protein film-forming materials derives from animal sources including collagen, gelatin, fish myofibrillar protein, keratin, egg white protein, casein and whey protein. Protein film-forming materials obtained from plant sources include wheat gluten, corn zein, soy protein, peanut protein, and cottonseed protein. Protein-based films possess moderate mechanical properties in comparison to conventional synthetic films, and good oxygen barrier properties at low or intermediate relative humidity exceeding those polyolefins (Balaguer, *et al.*, 2011). Zein, the major storage protein of corn, has been extensively used to produce biodegradable films. The success of zein from its discovery to nowadays is due to its ability to form tough, glossy, hydrophobic, greaseproof films that are resistant to microbial attack, with excellent flexibility and compressibility. An important factor that could promote the diffusion of zein as industrial polymeric material is the incorporation of antimicrobial compounds into the matrix (Del Nobile *et al*,. 2008).

Wheat gluten is a major functional food ingredient, which is inexpensive, biodegradable and whit good film-forming properties (Marcuzzo *et al.*, 2010); it presents important viscoelasticity, thermoplasticity and other properties which endow this biomaterial with a noteworthy potential for a wide range of technological application, particularly as a food packaging material (Balaguer *et al.*, 2011).

Protein films are formed through the partial denaturation of polypeptide chains by the addition of a solvent, alteration of pH, addition of an electrolyte to cause cross-linking and/or application of heat. Films are subsequently formed when the partially denatured peptide chains bond together primarily through hydrophilic and hydrogen bonds resulting in the formation of a protein matrix (Jong *et al.*, 2007).

Polysaccharide films have been receiving increasing interest because of low cost and availability of these film forming materials.

They include starch and starch derivatives, cellulose derivatives, alginate, carrageenan, chitosan, pectinate and various gums. Since their hydrophilic nature, only minimal moisture-barrier properties can be expected. Cellulose derivatives are interesting film forming compounds, as they are odorless, tasteless and biodegradable. Another biopolymer with excellent film forming ability is chitosan. This is non-toxic compound, obtained by the deacetylation of chitin, a structural components present in the shell of some crustaceans, and presents antimicrobial properties (Sánchez-González *et al.*, 2011).

However, certain polysaccharides when used in the form of high-moisture gelatinous coatings will retard moisture loss from some foods (e.g. meat products) during short-time storage. Moreover, polysaccharide based films can prevent from gases and lipid transfer. Because of these characteristics, polysaccharide based film could be applied to different food products, such as vegetable.

Lipid materials and waxes can be applied to food products by direct coating, dipping or pan coating. Lipid materials such as beeswax, candelilla wax, carnauba wax, triglycerides, acetylated monoglycerides, fatty acids, fatty alcohols and sucrose fatty acid esters, as well as resins such as shellac and terpene resin are used as edible film-forming materials. The primary advantage of lipid edible film is their high barrier characteristic due to their relatively low polarity. However, they have limited oxygen barrier properties and the use of fats as a protective covering for foods can have disadvantages, such as the onset of rancidity in the fats as well as the greasy surface coating that the fats may impart. Because these lipid and resin materials are not polymers, they do not generally form cohesive stand-alone films. However, along with often providing desirable gloss, they can be used to coat a food or drug surface to provide a moisture barrier or to provide a moisture-barrier component of a composite film (Jong, *et al.*, 2007).

According to Han (2000) and Quintavalla *et al.* (2002), several factors must be taken into the design or modelling of the antimicrobial film or package:

- *Chemical nature of films/coatings, casting process conditions and residual antimicrobial activity.* The choice of the antimicrobial is often limited by the heat lability of the component during extrusion or by the incompatibility of the component with the packaging material.
- *Characteristics of antimicrobial substances and foods.* Food components significantly affect the effectiveness of the antimicrobial substances and their release. Physicochemical characteristics of food could affect the activity of antimicrobial substances. For example, the pH of food influences the ionization (dissociation/association) of most active chemicals, and could change the antimicrobial activity of organic acids and their salts. The antimicrobial activity and chemical stability of incorporated active substances could be influenced also by the water activity of food. Moreover, each food has its own characteristic microflora. The release kinetics of antimicrobial agents has to be designed to maintain the concentration above the critical inhibitory concentration with respect to the contaminating microorganisms that are likely to be present.
- *Storage temperature.* Storage temperature can affect the antimicrobial activity of chemical preservatives. Generally, increased storage temperature can accelerate the migration of the active agents in the film/coating layers, while refrigeration slows down the migration rate.

The temperature conditions during production and distribution have to be predicted to determine their effect on the residual antimicrobial activity of the active compounds.

- *Mass transfer coefficients.* The simplest system is the diffusional release of active substances from the package into the food. A multilayer design has the advantage that the antimicrobial can be added in one thin-layer and its migration and release controlled by the thickness of the film layer or coating. In practice, a matrix of several layers is used to control the rate of release of the active substance. Control of the release rates and migration amounts of antimicrobial substances from food packaging is very important. Han (2000) summarized traditional mass transfer models and his own proposed models that may be used to describe the migration of active agents through food packaging systems consisting of single, double, or triple layers.
- *Physical properties of packaging materials.* When antimicrobial activity is added to packaging materials to reduce microbial growth, it may affect the general physical properties of the packaging materials. Han and Floros (1997) found that the transparency of the plastic film under study decreased with the addition of the active agent. The performance of the packaging materials must be maintained after the addition of the active substances, even though the materials contain ore heterogeneous formulation.

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PART II

EXPERIMENTAL WORK

Publication n. 1: FOOD SAFETY CONCERNS DERIVING FROM THE USE OF SILVER BASED FOOD PACKAGING MATERIALS

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Abstract

The formulation of innovative packaging solutions, exerting a functional antimicrobial role in slowing down food spoilage, is expected to have a significant impact on the food industry, allowing both the maintenance of food safety criteria for longer periods and the reduction of food waste. Different materials are considered able to exert the required antimicrobial activity, among which are materials containing silver. Silver has been used for centuries as an antimicrobial to fight infections and prevent spoilage, and it is well known that silver ions and silver-based compounds are highly toxic to Gram-negative and Gram-positive microorganisms. However, challenges exist in the application of silver to food contact materials due to knowledge gaps in the production of ingredients, stability of delivery systems in food matrices and health risks caused by the same properties which also offer the benefits. Aims of the present study were to test the effectiveness and suitability of two packaging systems, one of which contained silver, for packaging and storing *Stracchino* cheese, a typical Italian fresh cheese, and to investigate if there was any potential for consumers to be exposed to silver, *via* migration from the packaging to the cheese. Results did not show any significant difference in the effectiveness of the packaging systems on packaged *Stracchino* cheese, excluding that the active packaging systems exerted an inhibitory effect on the growth of spoilage microorganisms. Moreover, silver migrated into the cheese throughout the storage time (24 days). Silver levels in cheese finally exceeded the maximum established level for migration of a non-authorised substance through a functional barrier (Commission of the European Communities, 2009). This result poses safety concerns and strongly suggests the need for more research aimed at better characterizing the new packaging materials in terms of their potential impacts on human health and the environment.

KEYWORDS: food safety, food packaging, antimicrobial, silver migration.

1 INTRODUCTION

Food packaging technology is continuously evolving in response to the growing consumer demand for minimally processed, more natural, fresh and longer storable food.
Moreover, every year an increasing amount of edible food is lost along the entire food supply chain. The European Commission estimated that the annual food waste amounts to 89 million tons, or 179 kg per capita, varying considerably between individual countries and the various sectors, without even considering agricultural food waste or fish catches returned to the sea; furthermore, total food waste is expected to rise to approximately 126 million tons (a 40 % increase) by 2020, unless additional preventive actions or measures are taken (European Parliament, 2012; European Parliament – STOA, 2013). Thus, packaging optimisation strategies have been proposed to guarantee the maintenance of food safety criteria and reduce food waste.
This scenario has strongly inspired the packaging industry to go beyond the traditional functions of the package and to offer innovative solutions addressing the changing demands of the food industry and consumers as well as the increasing regulatory and legal requirements (Realini and Marcos, 2014). Therefore, the function of food packaging has evolved from being a simple physic barriers, aimed at avoiding food contact with the external environment (passive packaging), to exerting a functional role in slowing down food spoilage (active packaging) by means of specific action on the chemical, enzymatic and mechanical phenomena, thus extending the shelf life of food (Marsh and Bugusu, 2007).
Different active packages are considered able to exert antimicrobial activity, especially materials which contain silver (Quintavalla and Vicini, 2002; Coma, 2008). However, even though silver's antibacterial properties have long been proved (Silver *et al.,* 2006), the lack of standardization in terms of particles characterization and testing conditions makes it difficult to define its range of effectiveness and specificity against different bacterial species (Bae *et al.,* 2010).
Moreover, there is an expanding body of scientific studies demonstrating that silver, especially in its nanosize, could introduce new risks to human health (M. Ahamed *et al*., 2010; Gaillet and Rouanet, 2015). The use of silver in food contact materials could potentially increase the probability of consumers' exposure: silver could migrate from packaging into foods, even though preliminary results indicate that migration is expected to be minimal (Chaudry *et al.,* 2008); although these studies seem to give some reassurances about safety, the few migration studies published to date have been targeted to food simulants, so further investigation needs to be performed especially in the case of complex food matrices.

In the European Union, the main regulatory framework related to the use of food contact materials is still the Regulation (EC) No 1935/2004; it states that "*materials and articles, including active and intelligent materials and articles do not have to transfer their constituents to food in quantities which could endanger human health or bring about an unacceptable change in the composition of the food or bring about a deterioration in the organoleptic characteristics thereof*." However, the scientific literature has a complete lack of any data quantifying rates of migration of food package components into food.

The European Food Safety Authority (EFSA) stipulated, with reference to article 10 of Regulation (EC) No. 1935/2004, an opinion of the panel on food contact materials, enzymes, flavourings and processing aids (CEF) of the risks originating from the migration of substances from food contact materials into food is required [European Food Safety Authority (EFSA) 2009]. According to Commission of the European Communities (2009), Article 14, a maximum level of migration of 0.001 mg/kg should be observed for the migration of a non-authorized substance through a functional barrier. For some novel substances intended for the inclusion in food packaging materials, such as silver, adequate toxicological data is not yet available and so safety assessments are still in progress. Thus, silver must undergo an appropriate authorisation process and safety evaluation before it is introduced.

Aim of the present study were to test the effectiveness of two packaging systems, one of which contained silver, for the packaging and storage at 4°C of *Stracchino* cheese, a typical Italian fresh cheese, by assessing microbial, chemical and sensorial parameters. Moreover, the migration of silver from the packaging into the cheese was monitored during the chill storage period.

2 MATERIALS AND METHODS

2.1 Materials

Two food packaging systems were studied in the present research: the active packaging *Food-touch*® by Microbeguard Corp. (USA), containing silver zeolite for the purpose to exerting antimicrobial properties, and an innovative packaging *Ovtene*® by Arcadia Spa (Italy), containing calcium carbonate, talc and titanium dioxide, for the intent to extend food shelf life and supplied by a local producer. Both of these products were used in their original liner formats, as provided by suppliers. As control, a traditional passive packaging was also used.

Stracchino cheese, a commercially-available typical Italian fresh cheese with a declared shelf life of 20 days, preferentially consumed by children and the elderly, was selected as a perishable and high value food suitable for use in these packaging systems.

Stracchino pieces (250g) were hand-wrapped according to manufacturers' instructions in the selected packaging systems and then analyzed for a range of microbial, chemical and sensorial parameters. Packaged *Stracchino* cheese was stored at 4°C for 25 days.

2.2 Microbial, water activity and pH analyses

Microbial analyses were conducted to monitor the numbers of spoilage and indicator microorganisms in the cheese during storage in the three selected packaging systems as detailed in Table 1.

The following microbial determinations were performed:

Total Viable Count at 30°C (ISO 4833:2003; Plate Count Agar at 30±1°C for 72±3 hours in aerobic conditions), *Pseudomonas spp.* (Cetrimide-Fusidic acid-Cephalosporin Agar at 25±1°C for 44±4 hours), *Enterobacteriaceae* (ISO 21528-2:2004; Violet Red Bile Glucose Agar at 37±1°C for 24+2 hours), Lactic Acid Bacteria at 30°C (MRS Agar at 30±1°C for 72±3 hours in aerobic conditions), Moulds and Yeasts (Rose Bengal Chloramphenicol Agar at 25±1°C for 3-5 days). Additionally, water activity was measured (a_w, according to ISO 21807: 2004), as was pH (ISO 2917:1999).

Table 1: Sampling design for microbiological, chemical and sensorial determination.

		Number of samples		
Experimental time (days)	**Storage temperature (°C)**	**Traditional packaging**	**Innovative packaging (*Ovtene*®)**	**Active packaging (*Food-touch*®)**
0	0/+4	20		
8	0/+4	20	20	20
15	0/+4	20	20	20
21	0/+4	20	20	20
25	0/+4	20	20	20
Total number of samples		100	80	80

2.3 Chemical analyses

Chemical analyses were conducted on days 0, 7, 14, 20 and 24 of storage. In total, 20 replicate cheese samples were examined each day. The following chemical parameters were assessed:

- *Total Volatile Basic Nitrogen* (TVB-N), determined by the method prescribed in Regulation EC 2074/2005 for the evaluation of the TVN-B in fish;
- *Sulphides*, determined by Lead Acetate Test Strips (Sigma-Aldrich, Switzerland);
- *Peroxides* via iodometric titration (Biffoli, 1979);
- *Stamm test* (Hamm et al. 1965);
- *Thiobarbituric acid test* (TBA) (Fernandez et al. 1997).

2.4 Sensory evaluation

In order to detect any sensory difference during the storage period, sensory evaluation was conducted on *Stracchino* cheese packaged using only the *Ovtene*® system and the traditional packaging as control. Sensory evaluation on *Stracchino* packaged in the *Food-touch*® system was not conducted, due to the claimed presence of silver and the possibility of its migration into the cheese matrix. Two sensory evaluations were performed after 0 and 7 days of storage using fresh *Stracchino* as a standard reference. The sensory evaluation was performed as described by ISO 13299:2003. A panel of 15 judges, experts in sensory evaluation of cheese, was previously trained on the quantitative evaluation of the following selected descriptors: i) *Odor intensity*; ii) *Aroma intensity*; iii) *Saltiness*; iv) *Acidity*; v) *Bitterness*; vi) *Homogeneity*; vii) *Consistency*; viii) *Adherence*.

2.5 Silver migration test

Silver migration from the *Food-touch*® system into the cheese was determined on days 0, 7, 14, 20 and 24 of storage. In total, two independent determinations of 20 cheese samples were examined each day.

2.5.1 Atomic Absorption Instrumentation

Silver concentrations were determined using Electrothermal Atomic Absorption Spectrometry (ETAAS) on an M6 mkII Atomic Absorption Spectrometer (Thermo Electron, Cambridge, UK) with D_2 background correction, equipped with a GF95 Graphite Furnace atomiser.

2.5.2 Analytical methods

Cheese samples (2 g) were homogenised, then 8 ml concentrated HNO_3 and 2 ml H_2O_2 were added and the mixture digested in Teflon liners using a CEM (Mattews, NC, USA) Mars Xpress microwave oven. Digested cheese samples were then diluted to up to 25 ml in class A volumetric flasks with deionised water and analyzed. All calibration solutions for metal determination were made by dilution from Certified Standard Solutions (ULTRAgrade® ICP Standards, 1000 mg/mL) provided by Ultra Scientific (North Kingstown, RI, USA).

Trueness of analytical data was verified by means of Certified Reference Materials (NRCC DORM2) analysed concurrently with samples in each analytical batch. LOQ (6s) value were found to be 0.0015 mg/kg. Operating conditions are reported in Table 1S.

2.6 Statistical analysis

The microbial counts were logarithm transformed, and data distributions over time were represented by box and whiskers plots. To evaluate the effect of type of packaging, time and the interaction between the two independent variables on the dynamics of the microbial parameters, analysis of variance (ANOVA) was applied. The observation taken at point 0 (day 1), presenting the same values distribution for each type of packaging and the outliers, identified using Grubbs test, were not considered in the analysis (Dohoo et al., 2003; Agresti, 1990, Grubbs, 1969).

The assumption of homoscedasticity was verified using the Breusch-Pagan & Cook-Weisberg test and the residual plot (Breusch and Pagan, 1979; Cook, and Weisberg,1983).

The normality condition of residuals was verified using the Shapiro-Francia test (Shapiro and Shapiro, 1973), the graphical analysis of the residuals plotted against the normal probability distribution (q-q plot), and the histogram of residuals versus normal curve (Dohoo et al., 2003).

A post estimation analysis was performed to evaluate the significant paired contrasts taking into account the multi test correction in the evaluation of statistical significance (Dohoo *et al.*, 2003).
The software STATA 12.0 (Release 12) was used to conduct the statistical analysis of microbial data.
Analysis of variance was also performed to analyse the sensory data by SYSTAT software, in order to assess any significant sensory difference between cheese treated with the active or the traditional packaging systems, after checking the reliability and homogeneity of the obtained data.
P-value < 0.05 was considered significant in the statistical analysis.

3 RESULTS

3.1 Microbial analyses

The population dynamics of spoilage-related microorganisms (Total Viable Counts, lactic acid bacteria, moulds, yeasts, and *Pseudomonas* spp.) and *Enterobacteriaceae* in the *Stracchino* cheese are described in Figure 1.

The assumptions of homoscedasticity of data and the normality of residuals were satisfied for each analysed microbial group (data not shown).

ANOVA analysis showed that populations of each of the investigated groups of microorganisms were affected both by storage time (as expected) and by type of packaging ($p < 0.001$).

In particular, Total Viable Counts increased initially (between 0 and 15 days of storage), maintained a constant value during days 15 to 21, and then increased further during days 21 to 25 of storage (Figure 1, Panel A). In detail, in traditionally-packaged cheese, the Total Viable Counts were significantly higher than in *Ovtene*®-packaged cheese on days 8 and 25. Total Viable Counts in cheese packaged in the *Food-Touch*® system were slightly, but significantly, lower on each sampling day than in traditionally-packaged cheese. Total Viable Counts in cheese packaged in the *Food-Touch*® system were slightly but significantly lower than in cheese packaged in the *Ovtene*® system on each given day, except for days 8 and 25, when the cheese in the two packaging systems contained similar numbers of Total Viable Count bacteria.

Lactic acid bacteria showed a similar growth trend in cheese in the examined packaging systems (Figure 1, Panel B). Lactic acid bacteria numbers in cheese remained constant until day 8, and increased from day 15 until the end of storage. This trend was more evident in the case of the traditional packaging. At the end of storage, lactic acid bacteria numbers in cheese in *Ovtene*® packaging were lower than in traditionally-packaged cheese and in *Food-Touch*®-packaged cheese (Figure 1, Panel C).

No moulds were detected in *Stracchino* cheese during the study, so these data are not discussed further.

Ovtene®-packaged cheese had noticeably lower yeast counts than cheese in traditional packaging and *Food-Touch*®-packaged cheese (Figure 1, Panel D).

Pseudomonas spp. increased in numbers in the cheese in all three packaging systems in the first days of storage (up to around day 8), and remained constant thereafter, except for slight fluctuations (Figure 1, Panel E).

Finally in the case of *Enterobacteriaceae*, a marked increase in numbers was measured in the first 15 days of storage, then slight fluctuations were observed at the end of the storage time (Figure 1, Panel F). *Enterobacteriaceae* numbers were lower in cheese packaged in the *Ovtene*® system than in the traditional system after 25 days of storage.

Enterobacteriaceae numbers were lower in *Food-Touch*®-packaged cheese between days 21 and 25 than in cheese packed in the other two systems.

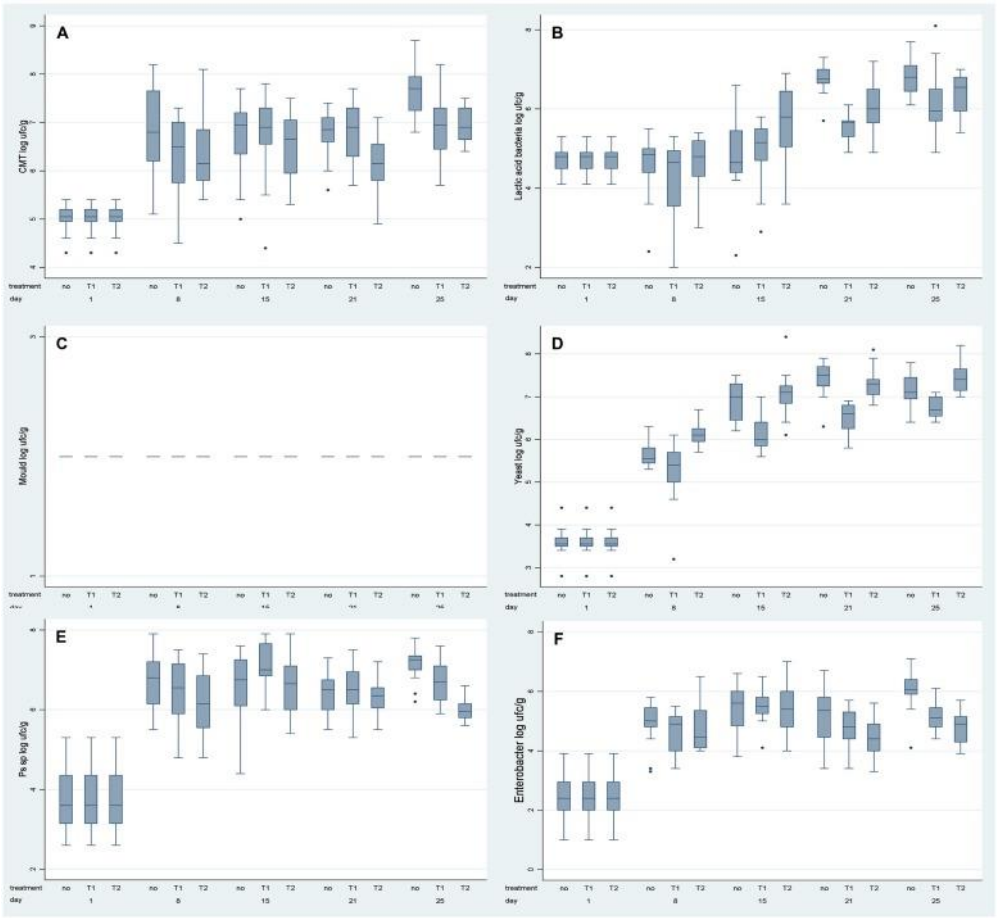

Figure 1. Box plot of the microbiological data per observation time and type of packaging: traditional (no), *Ovtene*® packaging (T1) and *Food-Touch*® system (T2). (A) Total Microbial Counts; (B) Lactic Acid Bacteria; (C) Moulds; (D) Yeasts; (E) *Pseudomonas* spp; (F) *Enterobacteriaceae.*

3.2 Chemical analysis

Table 2 shows the results of chemical analyses on cheese during storage. The only difference between the three examined packaging systems was the level of detected TVB-N. When the *Stracchino* cheese was wrapped with either one of the two active packaging options, higher levels of TVB-N were detected, compared with the traditional system. No differences were noticed for all the other investigated parameters.

Table 2. Chemical determinations obtained from analyses of cheese packaged in traditional, innovative and active packaging.

Traditional packaging					
Experimental Time (days)	**TVB-N (mgN/100 g)**	**Sulphides**	**Peroxides (MEQ O_2/Kg of fat)**	**Stamm test**	**TBA test**
0	2.00±1.02	negative	<5	negative	negative
7	3.00±1.27	negative	<5	negative	negative
14	4.30±6.30	negative	<5	negative	negative
20	0.10±0.31	negative	<5	negative	negative
24	13.00±8.97	negative	<5	negative	negative
***Ovtene*® packaging**					
Experimental Time (days)	**TVB-N average (mgN/100 g)**	**Sulphides**	**Peroxides (MEQ O_2/Kg of fat)**	**Stamm test**	**TBA test**
0	2.00±1.02	negative	<5	negative	negative
7	3.00±1.06	negative	<5	negative	negative
14	15.70±11.26	negative	<5	negative	negative
20	14.05±6.51	negative	<5	negative	negative
24	1.65±1.42	negative	<5	negative	negative
***Food-Touch*® packaging**					
Experimental Time (days)	**TVB-N average (mgN/100 g)**	**Sulphides**	**Peroxides (MEQ O_2/Kg of fat)**	**Stamm test**	**TBA test**
0	2.00±1.02	negative	<5	negative	negative
7	1.00±1.15	negative	<5	negative	negative
14	14.80±8.54	negative	<5	negative	negative
20	<0.10	negative	<5	negative	negative
24	2.55±1.93	negative	<5	negative	negative

Mean and standard deviation for each observation time are indicated.

3.3 Sensory evaluation

No significant difference due to the packaging characteristics, was displayed by the sensory evaluation tests for the identified parameters except for homogeneity and adherence, with *Ovtene*® packaging displaying the best performance (P>0.05; data not shown).

3.4 Silver migration test

The extent of Ag migration from the *Food-touch*® composite film into the cheese over time is shown in Table 3. The migration of Ag increased gradually from 0.053mg/kg after 7 days of incubation to 0.103mg/kg after 24 days (Table 3).

Table 3. Assessment of silver migration to cheese from traditional and active packaging.

Traditional packaging	
Time (days)	**Silver (mg/kg)**
0	<0.0015
7	<0.0015
14	<0.0015
20	<0.0015
24	<0.0015
***Food-Touch*® packaging**	
Time (days)	**Silver (mg/kg)**
0	<0.0015
7	0.053±0.025
14	0.245±0.053
20	0.047±0.023
24	0.103±0.038

Mean and standard deviation for each observation time are indicated.

4 DISCUSSION AND CONCLUSIONS

In this work, the effectiveness of two new active packaging systems on microbial, chemical and sensorial qualities of *Stracchino* cheese was evaluated. Moreover, the possibility that the cheese could contain chemicals deriving from the active food packaging systems (*Ovtene*® and *Food-Touch*®) was assessed.

Despite the *Food-Touch*® system resulting in lower bacterial growth at some given times throughout the cheese storage, the final results did not show any significant difference in the cheese microbiota examined, of any packaged *Stracchino* cheese samples, excluding that the investigated packaging systems exerted a different inhibitory effect on the growth of spoilage microorganisms.

On the contrary, a putative effect exerted by the *Ovtene*® system, which maintained two of the examined sensory characteristics, homogeneity and adherence, was observed. This effect may have been a consequence of the preservation of the functional cheese microbiota, known to be involved in the typical organoleptic properties of cheeses (Sgarbi *et al.*, 2013; Anniffy *et al.*, 2009).

These results are coherent with previously published research (Morsy *et al.*, 2014; Incoronato *et al.*, 2011; Incoronato *et al.* 2010), suggesting that although application of silver based antimicrobial systems in the food industry is a widespread phenomenon, appraisal of the full potential of silver as an antimicrobial and its possible implementation in food packaging technologies is still a challenging task (Losasso *et al.*, 2014; Berton *at al.*, 2014).

However, since health and safety properties of many food contact materials are not fully understood, food safety should be the main concern when formulating materials for food packaging applications. Thus, according to the European Regulation (EC) 1935/2004, efforts have to be devoted to investigate the overall migration of compounds from new packaging materials to the food, in order to elucidate the risks to humans consuming such packaged foods (Choudry, 2008; de Kruijf *et al*., 2002).

In this context, our results pose some safety concerns, as the level of silver migration from the active packaging system containing silver greatly exceeded the maximum established level for the migration of a non-authorised substance through a functional barrier (Commission regulation (EC) No. 450/2009).

Despite the relevance of the topic, to date, only a limited number of reports have studied the potential for silver migrating from plastic food containers, with most reports being focused on silver nanoparticles (Artiaga *et al.*, 2015, Echegoyen & Nerín, 2013; von Goetz *et al.*, 2013). In these studies, food containers were exposed to a number of food-simulating solutions (not real

foods) under a variety of experimental conditions in an attempt to determine the possible risks for human health. Conversely, our data investigated silver migration using a real food matrix as the acceptor, and clearly showed that silver levels in cheese reached unacceptable levels, up to around 250 times higher than the 0.001 mg/kg level prescribed by EU regulation.

Even though the published reports have revealed that silver has a low tendency to migrate from the investigated materials into solutions which mimic food, under regular use conditions, several discrepancies were found in these studies, particularly with regard both to the obtained results and to the analytical methodologies used. However, unambiguous methodologies to detect and quantify the chemicals migrated from packaging are currently lacking, making it difficult for us to produce an overall assessment of results published to date.

As far as the *Ovtene*® system is concerned, although this product did not display any effect in reducing the proliferation of all spoilage microorganisms, the preservation of some typical features of the cheese and the absence of any measured chemical migration into cheese could make this product interesting to the food industry.

In conclusion, the development of innovative and active packaging systems could provide important instruments to overcome existing challenges that are associated with packaging materials, positively affecting the shelf life and the quality of foods, which will ultimately benefit both the producers and consumers. However, more in-depth research is needed in order to characterize their potential impacts on consumer health and the environment.

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Publication n. 2: GLUTEN-BASED PACKAGING WITH AROMA COMPOUNDS: ANTIMICROBIAL ACTIVITY AGAINST *SALMONELLA* spp. AND *LISTERIA MONOCYTOGENES*

1 INTRODUCTION

Contamination of processed foods by foodborne pathogens such as *Salmonella* spp., *Listeria monocytogenes* and other pathogenic bacteria is an important safety issue for food business operators and consumers.

Salmonella spp. is one of the main causes of foodborne disease and in recent years has always been one of the most involved pathogens after *Campylobacter* spp. In the last years a reduction of the prevalence of *Salmonella* spp. in poultry and food of animal origin was been observed; nevertheless, according to the European plans to reduce it, *Salmonella* spp. remains among the most pathogenic microorganisms involved in cases of food poisoning, counting 82.694 human cases hospitalized in 2013, and 0,14% of deaths, as indicated by the last report of EFSA (EFSA 2015). *Salmonella* spp. is an important foodborne pathogen responsible for gastrointestinal illness worldwide. Humans infected with *Salmonella* spp. are reported to suffer from gastrointestinal symptoms such as diarrhea, vomiting, nausea and stomach cramps (Ravishankar *et al.*, 2009). Eggs and their products, pork, poultry meat, especially chicken, are considered the most contaminated kinds of food.

Despite the low number of reported cases compared to either *Salmonella* spp. or *Campylobacter* spp., *Listeria monocytogenes* has a very high mortality rate. In 2013, 1.763 human cases were reported with 15,6% of deaths. *Listeria monocytogenes* is a foodborne pathogen of high concern to immunocompromised individuals, especially pregnant women. The microorganism can cause mild flu-like illness and can pass the blood-brain barrier causing meningitis and encephalitis. It can also adversely affect the fetus, causing abortions and stillbirths (Ravishankar *et al.*, 2009). Fish and dairy products have been the most affected food products, in which *Listeria monocytogenes* is able to grow also at refrigeration temperatures. Because of the growing demand for consumption of fresh and minimally processed fish, research on the application of new preservation methods is required, not only for improving the product shelf life extension but also for ensuring their microbiological safety (Iturriaga *et al.*, 2012).

The antimicrobial properties of essential oils against pathogens such as *Salmonella* Enteritidis and Typhimurium, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Bacillus cereus* and

spoilage microorganisms were studied *in vitro* (Seydim, *et al*., 2006; Burt, 2004).

Different results were found depending on the test conditions, microorganisms or source of the antimicrobial compound. Among the essential oils, cinnamon and oregano essential oils are pointed out as the most efficient ones (Gutiérrez *et al.,* 2010). Oregano was the most effective essential oil against *Listeria monocytogenes, Salmonella* Enteritidis, *Escherichia coli 0157:H7, Staphylococcus aureus, Lactobacillus plantarum;* its major components are carvacrol and thymol (Fernández-Pan *et al*., 2012).

Cinnamon essential oil primarily contains cinnamaldehyde that is known to be inhibitive to growth of *Salmonella* Typhimurium and *Escherichia coli 0157:H7* (Burt, 2004).

Eugenol is the major component of clove oil and some studies showed the antimicrobial efficacy against *Listeria innocua*, *Salmonella* Enteritidis, *Staphylococcus aureus* (Fernández-Pan, *et al*., 2012). The incorporation of essential oils into edible films as natural bactericides might be an interesting option.

The objective of the present study was to determine the antimicrobial efficacy of an experimental gluten-based edible film added with carvacrol, cinnamaldehyde, and eugenol, in chicken breast artificially contaminated on the surface with *Salmonella* spp. and in smoked salmon inoculated with *L. monocytogenes*. Carvacrol, cinnamaldehyde, and eugenol are the main ingredients of oregano oil, cinnamon oil and clove oil respectively.

2 MATERIALS AND METHODS

2.1 *In vitro* testes

The first part of research had focused on selection of aroma compounds and determination of their antimicrobial activity against two potential pathogenic bacteria (*Salmonella* spp. and *Listeria monocytogenes*); subsequently a study of aroma compounds incorporation effects on antimicrobial and mechanical properties of gluten-based edible films was performed.

2.1.1 Bacterial strains

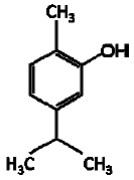
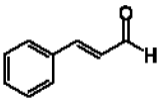
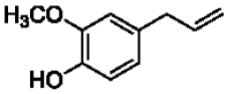
Reference strains were *Listeria monocytogenes* ATCC 13932 (American Type Culture Collection) and *Salmonella* Agbeni CNRS 463/S03 (National Reference Centre for Salmonella), while field strains were isolated from foodstuffs (fresh meat and fish).

2.1.2 Aroma compounds

Aroma compounds were carvacrol, cinnamaldehyde and eugenol (Sigma-Aldrich, USA).

Each aroma antimicrobial activity was tested against a reference strain and 15 field strains of *Listeria monocytogenes* and *Salmonella* spp. by agar disk diffusion method and by minimum inhibitory concentration (MIC) method. Physicochemical properties of aroma compounds selected for this study are presented in Table 1.

Table 1. Physicochemical properties of aroma compounds (Ben Arfa *et al.*, 2006; Munmaya, 2015).

	Carvacrol	**Cinnamaldehyde**	**Eugenol**
JUPAC name	2-isopropil-5-metilfenolo	(2E)-3-phenylprop-2-enal	2-metossi-4-(propen-2-il)- fenolo
Molecular formula	$(CH_3)_2CHC_6H_3(CH_3)OH$	$C_6H_5CH=CHCHO$	$4\text{-}(H_2C=CHCH_2)C_6H_3\text{-}2\text{-}(OCH_3)OH$
Smell	Oregano	Cinnamon	Clove
Molecular weight	150,217 (g/mol)	132,16 (g/mol)	164,2 (g/mol)
Vapour pressure 25°C (Pa)	6,4	2,6	3,9
Log*P*	3,52	1,90	2,73
Maximum solubilità in water	0,11 (g/l)	1,1 (g/l)	0,64 (g/l)
Density	0,976 g/ml at 20°C	1,05 g/ml at 25°C	1,067 g/ml at 25°C
Bolling Point	236-237°C	246°C	254°C
Molecular structure			

2.2 Agar disk diffusion

The agar disk diffusion method was employed for the determination of antimicrobial activities of three aroma compounds against *Listeria monocytogenes* and *Salmonella* spp. An inoculum of the tested bacteria composed by 100 µl of suspension containing 10^7 colony-forming units (CFU) per ml of each strain was spread on the surface of Tryptic Soy Agar (TSA) plates.

Cellulose filter paper disks (9 mm in diameter) impregnated with 100 µl of aroma compound were placed over each inoculated agar plate for 24 hours at 37°C, and, after incubation, the size of inhibition zone was measured with a calliper. In a parallel set of tests the impregnated filters were placed over the surface of plastic plate lids, rather than over the agar, in order to evaluate the effect of air spread aroma.

Then, squares of gluten-based films (20 mm x 20 mm) with aroma compounds at different concentrations (3, 5, 7, 10 %) were obtained and placed both in direct contact with the agar medium and on the lid of the plate.

2.3 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration is the lowest concentration of an antimicrobial that will inhibit the visible growth after overnight incubation. MIC are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to determine the potency of new antimicrobial agents.

MIC was determined according with Clinical and Laboratory Standard Institute (CLSI) guidelines. Multiple microtiter plates were filled with Tryptic Soy Broth (TSB). Serial dilutions of essential oil and the bacterial strain were added to the wells, and incubated at 37°C for 24 h; turbid wells were considered positive for bacterial growth and MIC was the lowest concentration of the essential oil that prevented visible growth of the microorganism in the plate.

2.4 Gluten-based film preparation (WG)

According to the method previously set up by Marcuzzo et al. (2010), gluten acid film forming dispersion was prepared dissolving 10 g of gluten (80%, Sigma-Aldrich, USA) in 50 g of ethanol (Carlo Erba, Italy). Glycerol (2,5 g, Sigma-Aldrich, USA), dispersed in 40 g of distilled water, was added to the alcoholic dispersion, and pH was adjusted to 4 by adding hydrochloric acid solution. Before stirring 15 min at 70°C, ultrasound treatment (UT) was applied to 50 ml of the film forming dispersion for exposure times ranging from 3 to 12 min were performed by using a UPS200S ultrasonic processor (Hielscher Gmbh, Germany) equipped with a sonotrode with 2 mm of tip diameter, at a power output of 200 W, an ultrasonic intensity of 600 W/cm^2 and a frequency of 24 kHz. Once the dispersion was prepared, a set of aliquots (3-5-7-10%, V/W protein) of pure aroma (carvacrol, trans-cinnamaldehyde and eugenol) was added and the mixture was emulsified with homogenizer (Ultra-Turrax model T25 IKA, Labortechnik, ODIL, France) at 24.000 rpm for 1 min. Film forming dispersion were spread on PVC-coated plates a thin layer chromatography spread (Desaga Heidelberg, Germany) and dried overnight a 25°C.

2.5 Testing of food matrices

In the second part of the research the antimicrobial effectiveness of experimental active materials with carvacrol (7% and 10% v/w protein) on selected meat and fish-based products artificially contaminated (challenge tests) was studied.

Two pathogenic microorganisms, *Salmonella* spp. and *Listeria monocytogenes*, were chosen to contaminate chicken and salmon matrix respectively.

2.5.1 Preparation of the inoculum

The inoculum was prepared according EURL *Lm* Technical Guidance Document (2014), and was adapted for *Salmonella* spp.

Firstly, the medium Brain Heart Infusion (BHI) was inoculated with a bead coated with each pathogen and was incubated at 37°C, which is the favourable temperature to optimal growth of *Listeria monocytogenes* and *Salmonella* spp., for a sufficient time for the organisms to reach the early stationary phase (15-18 h). This first subculture was mainly aimed at getting the cells in the same physiological state.

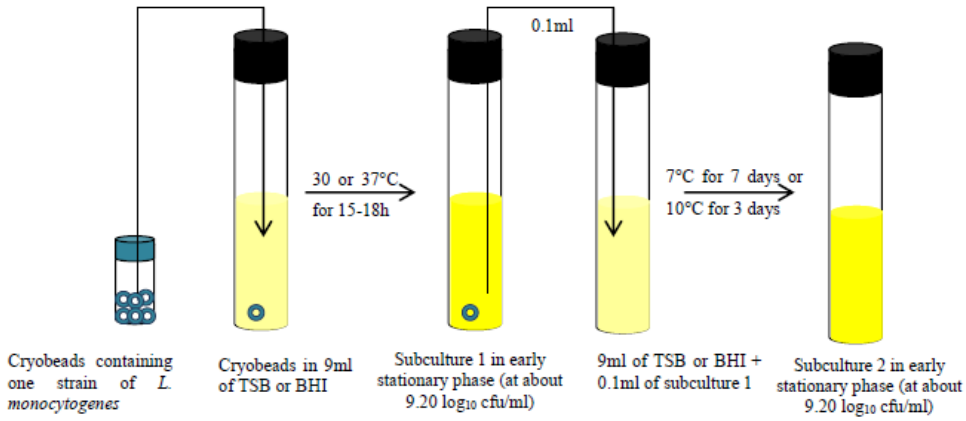


Figure 1. Preparation of the 2 subcultures for each strain (EURL *Lm*, 2014).

Secondly, the inoculated BHI was incubated at the temperature of 10°C, close to the storage temperature of the food product, for 3 days in order to adapt the strain to the storage condition of the product. This culture was incubated for a sufficient time necessary to reach the early stationary phase, to shorten the lag phase once inoculated in the products (Figure 1).

The microbial load selected for inoculation of the food matrix was 10^6 CFU/ml although it is little frequent in the real food products, but it allows greater emphasis on possible reductions.

2.5.2 Effect of WG film containing carvacrol on pathogenic bacteria survival/reduction

Chicken breast and smoked salmon were cut into 100 g pieces and placed in trays for food use. Chicken and salmon surface was contaminated uniformly with 1 ml of inoculum (10^7 CFU/ml) previously prepared. Absence of *Salmonella* spp. and *Listeria monocytogenes* in the chicken breast and smoked salmon respectively was verified before the inoculum with Real Time PCR assay (AFNOR BRD 07/06-07/04 and AFNOR BRD 07/10-04/05 respectively). After inoculation, samples were packaged by means of two systems. The WG packaging with carvacrol was placed both not and in

contact with the food. In order to verify if the film without aroma compound has an antimicrobial activity, two types of control samples were used: a gluten film prepared without the addition of aroma compounds and a conventional material packaging collected from a local market (polypropylene). Samples were stored in optimal thermal conditions (+4°C), and analyzed at different moments of the shelf life.
Microbial analyses were conducted to monitor the amount of *Salmonella* spp. and *Listeria monocytogenes* in the chicken breast and smoked salmon respectively, during storage in the two selected packaging systems as detailed in Table 2.

Table 2. Sampling design for microbial determinations.

		Number of samples			
Experimental time (days)	**Storage temperature (°C)**	**WG film (control) HS**	**WG film (control) DC**	**WG film with carvacrol HS**	**WG film with carvacrol DC**
1	0/+4	3	3	3	3
3	0/+4	3	3	3	3
5	0/+4	3	3	3	3
Total number of samples		9	9	9	9

WG film (control): gluten-based edible film without carvacrol; WG film with carvacrol: gluten-based edible film with carvacrol; HS: packaging with headspace; DC: packaging in direct contact with food matrix.

Analyses were conducted on days 1, 3, 5 of storage. In total, 3 replicates for every sample were examined each day for both packaging systems.
A single batch of chicken and salmon packaged with carvacrol 7 % WG film was tested, while two batches of both matrices packed with carvacrol 10% WG film with were analysed.
Listeria monocytogenes was enumerated according to ISO 11290-2:1998, while *Salmonella* spp. was counted using spread plate technique on Xylose Lysine Deoxycholate agar (XLD) after incubation at 37°±1°C for 24±3 h.

2.6 Mechanical properties

Tensile strength (TS) and elongation at break (E) of WG and WG with aroma compounds (previously equilibrated with magnesium nitrate at a_w=0.53) were measured with uniaxial tension testing at 25 °C, using a dynamometer (T. A. XT. Plus). The specimen dimensions were 8 x 2 cm, the initial distance between grips was 6 cm and the strain velocity was 1 $mm \cdot s^{-1}$. At least 10 specimens were analysed for each condition test.

2.7 Statistical analyses

Statistical differences in the film properties were determined by one-way analysis of variance (ANOVA) and Tukey multiple range test ($P<0,05$).

3 RESULTS

3.1 Agar disk diffusion

The agar disk diffusion tests showed inhibition of bacterial growth when pure essential oils were placed both on the agar surface and on the lid, except for eugenol that was active only if placed on the agar surface (Table 3).
The tests were conducted in triplicate for each strain (reference and field strain) and for each essential oil; the data reported in Table 3 represent the average values of the inhibition zone diameters measured. For *Salmonella* spp. and *Listeria monocytogenes* comparable inhibition haloes were observed.
Results were compared to those of the antibiotics (chloramphenicol and streptomycin), used as positive control. The inhibition zone diameter of the chloramphenicol and streptomycin ranged from 18-27 mm to 12-22 mm, respectively.

Table 3. Antimicrobial activity of pure essential oils for *Salmonella* spp. and *Listeria monocytogenes* (average values of inhibition zone diameter).

Essential oil	Lid (mm)	Surface of agar (mm)
Carvacrol	36	40
Cinnamaldehyde	36	44
Eugenol	0	23

Cinnamaldehyde resulted as the most effective against *Salmonella* spp. and *Listeria monocytogenes* when in contact of the surface of agar, based on the inhibition zone diameter values. The difference in the behavior of this aroma depending of the test conditions, in contact with the surface or on the lid, could be related to its hydrophobicity, represented by the log *P* value. In fact, it is possible to observe that cinnamaldehyde shows the lower log *P* value, compared to those of carvacrol and eugenol (Table 1). Therefore, this aroma is supposed to have affinity with the agar substrate (essentially hydrophilic), and thus to diffuse faster than the other aroma compounds. On the contrary, carvacrol showed an inhibitory effect similar to cinnamaldehyde, even if its hydrophobicity is the highest. This suggested that this aroma was the more effective against *Salmonella* spp. and *Listeria monocytogenes*, despite its physicochemical properties. The physicochemical properties of eugenol would suggest an intermediate behavior than the other two aroma compounds, that it was not confirmed by results that show a low effectiveness of this aroma against *Salmonella* spp. and *Listeria monocytogenes*, and only when placed in contact of the surface of agar (not on the lid).

Concerning the test conducted with gluten-based edible films containing essential oils, no effect was observed for films containing cinnamaldehyde or eugenol, placed both in contact with the agar surface and on the lid of the plate, regardless of the essential oil concentration incorporated. For this reason, determination of inhibition zone was not possible.
The WG film with the highest concentrations of carvacrol (7% and 10% v/w protein) demonstrated an antimicrobial activity only when the film was placed on the lid of the plate and more effective against *Salmonella* spp. than against *Listeria monocytogenes.* Observing the physicochemical properties of the tested aroma compounds, carvacrol is the one with greater volatility, and it tends to evaporate during the gluten-based film preparation. For this reason high concentrations are needed while to highlight an effect. In Figure 2, inhibition zones observed in agar plates with different amounts of *Salmonella* spp. are showed the inhibition zone corresponded to the surface area of the film samples.
Even if the determination of inhibition zone presumes a non-limiting diffusion of the aroma compound to the medium, the absence of effect in the case of antimicrobial packaging placed in contact with the agar surface could be related to the low water solubility of the aroma compounds or oil components added to the film, more than a low amount of the aroma retained by the film during the preparation process. The low affinity between the antimicrobial compounds and the agar medium could affect the agar- disk diffusion assay and also the antimicrobial activity. Even if agar-disk diffusion is probably affected by the low affinity between aroma compounds and agar medium, this system could better represent a real food product such as the matrices considered in this study, i.e. chicken breasts and salmon fillets. In fact, although these products represent complex matrices constituted by both hydrophilic and lipophilic zones, the weak tendency of lipophilic aroma compounds to diffuse to hydrophilic medium represent an important hurdle to the antimicrobial efficacy of the active films. Therefore, a vapor diffusion assay is more reliable in determining the antimicrobial effectiveness of lipophilic antimicrobial film (Sanla-Ead *et al.,* 2011).

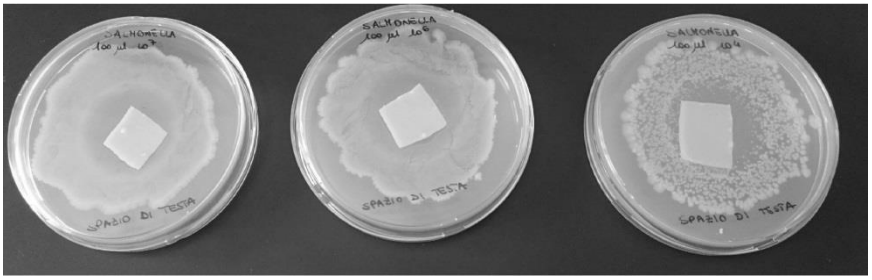


Figure 2. *In vitro* analysis of edible films with carvacrol.

3.2 Minimum inhibitory concentration

Minimun inhibitory concentration highlighted that *Salmonella* spp. and *Listeria monocytogenes* were sensitive to carvacrol, while they had an intermediate sensitivity to cinnamaldehyde and eugenol (Table 4).

Table 4. MIC of essential oils for *Salmonella* spp. and *Listeria monocytogenes.*

Essential oil	**MIC (µg/ml)**
Carvacrol	0,125
Cinnamaldehyde	8
Eugenol	8

3.3 Microbial analyses in food products

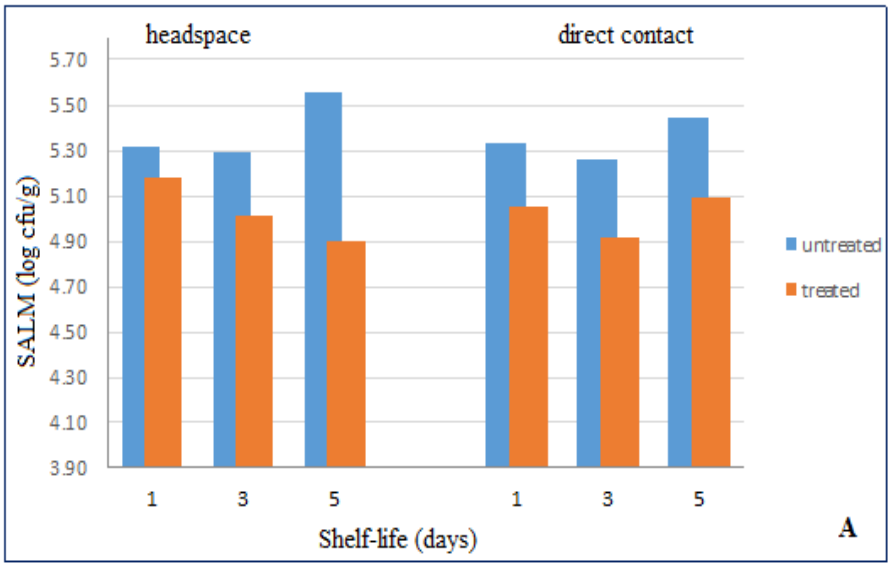
Since carvacrol resulted the most effective compound against *Salmonella* spp. and *Listeria monocytogenes* from agar disk diffusion test, films prepared with two different concentrations, 7 and 10%, were applied to real food matrices, chicken breast and salmon fillets. Antimicrobial activity resulted different as a function of the carvacrol concentration.

The tests performed on chicken and salmon packaged with WG film with carvacrol 7% did not show an antimicrobial effect against both pathogenic microorganisms considered (data not shown).

Concerning the films prepared with 10% of carvacrol, in the first batch of chicken breast packaged in direct contact and with headspace, *Salmonella* spp. count in the untreated samples remained more or less constant (P>0,05) during the first three days of shelf life and then increased on the fifth day. In headspace treated samples, the pathogenic load decreased during the storage time, reaching the lowest value after five days (Figure 3A); in the case of direct contact samples, *Salmonella* spp. counts kept unchanged during storage time with a lower concentration compared to control sample.

In the second batch, the amount of *Salmonella* spp. in treated samples was lower than in control samples at each analytical session and for both ways of packaging.

Just as for the first batch, the maximum inhibitory effect of carvacrol film was observed on the fifth day (Figure 3B).



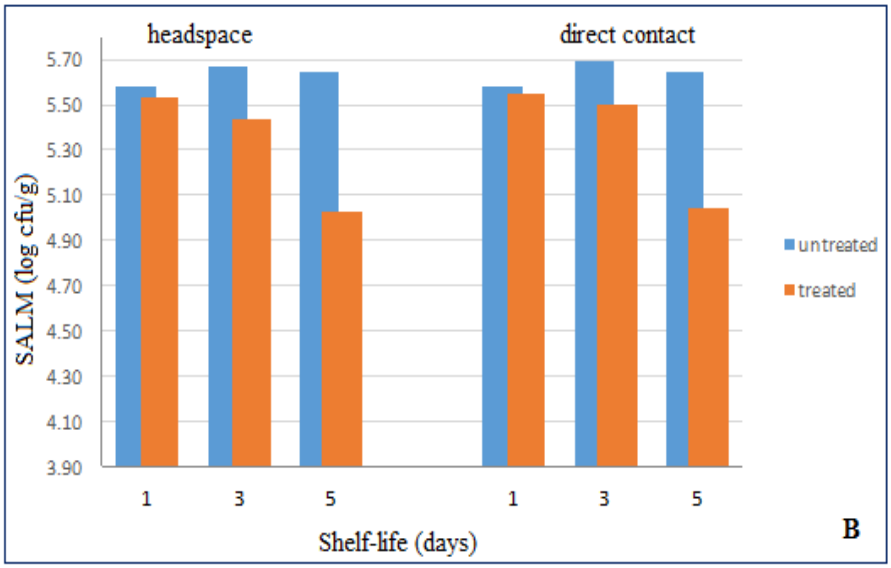


Figure 3. Enumeration of *Salmonella* spp. observed in chicken breast packaged with WG film (untreated) and with 10% carvacrol WG film (treated), batch 1 (3A) and batch 2 (3B). Means values obtained from three replicates.

As regard smoked salmon artificially contaminated with *Listeria monocytogenes,* two batches of samples were considered. In batch 1 (Figure 4A), a significant increase in pathogen counts in untreated samples was observed on the third day of shelf life, compared to the samples packed with films containing carvacrol, both in head space packaging and in direct contact with food. After three days of storage a decrease in microbial counts was observed also in the untreated sample. The antimicrobial effect was confirmed in batch 2 (Figure 4B), where an increase in microbial counts in untreated samples compared to treated samples was observed after three and five days of storage, for both headspace and direct contact samples. Results obtained show different behavior for treated and untreated samples: in fact, when for untreated samples *Listeria monocytogenes* counts increased as a function of storage time, for treated samples values are settled on a constant value. This evolution for treated samples instead of a decrease in microbial counts suggested an inhibitory effect of the aroma compounds: therefore, we could hypothesize that carvacrol cannot cause a diminution of microorganisms (i.e. sterilizing effect) but its presence in the headspace probably create a disadvantageous environment for microbial growth.
No differences between the two kinds of packaging both for controls and for treated samples were observed in the second batch. The load of *Listeria monocytogenes* observed in salmon packaged with WG film, increased significantly ($P<0,05$) after the third day of shelf life and maintained a constant value until the fifth day, while the pathogen counts in treated samples were constant during the whole observation period. The reduction of microbial loads induced by carvacrol packaging were more evident than in batch 1.

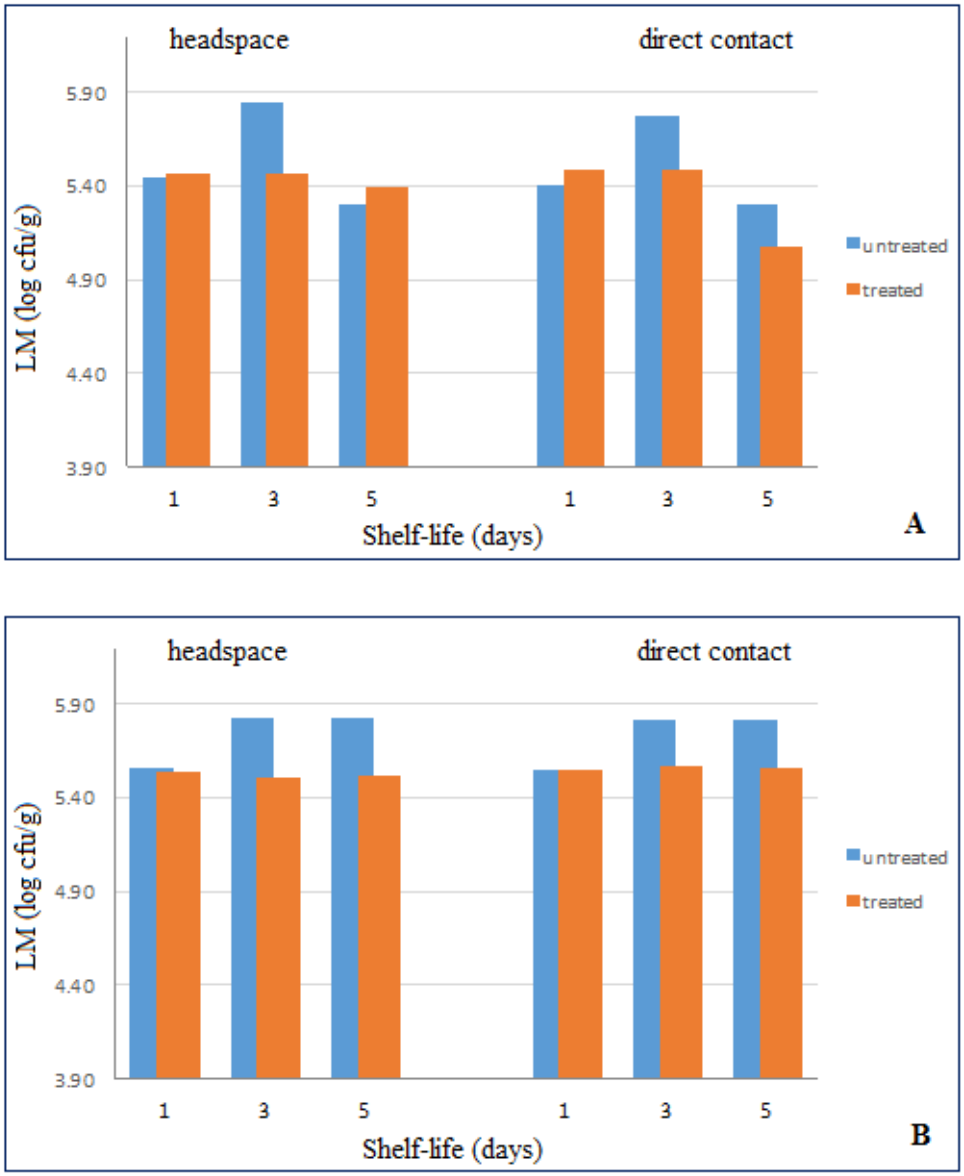


Figure 4. Enumeration of *Listeria monocytogenes* observed in smoked salmon packaged with WG film (untreated) and with 10% carvacrol WG film (treated), batch 1 (4A) and batch 2 (4B). Means values obtained from three replicates.

Two different packaging methods were compared in Table 5 and 6. Results showed a difference in microbial inhibition as a function of the WG film position: in particular, a greater microbial inhibition was found when the specimen was set on the lid, not in contact with the sample. Therefore, carvacrol seemed to have an effect against *Salmonella* spp. when it diffused in the headspace.

Table 5. Antimicrobial efficacy against *Salmonella* spp. in chicken breast as a function of 10% carvacrol WG film position in the package.

Food products	Shelf life (days)	WG film with carvacrol 10% (headspace)	WG film with carvacrol 10% (direct contact)
Chicken	1	5,18[a]	5,05[a]
breast	3	5,01[a]	4,91[b]
(batch 1)	5	4,90[a]	5,09[b]
Chicken	1	5,53[a]	5,55[a]
breast	3	5,44[b]	5,50[a]
(batch 2)	5	5,03[a]	5,05[a]

Table 6. Antimicrobial efficacy against *Listeria monocytogenes* in smoked salmon as a function of 10% carvacrol WG film position in the package.

Food products	Shelf life (days)	WG film with carvacrol 10% (headspace)	WG film with carvacrol 10% (direct contact)
Smoked	1	5,47[a]	5,49[a]
salmon	3	5,47[a]	4,48[a]
(batch 1)	5	4,39[a]	5,08[b]
Smoked	1	5,54[a]	5,55[a]
salmon	3	5,51[a]	5,57[a]
(batch 2)	5	5,52[b]	5,56[a]

3.4 Mechanical properties

Mechanical properties of an edible film are very important because they determine the properties of elasticity, strength and plasticity in order to maintain its integrity during handling and storage. In order to understand if the addition of essential oils or their components can affect the mechanical properties of the film as a function of the type of essential oil, mechanical properties of WG prepared with the same content of carvacrol, eugenol, trans-cinnamaldehyde were measured and compared with a sample prepared without EO addition (WG). Results of tensile strength and elongation are reported in Figure 5, A and B. Tensile strength (Figure 5A) was not affected by the type of aroma compounds, unlike what was for elongation (Figure

5B): WG containing eugenol was characterized by the highest elongation, where the sample prepared with the addition of cinnamaldehyde shown the lowest elongation. In the first case, results suggest a plasticizing effect of eugenol, contrary to cinnamaldehyde that seemed to reinforce the gluten network acting as a cross-linker, as previously observed by Balaguer *et al.* (2011).

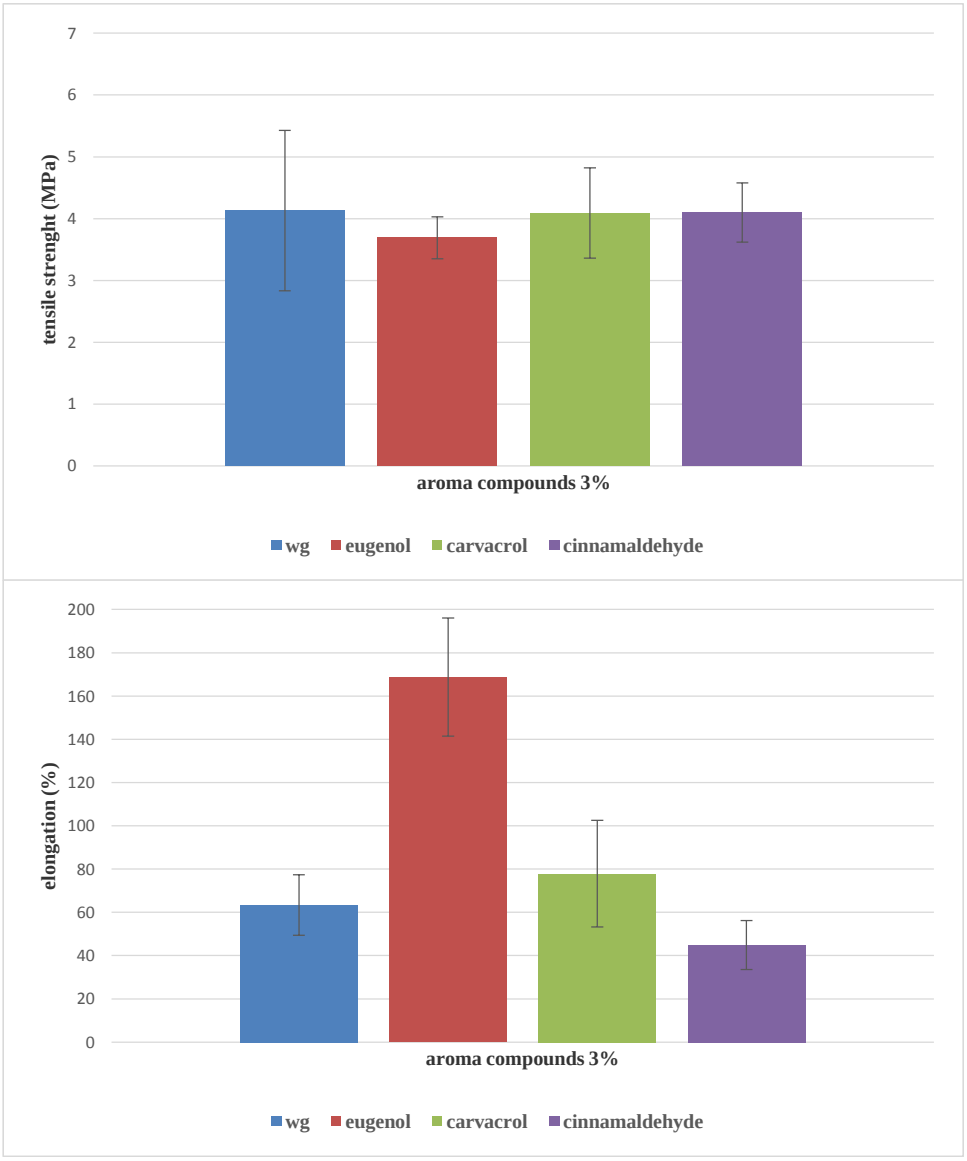


Figure 5. Tensile strength (A) and elongation (B) of WG films with and without aroma compounds.

Mechanical properties of WG prepared with different concentration of added carvacrol (3, 5, 7, 10%) are measured and compared. Results of tensile strength (Figure 6) showed no effect on film mechanical properties with EO initial concentration up to 7%. With EO concentration higher than 7%, it seemed that carvacrol had a cross-linker effect on the protein.

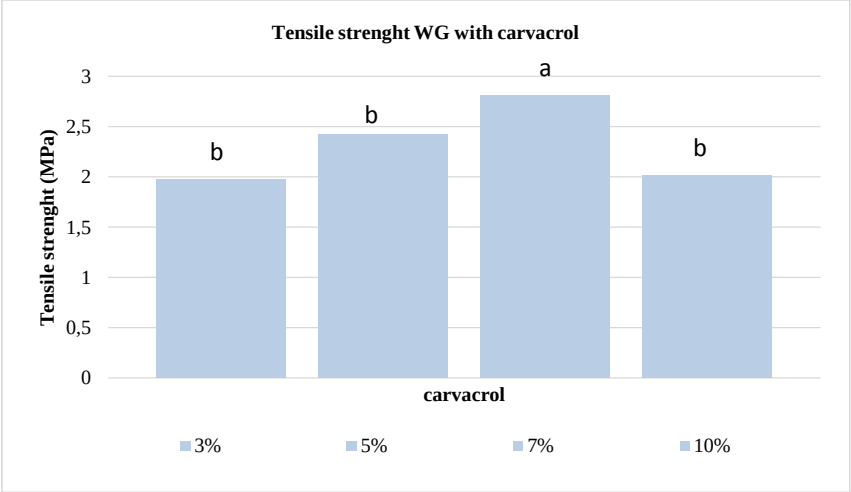


Figure 6. Tensile strenght of WG films at different concentration of carvacrol.

Elongation results (Figure 7) confirmed the data obtained for tensile strength, suggesting a reduction in plasticity and an increased rigidity of the structure.

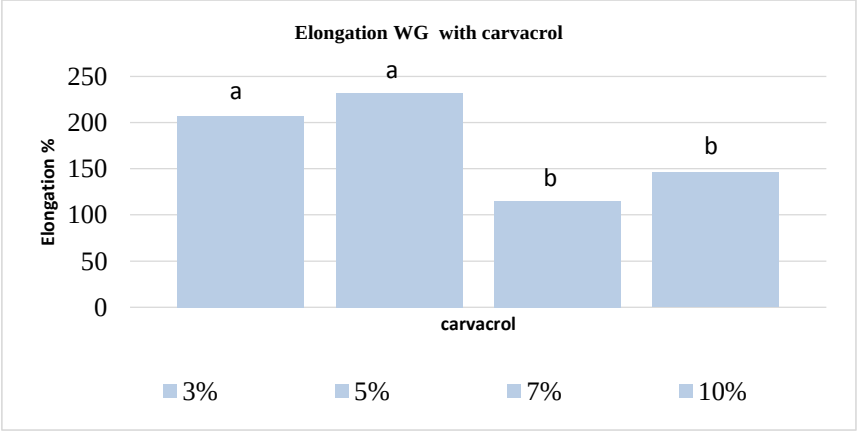


Figure 7. Elongation of WG films at different concentration of carvacrol.

4 DISCUSSION AND CONCLUSIONS

The preliminary study for the *in vitro* evaluation of the antibacterial activity of WG film with aroma compounds at different concentrations showed a positive effect only for WG film with carvacrol 7% and 10%. No effect was observed for gluten based edible films with cinnamaldehyde or eugenol, regardless the essential oil concentration incorporated. Films prepared with the lowest carvacrol concentrations (3-5%) did not show antimicrobial effect. Our results did not agree the data obtained by Fernández-Pan *et al.* (2012), that demonstrated effectiveness WPI films containing oregano against *Listeria innocua, Salmonella* Enteritidis and *Staphylococcus aureus* when the active substance concentration was 1%. Our results did not confirmed what found by Hosseini *et al*. (2008), in which edible films containing 0,5% clove essential oil were effectives against *Listeria monocytogenes* and *Staphylococcus aureus* but they resulted in non-effectiveness against *Salmonella* Enteritidis and *Pseudomonas aeruginosa.*

As concern the challenge tests with real matrices packaged with carvacrol film, a significant inhibitory effect on the growth of the pathogen was observed only if carvacrol concentration was at least 10%. The tests performed on chicken and salmon packaged with WG film with carvacrol 7% showed a not significant antimicrobial effect against both pathogenic microorganisms considered.

For chicken breast the maximum effect was obtained after five days both for packaging with head space and direct contact. In the first batch carvacrol incorporated in head space packaging seemed to be more efficient than if placed in direct contact with food matrix, but it was not confirmed in the second batch where the action was similar.

In smoked salmon the effect of reduction on *Listeria monocytogenes* loads exerted by experimental carvacrol film was more evident in the second batch and it seemed to be independent in the way of food packaging, i.e. with head space or direct contact.

The results from this work are coherent with previously published research (Ravishankar *et al.,* 2009) although the inhibition of pathogen growth is not so marked. In Ravishankar's study, carvacrol incorporated into edible apple film showed antimicrobial activity against *Salmonella* Enterica on the surface of chicken breast and *Listeria monocytogenes* on the surface of ham. At 4°C, films contain 0,5% carvacrol inducing about 0,8 log reduction of *Salmonella* Enterica. At 4 °C, film containing carvacrol showed about 2, 1, and 0,5 log reductions in *Listeria monocytogenes* populations and at 3%, 1,5%, and 0,5% concentrations, respectively.

The different results found by other researchers can be explained by the composition of the film matrices, which has also a fundamental effect on their antimicrobial activity. Emiroglu *et al.* (2010) observed antimicrobial

activity against *Staphylococcus aureus*, *Escherichia coli*, *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* and *Lactobacillus plantarum* with 1% oregano essential oil encapsulated in soy protein isolated based edible films, while Pellissari *et al*. (2009) inhibited the *Salmonella* Enteritidis and *Staphylococcus aureus* strains effectively by using a starch-chitosan matrix with a lower essential oil content (0,5%).

When a packaging material is developed, different properties have to be considered for final application. Among these, mechanical properties can be crucial, especially in the case of a bilayer or multilayer packaging, where the compatibility between the different materials is affected also by elongation and tensile strength, in particular when multilayer packaging is composed by a bio-based layer. Different papers discussed the effect of aroma compound addition on the bio-based film mechanical properties: in particular, the addition of trans-cinnamaldehyde to gluten-based films modified mechanical properties of final film, suggesting a role of the aroma in reinforcing the network formed by the protein (Balaguer *et al.,* 2011a). Along with the study of antimicrobial activity, mechanical properties of films containing aroma compounds were investigated, in view of the application to develop a real packaging system.

The addition of aroma compounds seemed to affect gluten films mechanical properties differently as a function of the type of the aroma compound: in particular the elongation resulted the highest when eugenol was added to the film. Since carvacrol resulted as the most effective against *Salmonella* spp. and *Listeria monocytogenes* growth, the effect of different concentrations of this aroma compounds on mechanical properties was deepened. Even if both tensile strength and elongation changed as a function of aroma compounds concentration, films prepared with 10% of initial content of aroma compound seemed to show mechanical properties not significantly different from those of film prepared with 3% and 5% of initial aroma compound content. Therefore, mechanical properties of film with increased antimicrobial activity were not affected by aroma compounds concentration.

Due to the microbiological results, we focused this study on the carvacrol and we observed that the addition of carvacrol in WG can modulate the structural characteristics of the mechanical properties of these films.

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CONCLUSIONS

Antimicrobial packaging is a promising and rapidly emerging technology in which antimicrobial agents are incorporated into or coated onto food packaging materials in order to prolong shelf life of the packaged food or to control the growth of pathogenic microorganisms. Even when antimicrobial films fail to remove completely higher numbers of unwanted microbes, they can act as an additional and post processing safety measure.

The first part of study aimed to verify the antimicrobial effectiveness of a commercial antimicrobial packaging (*Food-Touch®)* against the spoilage microorganisms of a fresh cheese. The microbial, chemical and sensorial characteristics of active packed *Stracchino* cheese were compared with ones observed in the cheese wrapped with a traditional passive packaging and in cheese packaged with an innovative film (*Ovtene*®), at that time regularly used in the production plant. The cheese manufacturer had previously replaced the traditional passive packaging with *Ovtene*® because the innovative film kept longer the cheese freshness and preserved its organoleptic characteristics, prolonging the shelf life. All the *Stracchino* samples, with active or not active packaging, belonged to the same batch of production and were hand-packaged by the producer as usually; so no production differences affected the results apart from the kind of film used as packaging.

The packaging products were analysed by mimicking a real-life scenario, in terms of time and temperature of storage as established by manufacturer, and also because a real food – *Stracchino* cheese – was used. Despite the *Food-Touch*® system resulting in lower bacterial growth at some given times throughout the cheese storage, the final results did not show any significant difference in the cheese microbiota examined, of any packaged *Stracchino* cheese samples, excluding that *Food-Touch*® and *Ovtene*® systems exerted a different inhibitory effect on the growth of spoilage microorganisms.

On the contrary, a putative effect exerted by the *Ovtene*® system, which maintained two of the examined sensory characteristics, homogeneity and adherence, was observed. This effect may have been a consequence of the preservation of the functional cheese microbiota, known to be involved in the typical organoleptic properties of cheeses.

These results are coherent with previously published research, suggesting that although application of silver based antimicrobial systems in the food industry is a widespread phenomenon, appraisal of the full potential of silver as an antimicrobial and its possible implementation in food packaging technologies is still a challenging task.

Our results pose some safety concerns, as the level of silver ions migration from the active packaging system containing silver exceeded the maximum

established level for the migration of a non-authorised substance through a functional barrier (Commission regulation (EC) No. 450/2009).
Despite the relevance of the topic, to date, only a limited number of reports have studied the potential for silver migrating from plastic food containers, with most reports being focused on silver nanoparticles.
In the second part of the thesis the antimicrobial activity of an experimental gluten-based edible film added with carvacrol, known for its antimicrobial capacity, was tested against two foodborne pathogens, *Salmonella* spp. and *Listeria monocytogenes* on fresh poultry and smoked salmon respectively.
Even if several studies about the efficacy of essential oils or their components against microbial spoilage were previously conducted, very few focused on the activity of these molecules once incorporated in a packaging material and applied to real food products.
As concerns the outcome of the study, a significant inhibitory effect on the growth of the pathogens was observed only if carvacrol concentration was at least 10%. The tests performed on chicken and salmon packaged with WG film with carvacrol 7% showed a not significant antimicrobial effect against both pathogenic microorganisms considered.
The results from this work are coherent with previously published research although the inhibition of pathogen growth is not so marked. The different results found by other studies can be explained by the composition of the film matrices, which has also a fundamental effect on their antimicrobial activity.
The final content of aroma compound incorporated in the film should be determined in order to investigate the real conditions under which the aromas are effective against the microorganisms selected. In fact the quantity of aroma compounds added to the packaging material should not be too pronounced to avoid a significant and unwanted change in the original sensory characteristics of the product.
The effect of the aroma compound incorporated in the packaging film seems to be linked to the ability of creating an unfavourable environment for the microorganism multiplication, saturating the internal packaging atmosphere, rather than a bactericidal action. So to ensure such effect throughout the food product shelf life it would be necessary to achieve a controlled release of the active molecule in order to have a constant concentration over time and to avoid the aroma consumption in the first phase of the storage.
In conclusion, in this study the efficacy of active packaging systems, both packaging material with silver zeolites and with aroma compounds, was evaluated in real food products stored at refrigeration temperature, 0-4°C. This temperature was selected in order to simulate the effective storage conditions of the products studied (*Stracchino* cheese, chicken and salmon fillets).

This represents an innovative approach both because we considered real food matrices and because the refrigeration temperatures are different compared to those used *in vitro* tests.
Moreover the volatility of the aromatic compounds is conditioned by the temperature, so the effectiveness of the experimental packaging systems is even more interesting because evaluated in unfavourable operating conditions.

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Research project:

- **Research project IZS VE 06/11** Health and hygienic analysis and correct identification of fish products in public canteens in the Triveneto area.

- **Research project IZS VE 17/11** Assessment of the consumers' food management in the domestic environment on the level of thermophilic *Campylobacter* spp. and *Listeria monocytogenes* contamination.

- **Research project IZS VE 17/12** Ethnic food and food safety: microbiological issues, adverse reactions, fraud and risk perception by the final consumer.

- **Research project IZS VE 19/12** Effectiveness of methods used in the home kitchen, in restaurants and in the canning industry for the inactivation of enteric pathogens such as viruses (Nv, Hav) in Manila clams and larvae of parasites (Anisakis) in fishery products.

- **Research project IZS VE 14/10** A microbiological risk assessment of small-scale traditional production of salami and other fermented pork products.