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TESI

"New approaches to Food Safety: study on food preservation and quality markers"

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I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale
(Marie Curie).

Index

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List of Abbreviations

(AC) Alternating current

(ADI) Acceptable Daily Intake

(AITC) Ally isothiocyanate

(ANS) Additives and Nutrient Sources

(BAs) Biogenic Amines

(CAD) Cadaverine

(COX) Cytochrome Oxidase

(D) Diffusion Coefficient

(DAD) Diode-arraydetector

(ECG) Electrocardiogram

(EEG) Electroencephalogram

(EFSA) European Food Safety Agency

(EOs) Essential oils

(ET) Engel Trichrome

(FCM) Food contact materials

(FDA) Food and Drug Administration

(FOX) Ferrous Oxidation-Xylenol Orange Method

(GMP) Good Practices Manufacturing

(GRAS) Generally recognized as safe

(H&E) Hematoxylin and Eosin

List of Abbreviations

(HPLC-DAD)	High performance liquid chromatographic with diodearray detection
(HV)	High-Voltage stunning
(IDF)	International Dairy Federation Method
(JECFA)	Joint FAO/WHO Expert Committee on Food Additives
(K)	Partition coefficient
(LPS)	Lactoperoxidase system
(MAP)	Modified atmosphere packaging
(MIC)	Minimum inhibitory concentration
(MV)	Mid-Voltage stunning
(Mw)	Molecular weight
(NS)	No stunned
(OIE)	Office International des Epizoties
(OML)	Overall migration limit
(PA)	polyamide
(PAS)	Periodic Acid- Schiff
(PDA)	Potato dextrose agar
(PE)	Polyethylene
(PEC)	Polyelectrolyte complex
(PEG)	Polyethylene glycol

List of Abbreviations

(PET)	Polyethylene terephthalate
(PP)	Polypropylene
(PS)	Polystyrene
(PUT)	Putrescine
(PVC)	Polyvinylchloride
(SCF)	Scientific Committee for Food
(SDH)	Succinic Dehydrogenase
(SML)	Specific Migration Limit
(SPF)	Specific Pathogen Free
(TiO2)	titanium dioxide
(TS)	Tensile strength
(WVP)	Water vapor permeability
(ZnO)	Zinc oxide.

List of Figures

List of Figures

List of Figures

List of Tables

List of Tables

Abstract

In the last decades, the food market globalization, the introduction of novel foods, and growing demand for minimally processed, fresh-cut and ready-to-eat products have made the food chain more complex, increasing the risk of microbiological contamination. To obtain healthy food and prevent human food borne diseases, research in the field of food science has focused on innovative food preservation technologies and study of quality markers. Actually, reduction in temperature, pH, water activity and the application of heat are the major preservation techniques employed to prevent or delay spoilage and are increasingly associated with hurdle technologies. The aim of the studies presented in this dissertation is to describe and analyze innovative food technologies and procedures to extent the shelf-life, assuring food hygiene and minimizing the impact on the quality properties of food.

The *Chapter I* was focused on the biopreservation that rationally exploits the antimicrobial potential of naturally occurring (micro-) organisms in food and/or their metabolites. As alternative preservation technology, the aim of the study was to develop active biobased packaging incorporated natamycin as antimicrobial agent. Two active films based on chitosan (1,5% w/v)and methylcellulose (3% w/v) enriched with natamycin were prepared by casting. The migration kinetics of the antimicrobial agent were studied in the food simulant 95% ethanol (v/v) at different temperatures. The diffusion (D_P) and partition coefficients were calculated by using a mathematical model based on Fick's Second Law. Natamycin was determined in the food simulant by reversed-high performance liquid chromatography with diode-array detection (HPLC-DAD). For microbiological analysis, cheese samples were completely covered with the

films, stored at 20°C for 7 days, and then analyzed for molds and yeasts. Results showed that the release of natamycin from chitosan based film was slower, if compared with methylcellulose film at the same temperature ($P<0.05$). Accordingmold/yeast count significant differences ($P<0.05$) were observed between chitosan film containing natamycin (7.91 log CFU/g) and polyethylene film, used as blank. Both chitosan and methylcellulose films seem to show good physical properties. Moreover, the good compatibility of natamycin with chitosan, low diffusion coefficient, and antimicrobial properties indicated that the film has great potential to be used in antimicrobial packaging, such as in cheese wrappings to inhibit mold spoilage. The application of antimicrobial agents to packaging materials could be useful to prevent the growth of microorganisms on the product surface and to improve microbial food safety.

Chapter II reviewed a research regarding the influence of the pre-slaughter stress on animal welfare and poultry meat quality. The study investigated the effects of electrical stunning parameters on the quality of poultry meat in Ross commercial broilers processed either without stunning (NS Lot), or by combining high (HV Lot), and mid-voltage (MV Lot), with two frequencies of 1000 and 800 Hz. As pre-slaughter stress markers and quality meat indicators, physicochemical (pH, peroxides) and histological parameters (glycogen reserve, muscle damages) were investigated. Results showed as the use of high frequencies stunning conditions increased lactate production, induced a gradual pH decline($P<0.05$), reduced the muscle oxidative activity($P<0.05$), improving meat quality. To assess animal welfare and quality poultry meat, pH monitoring and measurement of

superoxide radical production, might be considered markers easier to use under practical conditions at poultry slaughterhouse.

In *Chapter III*, new models and approaches applied to decontamination of the poultry meat issue were described. Treatments of decontamination of poultry carcasses might assure food safety and the reduction of human food borne infections. The aim of the study was to evaluate the effects of an experimental ozone gaseous treatment during the storage of chilled poultry carcasses by determination of biogenic amines as quality index. Physicochemical (pH determination), sensorial (panel test, questionnaire, and a scoring system), and chemical analysis (biogenic amines) were carried out. Amines were extracted with perchloric acid, derivative with dansylchloride, and separated by HPLC with a fluorescence detector. Results showed a gradual increase of pH values in poultry meat of treated and simply chilled carcasses. Ozonized carcasses showed acceptable sensory quality until to 20^{th} day and lower levels of putrescine (32.37 mg/kg) and cadaverine (132.30 mg/kg). On the contrary, simply chilled poultry carcasses showed unacceptable sensory quality and a significant increase of putrescine and cadaverine at 15^{th} day. Higher levels of putrescine (53.63 mg/kg) and cadaverine (175.20 mg/kg) were reached at 20t day of storage. If authorized, an ozone treatment during the storage of chilled poultry meat can induce a reduction of microbial contamination. Putrescine and cadaverine levels appeared to be useful to control the effectiveness of the ozone treatment on meat quality, to highlight, as quality index, the loss of poultry meat freshness, before sensorial meat changes.

Abstract

The novel and ambitious goal for optimal food preservation isthe multitarget preservation of foods, in which intelligently applied gentle hurdles will have a synergistic effect. The application of innovative preservation methods with the monitoring of food quality by the use of markers could contribute to reach the purpose.

La globalizzazione dei mercati, l'introduzione di nuovi alimenti e processi innovativi di trasformazione e la crescente domanda di alimenti “ready-to-eat” o sottoposti a un limitato processo di trasformazione possono aumentare il rischio della contaminazione microbiologica. Ai fini della sicurezza e della prevenzione delle malattie alimentari, numerosi studi si sono focalizzati sulla ricerca di tecnologie innovative di conservazione e di indicatori di qualità degli alimenti. Le diverse tecniche di conservazione degli alimenti si basano principalmente sulla riduzione di parametri come temperatura, pH, attività dell'acqua e applicazione del calore e, in molti casi, sull’associazione delle diverse tecnologie. Più recentemente l’industria alimentare si sta orientando verso tecnologie innovative finalizzate alla produzione di alimenti *naturali* e privi di additivi.

Lo scopo delle ricerche riportate in questo lavoro è stato quello di descrivere e analizzare tecnologie innovative di conservazione degli alimenti, tali da assicurare un prolungamento della shelf life e minimizzare gli effetti sulla qualità sensoriale e nutrizionale dei prodotti.

Il *Capitolo I* si è focalizzato sullo sviluppo di imballaggi alimentari attivi provenienti da fonti rinnovabili contenenti natamicina come agente antimicrobico. In campo alimentare, la bioconservazione, come metodo alternativo utilizza (micro-) organismi (e loro metaboliti) naturalmente presenti negli alimenti. In una prima fase sono stati preparati film attivi di chitosano (1,5% w / v) e metilcellulosa (3% w/v) contenenti natamicina mediante *casting*. Successivamente sono state studiate le cinetiche di migrazione dell'agente antimicrobico in un simulante alimentare(etanolo 95%) a diverse temperature. Il calcolo dei coefficienti di diffusione (D_P) e partizione è stato condotto utilizzando un modello matematico basato sulla

seconda Legge di Fick. La determinazione della natamicina è stata eseguita mediante analisi cromatografica con un sistema HPLC- DAD. Per le analisi microbiologiche, campioni di formaggio sono stati ricoperti con i diversi film, conservati a 20° C per 7 giorni e poi analizzati per la ricerca e numerazione di lieviti e muffe. I risultati hanno mostrato, alla medesima temperatura, una cinetica di rilascio della natamicina dal film di chitosano più lenta ($P < 0,05$) rispetto a quella del film in metilcellulosa. La crescita di lieviti e muffe ha mostrato differenze significative ($P < 0,05$) tra il film di chitosano contenente natamicina (7,91 log CFU / g) e il film di polietilene, utilizzato come bianco. Entrambi i film hanno mostrato buone proprietà fisiche. Inoltre la compatibilità della natamicina con il chitosano, il suo ridotto coefficiente di diffusione e le proprietà antimicrobiche mostrano che il film di chitosano può essere efficacemente utilizzato come imballaggio alimentare antimicrobico per prevenire la crescita di microrganismi sulla superficie degli alimenti e migliorare la sicurezza microbica del prodotto.

Il *Capitolo II* ha riguardato lo studio dell'influenza dello stress pre-macellazione sul benessere animale e sulla qualità della carne di pollame. In particolare, lo scopo del lavoro è stato quello di studiare gli effetti di differenti parametri elettrici di stordimento sulla qualità della carne di pollame in broiler Ross. Gli animali sono stati sottoposti a differenti modalità di macellazione :a) in assenza di stordimento;b) utilizzando elevata (1000 Hz)e c) ridotta frequenza (800 Hz). Come indicatori dello stress pre-macellazione e della qualità della carne sono stati valutati parametri fisico-chimici (determinazione del pH e dei perossidi) e istologici (riserva di glicogeno e danni muscolari). L'uso di elevate

frequenze ha determinato un aumento delle concentrazioni di lattato e una significativa riduzione del pHe dell'attività ossidativa muscolare (P <0,05) e una migliore qualità della carne. Alla luce dei risultati ottenuti il monitoraggio del pH e la valutazione dei livelli di radicali liberi potrebbero essere utilizzati,come parametri aggiuntivi e di facile utilizzo, per valutare il benessere animale e la qualità delle carni nei macelli avicoli.

Nel *Capitolo III* è stato studiato un nuovo approccio alla problematica alimentare relativa alla decontaminazione delle carcasse avicole. Dati bibliografici indicano che l'applicazione delle buone pratiche di produzione nelle carni di pollame da sola non è in grado di assicurare la qualità microbiologica delle carni, a causa del costante flusso di batteri nell'impianto di trasformazione e dell'inevitabile contaminazione crociata. I metodi di decontaminazione delle carcasse di pollame possono contribuire a ridurre le malattie alimentari legate al consumo delle carni. Pertanto lo scopo della ricerca è stato quello di valutare gli effetti di un trattamento sperimentale con ozono gassoso e ricercare la presenza di ammine biogene come indice freschezza, durante la conservazione delle carcasse di pollame refrigerate. Le ammine sono state estratte con acido perclorico, derivatizzate con dansil-cloride e separate usando un sistema HPLC associato a fluorimetro.I risultati hanno mostrato una riduzione della contaminazione microbica come effetto del trattamento con ozono. Le carcasse trattate hanno mostrato livelli più bassi di putrescina (32,37 mg/kg) e cadaverina (132,30 mg/kg) e un prolungamento della shelf life maggiore di 6 giorni. Nelle carcasse solamente refrigerate si è osservato un significativo aumento di putrescina e cadaverina a partire dal 15° giorno di conservazione, mentre livelli di putrescina (53,63 mg / kg) e cadaverina

(175,20 mg / kg) ancor più elevati sono stati raggiunti al 20° giorno di conservazione. Se autorizzato, un trattamento con ozono potrebbe ridurre il grado di contaminazione microbica durante lo stoccaggio di carcasse avicole refrigerate. Inoltre la determinazione di ammine, come putrescina e cadaverina, può essere considerata un valido indicatore dell'efficacia del trattamento con ozono e della qualità delle carni.

In conclusione l'utilizzo dell'associazione di diverse tecnologie alimentari con un effetto finale sinergico sembra essere la strada più percorribile per raggiungere l'importante obiettivo della conservazione ottimale degli alimenti. L'applicazione di metodi di conservazione innovativi unita al monitoraggio della qualità e l'uso di validi indicatori alimentari potrebbe contribuire a raggiungere lo scopo.

Food market globalization, the introduction of novel foods, new manufacturing processes and the growing demand for minimally processed, fresh-cut and ready-to-eat products may require a longer and more complex food chain, increasing the risk of microbiological contamination. Thus, novel and complementary food preservation technologies that comply with these demands from “farm to fork “are continuously seeked (Garcia et al., 2010).With few exceptions all foods lose quality and potential shelf life at some rate or other following harvest, slaughter or manufacture in a manner that is very dependent on food type, composition, formulation (for manufactured foods), packaging and storage conditions. Spoilage, or other changes that lead to loss of shelf life, may occur at any of the many stages between the acquisition of raw materials and the eventual consumption of a finished product (Gould, 1996).

Spoilage may be caused by a wide range of reactions essentially physical, chemical, enzymic and microbiological. Preservation is based firstly on the delay or prevention of microbial growth. It must therefore operate through those factors that most effectively influence the growth and survival of microorganisms. The major preservation techniques currently employed to prevent or delay spoilage are reduction in temperature, pH, water activity and the application of heat. However, these and other techniques are

increasingly used together in combination preservation or hurdle technologies. A further trend is towards the use of procedures that deliver products that are less heavily preserved, have higher quality, and are more natural, freer from additives and nutritionally healthier (Gould, 1996).

Among alternative food preservation technologies, particular attention has been paid to biopreservation to extent the shelf-life and to enhance the hygienic quality, minimizing the impact on the nutritional and organoleptic properties of perishable food products (Garcia et al., 2010). Natural compounds, such as essential oils, chitosan, nisin or lysozyme, are investigated to replace chemical preservatives and to obtain "green label" products (Devlieghere et al., 2004). Nowadays, research focuses on the incorporation of natural antimicrobial or antioxidant compounds such as plant extracts and bacteriocins in food packaging materials to control undesirable growth of microorganisms on the surface of foods (Vermeiren et al., 1999; Devlieghere et al., 2004). *Chapter 1* is focused on the development of active biobased packaging incorporated natamycin as antimicrobial agent. The main cause of spoilage of many refrigerated foods is microbial growth on the product surface. The application of antimicrobial agents to packaging materials could be useful to prevent the growth of microorganisms on the product surface and hence may lead to an

extension of the shelf-life and/ or improved microbial safety of the product (Collins- Thompson and Hwang, 2000; Devlieghere et al., 2004).

As mentioned above, spoilage may occur at any of the stages between the acquisition of raw materials and the eventual consumption of a food product. At the time the animals are put up for slaughter within the abattoir, the pre-slaughter stress can influence the animal welfare, as well as post mortem metabolism and quality meat (Gregory 1994; Ali et al., 2008). The aim of *Chaper 2* was to investigate the effects of electrical stunning parameters on quality of poultry meat. Results showed as the use of high frequencies stunning conditions increased lactate production, induced a gradual pH decline, reduced the muscle oxidative activity and, consequently, improved meat quality.

Hygiene intervention in the poultry meat processes alone does not lead to safe products, owing to the constant flow of bacteria entering the processing plant and unavoidable cross- contamination. Decontamination of meat and poultry carcasses can help to reduce human foodborne infections, and seems to be the only possibility to assure food safety. Decontamination treatment with ozone has been approved by the U.S. Food and Drug Administration (FDA) - Department of Health and Human Services, that has considered ozone as a food additives and GRAS

(Generally Recognized for Safe) substance (Bolder, 1997). The aim of *Chapter 3* was to evaluate the effects of an experimental ozone gaseous treatment and production of the biogenic amines, as freshness index, during the storage of chilled poultry carcasses. Results showed that putrescine and cadaverine may be a valuable and sufficiently rapid method for monitoring the effectiveness of a decontamination treatment. Moreover, if authorized, an ozone treatment during the storage of chilled poultry meat can induce a reduction of microbial contamination.

The innovative purpose for an optimal food preservation is the multitarget preservation of foods, in which intelligently applied gentle hurdles will have a synergistic effect.

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Chapter 1

Development of Chitosan and Methylcellulose films containing Natamycin as antimicrobial agent

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1. Introduction

The European Federation defines f*ood packaging* as products, made of any materials and of any nature, used for the containment, protection, delivery and presentation of foods, from raw materials to processed foods.

Food is often dynamic system with limited shelf life that needs very specific packaging functions. Food packaging confers convenience to food and communicating information about the product to consumers, and has a number of important functions. The principal roles are to contain and protect food from outside influences and damage, maintain the sensory quality, and provide to consumers information about ingredients and nutritional aspects. Consequently, food packaging confers convenience to food and communicating information about the product to consumers. Traceability, convenience, and tamper indication are secondary functions of increasing importance (Restuccia et al., 2010; Regattieri and Santarelli, 2015).

- **Protection/preservation**

Food packaging can retard product deterioration, retain the beneficial effects of processing, extend shelf-life, and maintain or increase the quality and safety of food. Keeping the contents clean, fresh, sterile and safe for the intended shelf life is a primary function. Packaging provides protection

from 3 major classes of external influences: chemical, biological, and physical. Chemical protection minimizes compositional changes triggered by environmental influences such as exposure to gasses (typically oxygen), moisture (gain or loss), or light (visible, infrared, or ultraviolet). Many different packaging materials can provide a chemical barrier. Glass and metals provide an early absolute barrier to chemical and other environmental agents, but few packages are purely glass or metal since closure devices are added to facilitate both filling and emptying. Closure devices may contain materials that allow minimal levels of permeability. Plastic packaging offers a large range of barrier properties but is generally more permeable than glass or metal.

Biological protection provides a barrier to microorganisms (pathogens and spoiling agents), insects, rodents, and other animals, thereby preventing disease and spoilage. Such barriers function via a multiplicity of mechanisms, including preventing access to the product, preventing odor transmission, and maintaining the internal environment of the package.

Physical protection shields food from mechanical damage, the objects enclosed in the package may require protection from mechanical shock, vibration, electrostatic discharge, compression, temperature, etc. encountered during distribution. Typically developed from paperboard and

corrugated materials, physical barriers resist impacts, abrasions, and crushing damage, so they are widely used as shipping containers and as packaging for delicate foods such as eggs and fresh fruits (Regattieri and Santarelli, 2015).

- **Containment and food waste reduction**

Inadequate preservation/protection, storage, and transportation have been cited as causes of food waste. Packaging reduces total waste by extending the shelf-life of foods, thereby prolonging their usability. Rathje and others (1985) found that the per capita waste generated in Mexico City contained less packaging, more food waste, and one-third more total waste than generated in comparable U.S. cities. In addition, they observed that packaged foods result in 2.5% total waste-as compared to 50% for freshfoods-in part because agricultural by products collected at the processing plant are used for other purposes while those generated at home are typically discarded. Therefore, packaging may contribute to the reduction of total solid waste (Marsh and Bugusu, 2007).

- **Marketing and information**

Marketers to encourage potential buyers to purchase the product can use packages. A package is the face of a product and often is the only product exposure consumers experience prior to purchase. Consequently,

distinctive or innovative packaging can boost sales in a competitive environment. The package may be designed to enhance the product image and/or to differentiate the product from the competition. Packaging also provides information to the consumer. For example, package labeling satisfies legal requirements for product identification, nutritional value, ingredient declaration, net weight, and manufacturer information (Marsh and Bugusu, 2007; Regattieri and Santarelli 2015).

- **Traceability**

The Codex Alimentarius Commission defines traceability as "the ability to follow the movement of a food through specified stage(s) of production, processing and distribution" (Codex Alimentarius Commission, 2004). Traceability has 3 objectives: to improve supply management, to facilitate trace-back for food safety and quality purposes, and to differentiate and market foods with subtle or undetectable quality attributes (Golan et al., 2004). Food manufacturing companies incorporate unique codes onto the package labels of their products; this allows them to track their products throughout the distribution process. Codes are available in various formats (for example, printed barcodes or electronic radio frequency identification) and can be read manually and/or by machine (Marsh and Bugusu, 2007).

- **Convenience**

Convenience features such as ease of access, handling, and disposal; product visibility; resealability; andmicrowave ability greatly influences package innovation. Consequently, packaging plays a vital role in minimizing the effort necessary to prepare and serve foods. Oven-safe trays, boil-in bags, and microwavable packaging enable consumers to cook an entire meal with virtually no preparation. Advances in food packaging have facilitated the development of modern retail formats that offer consumers the convenience of 1-stop shopping and the availability of food from around the world. These convenience features add value and competitive advantages to products but may also influence the amount and type of packaging waste requiring disposal (Marsh and Bugusu, 2007).

- **Tamper indication**

Willful tampering with food and pharmaceutical products has resulted in special packaging features designed to reduce or eliminate the risk of tampering and adulteration. Although any package can be breeched, tamper-evident features cannot easily be replaced. Tamper-evident packaging usually requires additional packaging materials, which exacerbates disposal issues, but the benefits generally outweigh any drawback (Marsh and Bugusu, 2007).

A questionnaire distributed to Italian users analyzed the perception of Italian consumers on packaging quality attributes using the Theory of Attractive Quality, developed by Kano et al. (Regattieri et al., 2012; Kano et al., 1984). Italian consumers consider the ergonomic quality characteristics as the most significant packaging attributes and the protection of the product the most important packaging function. They also perceive the use of recyclable material another important packaging attribute, in line with the growing importance of environmental considerations (Regattieri and Santarelli, 2015).

Packaging is built up as a system usually consisting of a primary, secondary, and tertiary level (Saghir, 2002) (Fig.1.1).

- The primary package concerns the structural nature of the package; it is usually the smallest unit of distribution or use and is the package in direct contact with the contents.
- The secondary package relates to the issues of visual communication and it is used to group primary packages together.
- The tertiary package is used for warehouse storage and transport shipping (Long, 1982; Regattieri and Santarelli, 2015).

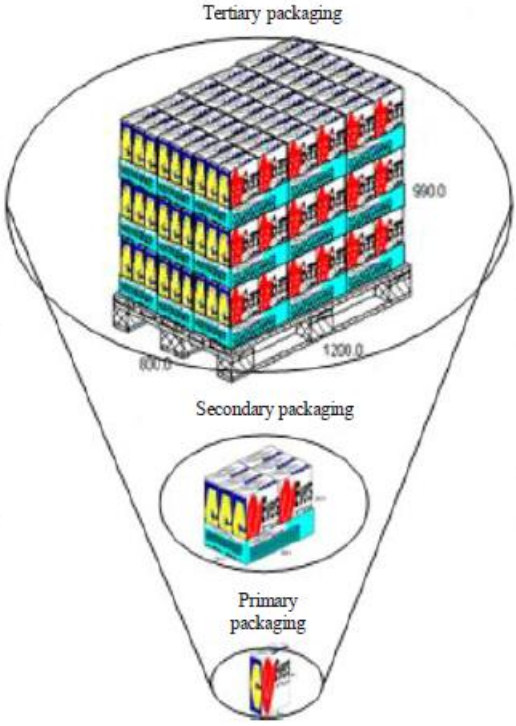


Fig. 1.1.Graphical representation of the packaging system (Regattieri and Santarelli, 2015).

The product packaging system (overall packages and accessories) is highly relevant in the supply chain, because of the necessity to minimize costs, to the development of web operations (i.e. electronic commerce) and reduce the environmental impact. All products moved are contained in packages. For this reason, the analysis of the physical logistics flows and the role of

packaging is an important issue for the definition and design of manufacturing processes, improvement of layout and increase in companies ‘efficiency (Regattieri and Santarelli, 2015).

Considering the sustainability, environmental responsibility, and recycling regulations, the packaging system plays an increasingly important role. Packaging issues affect several environmental aspects:

- Waste prevention: packages should be used only where needed. Usually, the energy content and material usage of the product being packaged are much greater than that of the package.
- Material minimization: the mass and volume of packages is one of the criteria to minimize during the package design process. The use of “reduced” packaging helps to reduce the environmental impacts.
- Re-use: the possibility to re-use of a package or its component for other purposes is encouraged.
- Recycling: the emphasis focuses on recycling the largest primary components of a package (steel, aluminum, paper, plastic, etc.).
- Energy recovery: waste-to-energy and refuse-derived fuel in facilities are able to make use of the heat available from the packaging components.

- Disposal: incineration and placement in a sanitary landfill are needed for some materials.

Users and companies have shown an interest in the environment and its link with the packaging system. Indeed, they believe that careful use of packaging can lead to an important reduction in environmental impact (Regattieri et al., 2012a; Regattieri et al., 2012b).

There is no such thing as the ideal packaging. Packaging should be such that we could come close to the ideal and the criteria of ideal packaging are listed as (Driscoll et al., 1999):

- zero toxicity
- high product visibility
- strong marketing appeal
- ability of moisture and gas control
- stable performance over a large temperature range
- low cost and availability
- suitable mechanical strength (i.e., strength in compression, wear, and puncture characteristics)
- easy machine handling and suitable friction coefficient

- closure characteristics, such as opening, sealing and resealing, pouring
- ability to include proper labeling
- resistance of migration or leaching from package
- protection from loss of flavor and odor
- controlled transmission of required or unwanted gasses.

Companies have begun to use recyclable materials (e.g. cardboard, paper, and plastic) (Regattieri and Santarelli, 2015) and innovative packing as biobased packaging, edible films, and coatings (Campos et al, 2011).

Progress has been made in the development of diversified packaging materials and in the packaging equipment. However, most packaging materials used for foods are not inert and reaction may occur between food and package material. Food may interact with the packaging materials and this may change the initial mechanical and barrier properties, as well as the safety of the product (Sablani and Rahman, 2007).Food and packaging interactions can be defined as interplay between food, packaging, and the environment, which produces an effect on the food and/or package. Interactions between foods and packaging can be detrimental to quality and/or safety. Examples of such undesirable inter actions include the

transfer of potential toxicants and/or flavor taints from the package to the food. The scalping of desirable flavors from the food by the package, gain or loss of moisture due to permeation, ingress of oxygen resulting in product oxidation, reduction in the physical properties of the packaging material, or loss of carbonation (Hotchkiss, 1997).

Careful consideration must be given to those factors affecting such interactions when selecting packaging materials in order to maximize product quality, safety, and shelf life while minimizing undesirable changes.

Direct interactions between food and packaging are not necessarily detrimental. The same principles governing undesirable interactions can be used to affect desirable outcomes. Examples include films, which directly interceptor absorb oxygen, inhibit microorganisms, remove undesirable flavors by sorption, or indicate safety and product shelf life.

Classical food-package interactions can be categorized into three types: migration, permeation, and sorption (Hotchkiss, 1997).

In packaging regulations, the term *migration* is used to describe the transfer of package components from the package to the contained food product. A distinction is usually made between global migration and specific migration. Global migration refers to the total transfer, i.e., the

quantity of all substances migrating from the package into the packaged food, whereas specific migration relates to the transfer of one or more identifiable substances that is a constituent of the packaging material (Giacin and Brzozowska, 1987; Sablani and Rahman, 2007) (Fig.1.2).

The volatiles such as flavors and aromas are lost by means of permeation and absorption, and this may directly affect the food quality. The permeation of polymeric film depends on the solution and the transport behavior of gas and vapor.

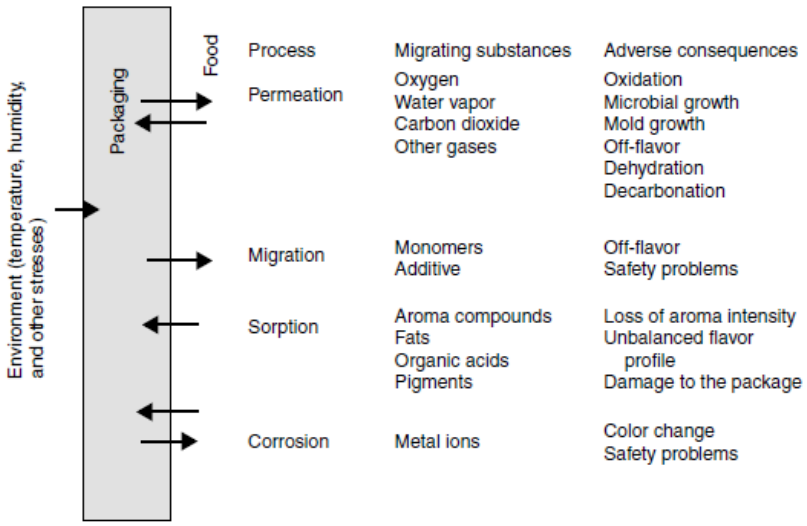


Fig. 1.2. A complete scenario of product–package interaction resulted from several modes (Sablani and Rahman, 2007).

The packaging materials can also absorb flavor compounds from products, the loss of which results in a reduced perception of quality (Sablani and Rahman, 2007).

In specific situations, product components may penetrate the structure of the packaging material, causing loss of barrier and mechanical properties. Further, the migration of low-molecular weight components from packaging material to a contained product can result in flavor loss or color change. In addition, a packaging material undergoing oxidation can also accelerate the oxidation of products in contact with that material. Packaging materials can absorb flavor compounds from products, the loss of which results in a reduced perception of quality; thus, being packaged, scalping of flavor compounds is a concern for many aseptic products currently (Sablani and Rahman, 2007).

Specific examples include migration of plasticizers or contaminants from recycled polymers, which are regulatory, and safety issues or migration of functional food additives, which can enhance quality. In addition, permeation or gasses such as oxygen or carbon dioxide, which may be a benefit in modified atmosphere packaging (MAP), but undesirable in carbonated beverages; and sorption of components such as flavors, odors, or taints, which can result in organoleptic changes, are included. Studies of

potentially toxic migrants should likewise be based on biological or toxicological considerations. For example, the US FDA'sthreshold of regulation policy is based on a *toxicological index*, which was developed by surveying compounds whose toxic potential has been investigated in detail (Federal Register, 1995). Using the index it is possible to estimate, with a known degree of confidence, the potential maximum toxicity of a compound whose toxicity has not been investigated. Then the estimated toxicity can be translated into a level of risk and maximum allowable level in the diet. Migrants which are below this dietary concentration are then of little regulatory concern. The next effect of this policy is that 'zero' is defined for packaging migrants, ending the constant lowering of 'zero' as analytical chemistry has improved (Rulis, 1996).

However, there is a considerable discussion of packaging, which can directly interact with the food and environment to enhance safety and quality (Tab.1.1).

Tab. 1.1 Active packaging interactions influencing food quality and safety (Hotchkiss, 1997).

Principle	Objective and Method	Examples
Barrier control in films	a. Pore size/dimensions	Map/gas press release, rapid adjustment
	b. Selective/adjustable permeation	MAP, control atmosphere composition, temperature compensation
Energy modulation of microwaves	a. Defletcing/shielding	Non-hetaing regions
	b.Energy conversion/transfer	Crisping (susceptors)
	c. Expansion	Done-ness indicators
Reduce/inhibit microbial growth	a. Migrants	Inhibit surface spoilage
	b. Metal ions	Inhibit spoilage
	c. Enzymes/proteins	Film sterilization/sanitation
Generation, release of active agents	a. CO_2 generation	MAP
	b. Hydrolysis to release SO_2, acids, etc	Microbial-control fruits
	c. Desorption to release EtOH	Antimycotic- in baked products
Adsorption, absorption	a. removal of undesirable odors	Aldehyde sorption, rancidity reduction
	b. moisture drip control	Control relative humidity, free water
	c. CO_2 absorption	MAP
Chemical reactions	a. Oxidations	O_2 scavengers
	b. acid/base reactions	CO_2 sorption/generations
	c. Organic reactions	O_2 removal

Examples of several potentially beneficial food-packaging interactions are active antimicrobial films, flavor- enhancing packaging, and selective barrier films (Hotchkiss, 1997).

1.1 Active Packaging

Active Packaging is an innovative concept that can be defined as a mode of packaging in which package, product, and environment *interact* to prolong shelf life or enhance safety or sensory properties while maintaining the quality of the product (Suppakul et al., 2003; de Oliveira et al., 2007).

Definitions stated in Regulations 1935/2004/EC and 450/2009/EC, consider that "active materials and articles are intended to extend the shelf life, maintain or improve the condition of packaged food" (Campos et al., 2011).

Packaging materials protect food from surrounding challenges. Changes in industrial procedures (e.g. introduction of combined techniques to obtain medium- and high-moisture food products), research on the application of emergent stress factors (e.g. high pressures, development of convenient food products with longer shelf life), and changing in retailing practices and/or in way of life, have promoted the development of new packaging materials (Camps et al., 2011).

Consumer demands for more natural foods, and for environmental protection, catalyzed, during past decades, the development of new packaging materials. Active, intelligent, and edible packaging is emerging factors of all this background (Campos et al., 2011).

Active food packaging is based on a deliberate interaction of the packaging with the food and its direct environment. They contain components intended to release or absorb substances into/from the packaged food or the environment surrounding the food. Active materials and articles are allowed to bring about changes in the composition or the organoleptic characteristics of the food on condition that the changes comply with the provisions foreseen in the Community or national food legislations (Reg.1935/2004) (Dainelli et al., 2008).Firstly introduced in the market of Japan in the mid-1970s, active and intelligent packaging, only in the mid-1990s raised the attention of the industry in Europe (Fig.1.3) (Restuccia et al., 2010).

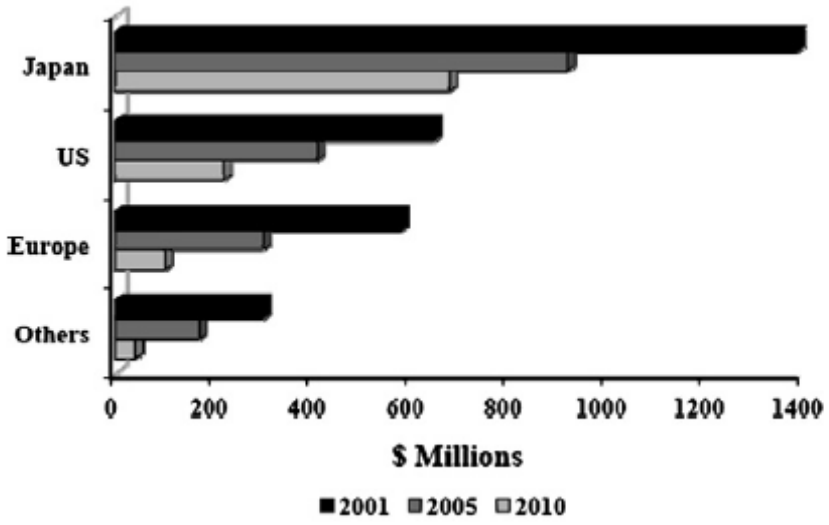


Fig.1. 3. Global markets for active packaging 2001–2010 (Anon. August, 2007).

Their low diffusion in EU countries has been related to two main reasons: cost and acceptance (Dainelli et al., 2008).Considering costs, it is obvious, that the packaging cost will drastically be reduced with broader application and thus scaling-up of production. Discussions are ongoing as to whether consumers will be ready to pay the extra costs for the extra safety/quality tools (Lähteenmäki and Arvola, 2003).Furthermore, consumers are demanding food-packaging materials that are more natural, disposable, potentially biodegradable, as well as, recyclable. For this reason, there is a growing interest in the study and development of renewable source-based biopolymers able to degrade via a natural composting process for antimicrobial active packaging applications (Cha and Chinnan, 2004; Kumar et al., 2004; Lopez-Rubio et al., 2004; Chiellini, 2008; Spizzirri et al., 2010).

The second problem is acceptance. Paradoxically, whereas the concept of active and intelligent packaging is now considered as ‘modern’, the concept belongs to ancestral traditions in all tropical areas of the world. In some regions of Africa, Asia, and South America, vegetal leaves were and still are traditionally used for food packaging in markets dedicated to leaves commercialization. In fact, beyond their use as a simple ‘barrier’, numerous varied vegetal leaves are used for their ability to transfer

aromatic, coloring, enzyme or antimicrobial substances (e.g. essential oils) to foods. Vegetal leave packaging interacts with foods for modifying their texture and organoleptic properties or slowing down microbial spoilage. They are also used for their ability to change colour with temperature and/or time, thus playing the role of cooking or freshness indicators. Up to four different types of leaves are used as successive layers, each of them having a very specific function. Leaves have also been used for ages in Mediterranean regions of Europe, e.g., to wrap traditional cheeses for allowing good maturation process. In developed countries, moisture and oxygen absorbers were among the first series of active and intelligent packaging, to be developed and successfully applied for improving food quality and shelf-life extension (e.g. for delicatessen, cooked meats etc.). Next to these, numerous others concepts such as ethanol emitters (e.g. for bakery products), ethylene absorbers (e.g. for climacteric fruits), carbon dioxide emitters/absorbers, time/temperature and oxygen indicators etc. have been developed. If compared to Japan, USA or Australia, the penetration of active and intelligent packaging in the European market is limited thus far (Fig.1.4).This time lag was mainly attributed to a complex and not enough flexible European regulation that could not keep up with

technological innovations in the food-packaging sector (Dainelli et al., 2008).

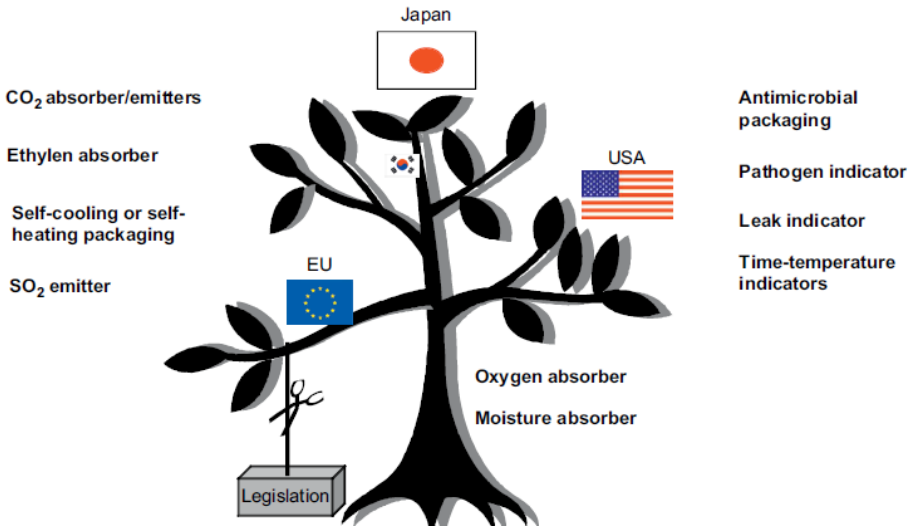


Fig.1.4. World diffusion of active packaging (Gontard, 2000).

The principles behind active packaging are based either on the intrinsic properties of the polymer used as packaging material itself or on the introduction (inclusion, entrapment etc.) of specific substances inside the polymer. An active agent can be incorporated into the packaging material or onto its surface, in multilayer structures or in particular elements associated with the packaging such as sachets, labels or bottle caps (Gontard, 2000). The nature of the active agents added is very diverse (organic acids, enzymes, bacteriocins, fungicides, natural extracts, ions, ethanol etc.), as well as the nature of the materials into which they are

included such as papers, plastics, metals or combinations of materials. The active systems can be placed outside the primary packaging, in between different parts of the primary packaging or also inside the primary packaging. In this last case, the systems can be in contact only with the atmosphere surrounding the food, with the food surface or placed inside the food itself (for liquid foods). Active packaging can be classified into two main types: *non-migratory* active packaging acting without intentional migration, and *active* releasing packaging allowing a controlled migration of non-volatile agents or an emission of volatile compounds in the atmosphere surrounding the food (Fig.1.5) (Dainelli et al., 2008).

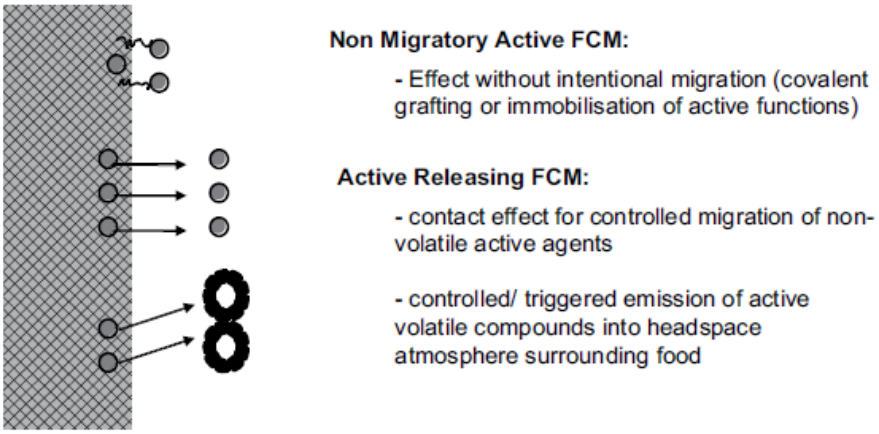


Fig.1.5. Two different types of active food contact materials (FCM) classified as a function of intentional or unintentional migrations (Dainelli et al., 2008).

- *Non-migratory active packaging* is a packaging, which elicits a desirable response from food systems without the active component migrating from the packaging into the food. The most well-known examples of non-migratory active packaging are moisture absorbers, mostly based on the adsorption of water by a zeolite, cellulose and their derivatives. In the market of moisture absorbers systems, the tendency is to introduce the absorbing substances inside the packaging material in order to make the active system invisible for the consumer (Dainelli et al., 2008). Another example of non-migratory packaging is the antimicrobial packaging based on the entrapment in a silicate network of silver ions, a widely marketed antimicrobial agent. Nevertheless, even food contact antimicrobial systems with an assumed non-intended migration (e.g. silver or silver-based systems, other immobilized or grafted biocides), are known to exhibit some degree of migration.
- *Active releasing packaging* elicits a desirable response from food systems, by migration of active component into the food. In the field of active releasing packaging, one of the best-known products is an ethanol releaser, which is able to slow down mold growth and increase the shelf-life of bakery products. Water-vapour releasing

sachets are a key element of successful innovative packaging for ready-to-cook fresh vegetables. Another example of active releasing materials is a plastic film containing ally isothiocyanate (AITC), a strong antimicrobial substance extracted from mustard or wasabi. AITC is entrapped in cyclodextrins for protecting the volatile active agent from being thermally degraded during extrusion. When exposed to high moisture conditions after the packaging of the food product, cyclodextrins have the ability to change in structure and to release the antimicrobial agent in the atmosphere surrounding the food (Lee, 2005). Much research is devoted to the design of antimicrobial packaging containing natural antimicrobial agents for specific or broad microbial inhibition, depending on the nature of the agents or on their concentration. Different types of antimicrobial delivery systems and combination of packaging material food are developed to maximize the efficacy of the system (Gontard, 2007; Dainelli et al., 2008).

Thus, there is a strong need to better understand the active agent principles and mechanisms as well as to optimize their use in order to design active packaging elements that:

(i) are sufficiently effective and reduce detrimental side effects;

(ii) assure an accurate, knowledge- based, assessment of potential risks.

Bibliographic data showed that antimicrobial systems are often not reproducible in the challenging field of antimicrobial packaging or even contradictory. It is the case of chitosan or chitosan-based systems of which the biocide phenomenology and optimum efficiency have just recently been understood, because of the positive migration of glucosamine fractions and food composition (Fernandez-Saiz et al. 2006; Fernandes et al., 2008).

1.2 Antimicrobial Packaging

Antimicrobial packaging is a type of active packaging that acts reducing, inhibiting or retarding microorganism growth contaminating the packaged food (Appendini and Hotchkiss, 2002; Santiago-Silva et al., 2009). Antimicrobial packaging can take several forms:

- Addition of sachets pads containing volatile antimicrobial agents into the package.

The most successful commercial application of antimicrobial packaging has been sachets that are enclosed loose or attached to the interior of a package. Three forms have predominated: oxygen absorbers, moisture

absorbers, and ethanol vapor generators. Oxygen and moisture absorbers are used primarily in bakery, pasta, and meat packaging to prevent oxidation and water condensation. Although oxygen absorbers may not be intended to be antimicrobial, a reduction in oxygen inhibits the growth of aerobes, particularly molds. Ethanol vapor generators consist of ethanol absorbed/ encapsulated in carrier materials and enclosed in polymer packets. Absorbing pads are used in trays for packaged retail meats and poultry to soak up meat exudates. Organic acids and surfactants have been incorporated into these pads to prevent microbial growth in the exudates, which are rich in nutrients (Hansen et al., 1989).

- Use of polymers that are inherently antimicrobial.

Packaging materials which are inherently antimicrobial could be used in at least three ways to package foods. First, they could extend shelf-life and promote safety by reducing the rate of growth of specific microorganisms in non-sterile foods, such as refrigerated milk. Secondly, antimicrobial packaging could be self-sterilizing or sanitizing. Such a material would greatly reduce the potential for recontamination of processed products and might greatly simplify the aseptic packaging process, by eliminating the need to treat the packaging material prior to filling. Lastly, this concept could result in self-sterilizing foods, especial liquids. This might be

particularly useful for high acid products such as fruit juices. Anti-microbial polymers might also be used to treat surfaces of food processing equipment so that they might self-sanitize during (Appendini and Hotchkiss, 2002).

- Coating or adsorbing antimicrobials onto polymer surfaces.

Antimicrobials, that cannot tolerate the temperatures used in polymer processing, often are coated with the material after forming or added to cast films. Cast edible films, for example, are used as carriers for antimicrobials and applied as coatings onto packaging materials and/or foods. Examples include nisin/methylcellulose coatings for polyethylene films (Cooksey, 2000) and nisin/zein coatings for poultry (Food Safety Consortium Newsletter, 2000; Appendini and Hotchkiss, 2002).

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

This type of immobilization requires the presence of functional groups on both the antimicrobial and the polymer. In addition to functional antimicrobials and polymer supports, immobilization may require the use of *spacer* molecules that link the polymer surface to the bioactive agent. These spacers allow sufficient freedom of motion, so the active portion of the agent can contact microorganisms on the food surface. Spacers used for

food antimicrobial packaging may include dextrans, polyethylene glycol (PEG), ethylenediamine and polyethyleneimine, due to their low toxicity and common use in foods. The potential reduction in antimicrobial activity due to immobilization must be considered (Appendini and Hotchkiss, 2002).

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

The incorporation of antimicrobial agents in flexible films is a promising technology, as the majority of solid or semi-solid foods present high microbial growth on their surface. The rationale for incorporating antimicrobials into the packaging is to prevent surface growth in foods, where a large portion of spoilage and contamination occurs (Silveira, 2005).The gradual release of an antimicrobial, from a packaging film to the food surface may have an advantage over dipping and spraying (Oliveira and Oliveira, 2004; Santiago-Silva et al., 2009).Additives released from active packaging increase the consumers' safety since these compounds, instead of being directly added to the food, are released in a controlled way, in smaller amounts and only where they are necessary, such as on the product surface (de Oliveira et al., 2007).

Antimicrobials in food packaging that may migrate to food are considered food additives, so must meet the food additive standards (Appendini and Hotchkiss, 2002). Various natural and synthetic compounds are being used to produce antimicrobial food film. Some of them include metallic ions, organic acids, benzoates, sorbates, isothiocyanates and bacteriocins (Pires, 2006). The bacteriocins are an attractive option of antimicrobial compounds, as they constitute natural preservatives, avoiding the addition of synthetical compounds to food (Cotter et al., 2005; Santiago-Silva et al., 2009).For the selection of an antimicrobial, it must be considered the effectiveness against the target microorganism and the possible interactions among the antimicrobial, the film-forming biopolymer, and others food components present. These interactions can modify the antimicrobial activity and the characteristics of the film being these key factors for the development of antimicrobial films and coatings (Campos et al., 2011).

The most common antimicrobial agents used in edible films and coatings are:

- Organic Acids.

Organic Acids such as lactic, acetic, malic, and citric acids, among others, are present in the composition of many foods and widely used for preservation. The antimicrobial activity is based on pH reduction,

disruption of substrate transport, and reduction of proton motive force (Campos et al., 2011).

Acildulants.

The most common acidulant agents are acetic, lactic, and malic acids. They are obtained by fermentation and are effective against the main pathogen bacteria encountered in foods (Samelis and Sofos, 2003).Organic acids have been used as acidulants in edible films made from carbohydrate, proteins, and chitosan. The type and concentration of organic acid modified the mechanical properties of films. From the point of view of the antimicrobial activity, organic acids exert different effects depending on the type of acid, its concentration, the systems composition, the environmental conditions, and the target microorganism. As a consequence, it is not possible to predict the inhibitory activity taking into account the data obtained from in vitro experiments using culture media (Campos et al., 2011).

Weak Lipophilic Acids.

Weak lipophilic acids have the ability to penetrate the cell membrane when they are in the undissociated form; they act acidifying the cytoplasm and inhibiting the growth. Its antimicrobial action is enhanced by the addition of anacidulant which decreases the pH (Sofos,

2000). Within the lipohilic acids, sorbic acid and its potassium salt are themost frequently used in edible films. The potassium salt and the acid, commonly named as sorbates are usually employed because of their high solubility in water (Sofos, 2000). Sorbates inhibit the growth of bacteria, yeast, and molds, being more effective against the latter. The antimicrobial action is more pronounced at pH below 5.0and depends on system composition and environmental conditions. Potassium sorbate presence changed the physical properties of films based in different biopolymers such as whey proteins (Cagri et al., 2001) or polysaccharides (Vásconez et al. 2009; Shen et al. 2010).In relation to mechanical properties, sorbates, as well as other organic acids, act as plasticizers and, as expected, they increase the elongation and decrease tensile strength (Campos et al., 2011).

- Bacteriocins.

The bacteriocins are antimicrobial proteins/peptides produced by bacteria that acts inhibiting or retarding the growth of other bacteria. Lactic acid bacteria of food origin produce some bacteriocins. This makes possible their application to control of some specific bacteria growth in food (Cotter et al., 2005). Bacteriocins are nontoxic and non-antigenic to humans and

have status GRAS(generally recognized as safe)(Halami and Chandrashekar, 2005; Santiago-Silva et al., 2009).

Nisin.

Nisin is a small antimicrobial peptide produced by lactic acid bacteria; it inhibits gram positive bacteria such as L. *monocytogenes* and Staphylococcus *aureus* and gram-negativebacteria when the bacteria cell wall was previously weakened by a permeabilising agent such as EDTA or lysozyme. Nisin is generally recognized as safe and is permitted for use in over 50 countries (Campos et al., 2011).This bacteriocin has been widely used as a safe and natural preservative in thefood industry (Cè et al., 2012).Nisin has been tested on meat and meat products, dairy foods, and vegetarian foods (Thomas et al., 2000). It has been used in films made by tapioca starch (Sanjurjo et al., 2006), whey protein (Pintado et al., 2009), sodium caseinate (Kristo et al., 2008), soy protein (Eswaranandam et al., 2004), methylcellulose, and hydroxypropyl methylcellulose (Campos et al., 2011).

Lactoperoxidase.

The lactoperoxidase system (LPS) is a natural antimicrobial present in milk and in mammalians saliva and tears. It presents a broad antimicrobial spectrum since it shows bactericidal effect on Gram (−)

bacteria, bacteriostatic effect on Gram (+) bacteria, and antifungal activity (Naidu, 2000).The LPS system consists of three components: LPS,thiocyanate, and hydrogen peroxide (H_2O_2). The enzyme catalyzes the oxidation of thiocyanate by the use ofH_2O_2 and produces hypothiocyaniteand hypothiocyaniteacid. These products inhibit microorganisms by the oxidation of sulphydryl (–SH) groups in their enzyme films systems and proteins (Seifu et al., 2005).Lactoperoxidase and its components have been used in whey protein (Min et al., 2005) and in alginate films (Yener et al., 2009;Campos et al., 2011).

Natamycin.

Natamycin is a naturally occurring antifungal agent classified as a macrolide polyene, produced during fermentation by the bacterium Streptomyces natalensis (Cè et al., 2012). Natamycin kills yeasts inhibiting vacuolar fusion through the specific interaction with ergosterol (Ollè Resa et al., 2013). It has been used in the food industry as a hurdle to fungal outgrowth in dairy products, meats, and other foods (Delves-Broughton et al., 2006). More specifically, natamycin is commonly used in products such as cottage cheese, sour cream, and yogurt (Cè et al., 2012).The use of natamycin is allowed by the Brazilian legislation, Mercosul (South Economic Community) and the

European Community as an additive for food preservation. (de Oliveira et al., 2007). According to Directive 95/2/EC, natamycin may be used for the surface treatment of semi-hard and semi-soft cheese and dry cured sausage at maximum level of 1 mg/dm^{2}in the outer 5 mm of the surface (EFSA, 2009).

- Antimicrobials from Herbs and Spices.

Essential oils (EOs) are aromatic oily liquids obtained from individual or integrated plant material: flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots (Burt, 2004). EOs are commonly obtained by steam distillation of plants. Chemical composition of EOs is complex and strongly dependent on the part of the plant considered (e.g., seed vs. leaves), the moment of the harvest (before, during, or after flowering), the harvesting season and the geographical sources. Major components in EOs are phenol substances, which are thought as the responsible of the antimicrobial properties, and many of them are classified as GRAS.The antimicrobial activity of the EOs can be attributed to their content of monoterpenes that, due to their lipophilic character, act by disrupting the integrity of microbial cytoplasmic membrane, which thus loses its high impermeability for protons and bigger ions. Lipophilic compounds accumulate in the lipid bilayer, leading to disruption of the membrane

structure (Zhang et al., 2009). Then, membrane functions are compromised, not only as a barrier but also as a matrix for enzymes and as an energy transducer (Liolios et al., 2009). Some disadvantages of EOs are their biological and chemical instability, reduced solubility in water and poor distribution to target sites. In general, levels of EOsand their compounds necessary to inhibit microbial growth are higher in foods than in culture media. This is, in part, due to interactions between phenolic compounds and some components of the food matrix like proteins and fat. Moreover, regarding the performance of someEOs incorporated in edible films, it is important the study of their stability. Du et al. (2008) determined the destruction of carvacrol, the main constituent of oregano oil, during the preparation and storage of apple-based films made by continuous and batch casting methods (Campos et al., 2011).

- Antimicrobial packaging with inorganic nanoparticles.

The utilization of inorganic nanoparticles as antimicrobial agents has recently gained popularity which attributed to the good stability of these materials to withstand harsh process conditionssuch as high pressures or temperatures in plasticfabrication process. The most extensively studied inorganicnanoparticles for antimicrobial purposes were titanium dioxide (TiO_2) and zinc oxide (ZnO). Titanium dioxide (TiO_2) isnon-toxic and

approved by FDA for used in foods, drugs,and food contact materials.TiO_2generated hydroxyl radicals and reactive oxygen species via light reaction which can inactivate microorganisms by oxidizing the polyunsaturated phospholipids component of the cell membrane. The applications of ZnO nanoparticles coating systems have recently attracted a great deal of attention due to its antimicrobial activity towards both the gram-positive and gram-negativebacteria. Kinetics of bacterial growth showed that the growth rate of bacterial was suppressed appreciable in the solution containing ZnO-coated film (Sung et al., 2013).

The incorporation of antimicrobial agents into polymer can adversely affect the physical properties, mechanical integrity and thermal stability of packaging when the antimicrobial agents used are not compatible with the polymer. The study of polymer chemistry and structure are important to predicting the influence of some antimicrobial agents on the packaging. Subsequently, the selection of antimicrobial agents, packaging polymers, and incorporation methods can be more effective.

Mechanical properties of film, as tensile strength (TS) and elongation at break, are the most important characteristics to be analyzed and benchmarking. These properties can measure film strength and stretch ability prior to breakage when extension force applied. The incorporation

of additives into polymers, other than cross linking agents, generally detriment the packaging tensile strength. Additives are usually small components that tend to occupy the space between polymer chains and cause slippage when external forces being applied. This phenomenon is called “plasticizing effect”, and can be proven by comparing antimicrobial agent incorporation methods between both coating method and extrusionmethod. Generally, TS did not reduce significantly when small amount of antimicrobial additives applied. Another property that should take into accounts water vapor transmission that represents the ease of moisture to penetrate and pass through material (Li et al., 2006). It is important for a packaging to have good water vapor barrier. Water can accelerate microorganisms’ growth and reduce shelf-life of foods. Water vapor transmission of antimicrobial packaging is very much dependent on the hydrophilic and hydrophobic ratio of the antimicrobial-matrix material. Hydrophilic material tends to increase packaging water vapor transmission (Sung et al 2013).

1.3 Active Packaging Legislation

The European Union’s Regulation 1935/2004/EC offered for the first time the opportunity for active packaging to be used in Europe by allowing the

application of materials with agents that could migrate into foods. The Regulation, regarding all materials and articles intended to come into contact with food, contains also general provisions on the safety of active and intelligent packaging and sets the framework for the European Food Safety Agency (EFSA) evaluation process. The Regulation 450/2009/EC can be considered a measure that lays down specific rules for active and intelligent materials and articles to be applied for their safe use in addition to the general requirements established in Regulation 1935/2004/EC. The new regulation represents a partial answer to the lack of penetration of active and intelligent packaging in the European market. In fact, Japan, USA, and Australia in past years were more adequate, because of the flexible regulations permitted technological innovations in food packaging(Restuccia et al., 2010).Definitions stated in Regulation 1935/2004/EC and in Regulation 450/2009/EC consider active materials and articles: ‘‘*materials and articles that are intended to extend the shelf-life or to maintain or improve the condition of packaged food*”. They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food (Floros et al., 1997, Brody, 2001).To guarantee the conformity of the used materials the Framework Regulation 1935/2004/EC

demands in article 16 the preparation of a declaration of compliance. This documentation, shall contain the description and the results of the analysis carried out to demonstrate the compliance of the material and article, and in particular the compliance with quantitative restrictions in the use of the substances such as OML, SML, etc (Fig.1.6) (Restuccia et al., 2010).

- **Specific Migration Limit (SML)**means the maximum permitted amount of a given substance released from a material or article into food or food stimulants;
- **Overall migration limit (OML)**means the maximum permitted amount of non-volatile substances released from a material or article into food stimulants.

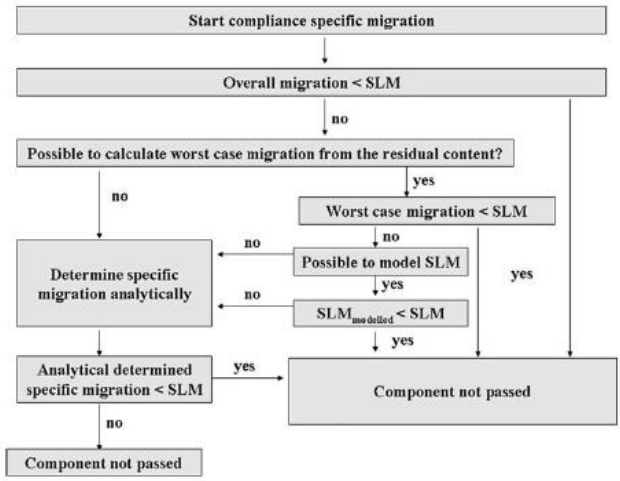


Fig.1.6. *General scheme adopted for safety assessment applied to every component with a specific migration limit in a food- contact material(Restuccia et al., 2010).*

Regulation 10/2011/EC set requirements for testing the migration from food contact materials. To determine the extent of chemical transfer from packaging into food migrants are measured in food stimulants. Food simulants are used as substitutes for food due to the simplification of chemical analysis. Chemical detection and quantification require specific analytical methods for each chemical of interest, specially developed for each food or food simulant type. Food simulants vary in terms of their chemical properties, thus representing several different food types: hydrophilic (water-based), lipophilic (fatty foods) or amphiphilic (foods with both watery and fatty properties). For example, migration into an oily food is measured with the food simulant vegetable oil (substitute of the simulant95% ethanol). The food simulants 10% ethanol or 3% acetic acid are used for water-based and acid foods and drinks. Dry foods are simulated by a synthetic polymer with a defined pore size (trade name "Tenax"). Butter and other foods that are amphiphilic are simulated by 50% ethanol solution.

As regards the non-active part, e.g. the packaging of the active constituents, it should comply with the applicable food contact legislation (Fig.1.7).

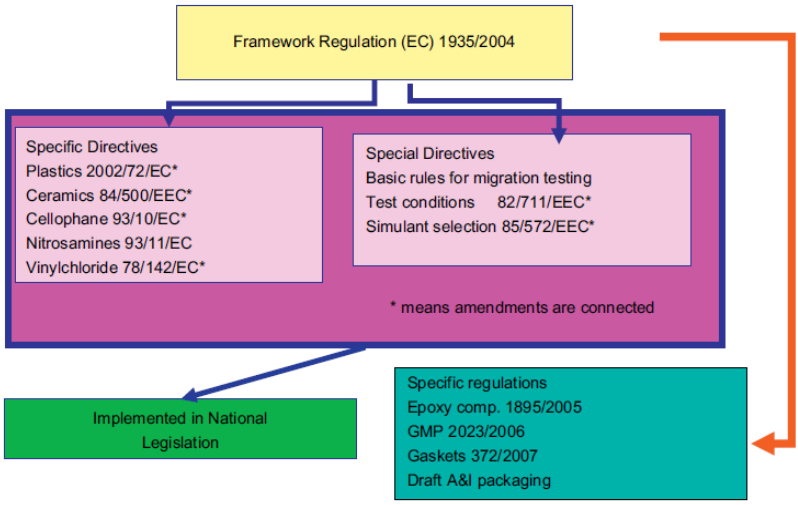


Fig.1.7. Food contact material legislation in EU(Dainelli et al., 2008).

This is the Framework Regulation 1935/2004/EC for all food contact materials. For the active/intelligent component in active and intelligent packaging, there is no specific Directive at this moment and they have to comply with the Framework Regulation 1935/2004/EC. Important articles are Articles 3(applicable to the packaging of the active or intelligent components) and 4 (on active and intelligent components).The first one states that food contact materials shall not transfer constituents to food in quantities, which could:

- endanger human health;

- bring about an unacceptable change in the composition;

- bring about deterioration composition in organoleptic characteristics thereof.

Article 4 describes special requirements for active and intelligent materials in which the main issues are:

- active materials may bring about changes in the composition or organoleptic characteristics of food on the condition that the changes comply with the community or national provisions applicable to food;

- substances that are released from active packaging shall be authorized and used in accordance with the relevant community provisions applicable to food;

- active materials shall not bring about changes in the composition or organoleptic characteristics of the food, for instance by masking the spoilage of food, which could mislead the consumer;

- intelligent materials shall not give information about the condition of the food which could mislead the consumer;

- adequate labeling to allow identification of non-edible parts;

- adequate labeling to indicate that the materials are active and/or intelligent(Dainelli et al., 2008).

The authorization of active and intelligent components has to be granted in accordance with *Articles 7–9* of Regulation 1935/2004/EC, upon

submission of application. Application shall comprise a technical dossier containing specified information and EFSA shall give an opinion within 6 + 6 months providing an explanation for the delay.

Article 15 provides authority to require suitable labeling where needed to advance traceability or safety of use; it contains a great deal of detail about multi-language labeling and what the states

can do to accomplish any local labeling purposes. A declaration of compliance with the Regulations, and the making of all data available to competent authorities is required (*Article 16*).In addiction,all materials and articles must be labeled or otherwise identified so that traceability can be accomplished(*Article 17)*(Restuccia et al., 2010).

General requirements stated in Regulation 1935/2004/EC for the safe use of active and intelligent packaging have been integrated by Regulation 450/2009/EC.

Commission Regulation 450/2009/EC is the specific measure under the Framework Regulation that regulates active and intelligent materials and articles. This Regulation includes additional provisions:

-the individual substance or group/combination of substances which make up the active or intelligent component should be safe and comply with the

requirements in the Framework Regulation 1935/2004/EC and the Regulation 450/2009/EC;

- substances should undergo a safety assessment by EFSA before they are authorized for use,

- a Union list of substances or group/combination of substances to be used in active and intelligent components should be drawn up following risk assessment of these substances by EFSA;

- substances released from active releasing materials should comply with any restrictions in the existing food law (e.g. as authorized food additives) thus complying with the safety requirement;

- the overall migration from active releasing materials can exceed the overall migration limits described in EU or national legislation as long as the levels transferred to the food comply with restrictions in the existing food law (e.g. as authorized food additives). The transfer of these active substance/substances should not be included in the calculation of the OML;

- as well as complying with the Framework Regulation the passive parts of the active and intelligent packaging materials must also comply with the rules applicable to the same materials and articles when they do not contain the active component, such as the Plastics Regulation 10/2011/EC. For

materials such as paper and board for which the specific requirements are not regulated at EU level existing national legislation should be applied;

- intelligent systems that are on the non-food contact surface of the package can be separated from the foodstuff by a functional barrier, i.e. a barrier to any migration. If it is demonstrated that the packaging material acts as a functional barrier to migration then non-authorised substances can be used providing they meet specific criteria defined in the Regulation.

Active packaging systems that intentionally release substances into the package must comply with the (direct) food additives legislation (Reg.1333/2008/EC)(Restuccia et al., 2010).

2. Biodegradable Polymers for Food Packaging and Edible Films

2.1 Biobasedmaterials for Food Packaging application.

Synthetic packaging films have led to serious ecological problems due to their non biodegradability. The major concern of the consumer for environmental protection led food and packaging industries to increase research in biodegradable food packaging materials. In this context, biopolymers can be an alternative source for packaging materials development due to their biodegradability (Fajardo et al., 2010).

Owing to consumer health concern and environmental problems derived from packaging wastes, various research groups have been looking toward green polymers as biodegradable alternatives to synthetic plastic packages (Imran et al., 2010; Jamshidian et al., 2010; Mastromatteo et al., 2010). To acquire this ‘green-tag’ by using bioactive preservative in biodegradable packaging material, researchers have studied incorporation of different bioactive (Imran et al., 2014). The current global consumption of plastics is more than200 million tons,with an annual grow of approximately5%, which represents the largest field of application for crudeoil.Until now petrochemical-based plastics such as polyethyleneterephthalate (PET), polyvinylchloride (PVC), polyethylene(PE), polypropylene (PP), polystyrene (PS) and polyamide (PA) have been increasingly used as packaging materials because their large availability at relatively low cost and because their good mechanical performance such as tensile and tear strength, good barrier to oxygen, carbon dioxide, anhydride and aroma compound, heat sealability, and so on. But nowadays their use has to be restricted because they are not non-totally recyclable and/or biodegradable so they pose serious ecological problems (Sorrentino et al., 2007). Recycling this material is impracticable and most of the times economically not convenient. As a consequence, several thousands of tons

of goods, made on plastic materials, are land filled, increasing every year the problem of municipal waste disposal. The growing environmental awareness imposes to packaging films and process both user-friendly and eco-friendly attributes (Siracusa et al., 2008).

Biobased packaging materials do not pretend to replace traditional packaging materials; they can help to reduce the cost and also the amount of traditional packaging used. They are intended to lengthen shelf life and also to respond to consumer demand for even more natural products and for the lower contamination of the environment (Campos et al., 2011).

Biobased packaging materials can be created on the basis of polymers directly extracted/removed from natural materials, such as polysaccharides (starch and cellulose) or proteins like casein and gluten. Elaboration of these films and coatings has been possible thanks to the *filmogenic* capacity of natural biopolymers. For their formulation, can be used polysaccharides, proteins, and lipids and they must result neutral with respect to color and flavor. Hydrocolloids have good aptitude to form a continuous and cohesive matrix with adequate mechanical properties (Tab.1.2) (Bourtoom, 2009).

Such ability is related to the chemical structure of these compounds, which allows the association through hydrogen bonding of their polymeric chains.

The most common biopolymers used for antimicrobial film elaboration are polysaccharides (single or blend of several types), proteins (single or mixtures from different sources), and blends of carbohydrates and proteins. The combination of polysaccharides and/or proteins with lipids in blends, emulsions, or multilayer structures has been also studied for controlling film properties. Lipids are often supported on a polysaccharide matrix to provide a film with mechanical strength (Bourtoom, 2009). Film forming conditions and film composition affect additive migration and, as a consequence, the packaging effectiveness (Campos et al., 2011). To obtain biobased films, in a first step, the material must be properly dispersed and/or dissolved in a solvent like water, alcohol, diluted acids solutions, or mixtures of solvents. In some cases, it is necessary to heat or adjust the pH of the slurry containing the hydrocolloids; in order to dissolve the macromolecule (Vargas et al., 2008).The addition of substances with plasticizing provides the films with good mechanical behavior in terms of flexibility. The plasticizer most used is glycerol because of its better stability and compatibility with hydrophilic biopolymeric chains, if compared to sorbitol, PEG, and sugars (Fernández Cervera et al., 2004).

Tab. 1.2. *Hydrocolloids used in edible films elaboration, film mechanical properties and water vapor permeability (Campos et al., 2011).*

Hydrocolloid	**Concentration in film forming solution**	**Mechanical and permeability properties**			**References**
		Tensile strength (MPa)	**Strain at break (%)**	**Water vapor permeability (g mm m-2 day-1 KPa-1)**	
Cellulose ethers	HPC 3% w/w	16-18	60-110	0.043-0.056 (0/50-23 °C)	Belalia et al., 2008
	HPMC 6% w/v	20-43	26-41	3.6-89.9 (0/100-38°C)	Imran et al., 2010
	MC 1.5-6% w/w	17-44	14-97	2-10 (0/52-25°C)	Thuran et al., 2004
Starches	Tapioca 5% w/w	0.16-2.3	70	54-139 (0/70-25°C)	Flores et al., 2007
	Cassava 5% w/w	1.0-4.7	39-164	1.5-7.2 (0/75-23°C)	Kechichian et al., 2010
	Sweet potato 4% w/w	8-43	0.6-3.2	14-86(0/75-23°C)	Shen et al., 2010
Seaweed extracts	k- Carrageenan 0.1-1% w/w	57	7	-	Lafargue et al., 2007
	Alginate 1% w/w	39-66	2.7-4.8	18.7-30.9	Pranoto et al., 2005
Gums	Locus bean 0.9% w/v	4-40	0.5-10	-	Aydinli et al., 2004
	Gellan 0.5% w/v	-	-	18-23 (100/33-25°C)	Tapia et al., 2007

Pectin	Pectin citric fruit 2.5-5.8 mg/cm2	13-25	0.8-1.2	1.6-4.7 (0/84-25°C	Giancone 2006
Chitosan	2% w/v	5.5-21.3	7-43	4.8-7.3 (0/75-25°C)	Hosseini et al., 2009
	1% w/w	7-12	11-17	60-138 (100/59-5°C)	Vargas et al., 2009
	1.5% w/w	18-106	5-20	14-80 (100/50-25°C)	Zivanovic et al., 2005
Proteins	Sodium caseinate 5% w/w	3.4-4.1	78-125	-	Mendez et al., 2012
	Soy protein isolate 10% w/w	4.7-10-7	-	-	Sivarooban et., 2008
	Whey protein isolate 5% w/w	4-16	1-10	204-264 (100/53-25°C)	Zinoviadou et al., 2009
Blends	Whey protein/HPMC	4-61	16-112	-	Brindle et al., 2008
	Starch/ chitosan	0.36-19.7	61-152	0.15-3.3 (0/70-25°C)	Chillo et al., 2008

When the hydrocolloids are dispersed, it is possible to add other substances like antimicrobials, antioxidants, flavorings, and colorants to the film-forming solution, in order to confer the desired functional property. The removal of the solvent in excess is the following step. The drying rate and environmental conditions will determine the final thickness and structural

characteristics of the resultant films. It is important to remark that film forming conditions and film composition affect additive migration and, as a consequence, its effectiveness (Campos et al., 2011).

It is desirable that films have selective barrier properties to several gasses. Biobased films have a very low oxygen permeability, even if hydrocolloid-based films possess high water vapor permeability (WVP)(Buonocore et al., 2005).Generally, lipid addition is the strategy selected to reduce the water vapor transmission rate (Ayranci and Tunc, 2003). Another possibility is the modification of polymer structure by cross linking reaction, photocrosslinking,gamma-irradiation, or reaction with polyvalentions (Marques et al., 2006).The resistance of biofilms to water, determined by the solubility in water test, is critical for the potential food application. Sometimes, high water solubility is desired, for example when the film or coating will be consumed simultaneously with the food (edible films). However, in other technological situations such as packaging application of films, a low solubility in water molecules is extremely necessary (Campos et al., 2011).

Biobased food packaging can be prepared from different renewable sources, such aspolysaccharides, proteins and/or lipids (da Silva et al., 2012). Polysaccharides (cellulose and derivatives, starches, seaweed

extracts, gums, pectins, chitosan) based films are transparent and homogeneous with moderate mechanical properties. However, their application is limited by their water solubility and WVP. To solve this shortcoming, blending with different biopolymers (Xu et al., 2005), addition of hydrophobic materials, such as oils or waxes (Ayranci and Tunc, 2003), or chemical modification of polymer structure (Marques et al., 2006) have been proposed (Campos et al., 2011).The ability of different proteins (milk protein) to form films and coatingsdepends on their molecular characteristics:molecular weight, conformations, electrical properties(charge vs pH), flexibilities, and thermal stabilities (Vargas et al., 2008). Film formation involves heattreatment to denature the protein, followed by solventevaporation (casting). Protein-based films potentially havea good gas barrier and mechanical properties, ifcompared with those obtained from polysaccharides andfat-based films. Proteins, in fact, have a high intermolecular binding potential, even if the poor water vapor resistance limits their application.Fortunately, improvement of protein film properties couldbe attained by modifying the properties of protein bychemical and enzymatic methods, combining them withhydrophobic materials or some polymers, or using aphysical method (Bourtoom, 2009; Campos et al., 2011).In general, biobased polymers may be divided

intothree main categories based on their origin andproduction (Fig.1.8)(Haugaard et al., 2001; Weber et al., 2010).

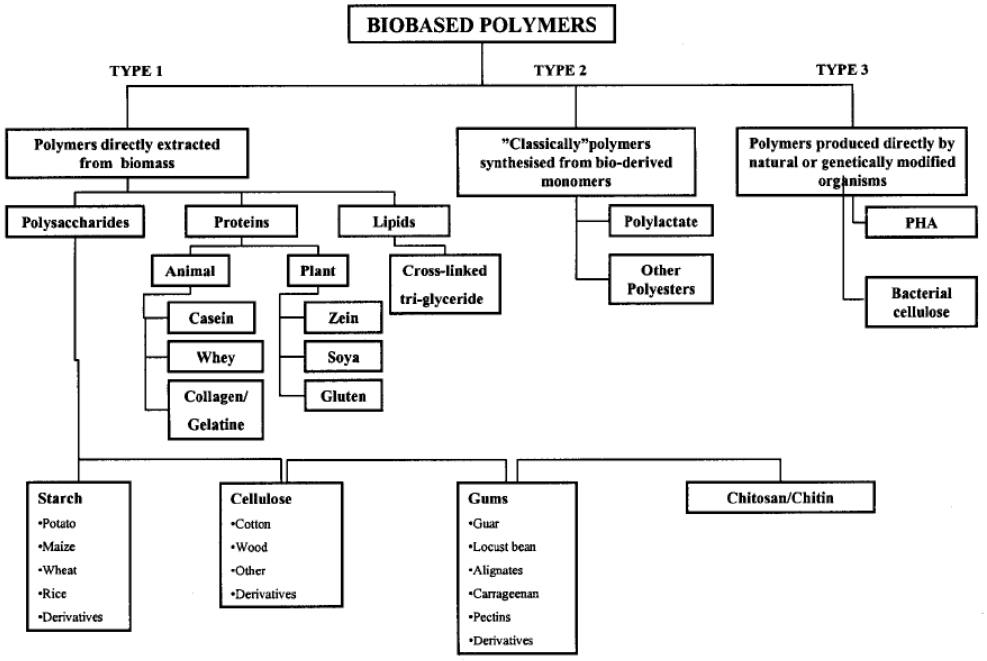


Fig.1. 8.Origin of different categories of biologically based materials (Weber et al., 2010).

- Category 1**:** Polymers directly extracted/removedfrom biomass. Examples are polysaccharides, suchas starch and cellulose, and proteins, like casein and gluten.

- Category 2: Polymers produced by classical chemical synthesis using renewable biobased monomers. A good example is polylactic acid, a biopolyester polymerized from lactic acid monomers.
- Category 3: Polymers produced by microorganisms or genetically modified bacteria. This group of biobased polymers consists mainly of the polyhydroxyalkanoates, but developments with, for example, bacterial cellulose are in progress.

The markets of biobased food-packaging materials are expected to start out as niche markets, where the unique properties of the biobased materials apply an advantage to the packaging concept. In the following, a few niche markets have been pinpointed, but certainly more possibilities will open up for these materials as the developments seem to go very fast. Biobased packaging can be used to preserve either vegetable or animal origin products. Fresh or minimally processed fruits and vegetables are very perishable and required of some combined techniques to extend properly the shelf life. Fruit and vegetable tissues may remain biologically active, suffering many physiological changes from post-harvest, during storage, and until they are consumed or processed. In addition, operations like washed, sorting, trimming, peeling, slicing, coring, etc., are usually carried

out on these products, promoting cell tissue disruption and membrane collapse (Weber et al., 2010).

Edible films and coatings have long been known to protect perishable fruits and vegetables from deterioration by retarding dehydration, suppressing respiration, improving textural quality, helping to retain volatile flavor compounds, and reducing microbial growth (Tab.1.3) (Haugaard et al., 2001; Lin and Zhao, 2007; Maftoonazad et al., 2007).

In addition, they can be used as a vehicle for incorporating functional ingredients, such as antioxidants, flavor, colors, antimicrobial agents, and nutraceuticals (Garcia et al., 2008). Garcia et al. (2008) wrapped pumpkin cylinders with tapioca starch edible films containing potassiumsorbate and demonstrated that films could act as a physical barrier to exclude the entrance of microorganisms, provided a source of preservative available to prevent microbial growth at the surface, and, at the same time, controlled spoilage flora in the pumpkin tissue, since part of the antimicrobial was released to the food.

Tab.1.3.Example of application of biobased packaging materials and edible films/coatings on fruit and vegetables (Haugaard et al., 2001).

Product example	Function of packaging	Value added function	Examples of materials
BIOBASED PACKAGING	containment	-	Starch based plastics, pulp trays, cellophane (Germany)
Fruit and vegetables			
Soft fruits	containment	-	Nitrocellulose coated cellophanes (Belgium)
Fruits	containment	-	Pulp containers (Germany)
Fresh products (lettuce, broccoli, etc)	Moisture barrier, oxygen barrier, carbon dioxide barrier, mechanical protection	-	Laminate of chitosan-cellulose, polycaprolactone, protein
Cut vegetables	-	Anti-fogging	Starch laminate
EDIBLE COATING			
Fruit and vegetables	Oxygen barrier, Carbon dioxide barrier	-	Chitosan, carnauba, wax, zein

Other authors used chitosan-based coating to improve the quality of mango slices and verified the effectiveness of coating for the inhibition of mesophilic aerobic bacteria (Chien et al. 2007). Durango et al. (2006) evaluated the application of a starch–chitosan coating on minimally

processed carrots and observed a substantial inhibition of total viable count, lactic acid bacteria, psychrotrophictotal coliforms, and yeasts and molds. Edible films have also demonstrated their effectiveness for the organoleptic and nutritional preservation of fresh vegetables. Methylcellulose coating containing ascorbic, citric, and stearic acids lowered the browning rate and the reduction of vitamin C in mushrooms and cauliflower (Ayranci and Tunc 2003; Campos et al., 2011).

On the other hand, specific properties are required for packaging materials to assure the shelf life of meat and meat products (Tab.1.4), because of most outbreaks of contamination are associated with the consumption of meat products (Coma, 2008).

Contamination with pathogens may occur during further processing or packaging. Minimally processed ready to eat meats are a potential source of food-borne pathogens such as Salmonella *typhimurium*, L. *monocytogenes*, and E. *coli*O157:H7(Haugaard et al., 2001; Quintavalla and Vicini, 2002; Campos et al., 2011).Edible films and coatings carrying antimicrobials are a promising tool for decreasing the risk of pathogenic bacteria and also for extending product shelf life. In meat products, application of a coating or film not only is useful as a carrier of the antimicrobial but it can also prevent moisture loss during storage of fresh

or frozen meats, hold juices of fresh meat cuts when packed in plastic trays, reduce the rate of rancidity, and restrict volatile flavor loss and the uptake of foreign odors (Haugaard et al., 2001; Quintavalla and Vicini, 2002; Campos et al., 2011).

Tab.1.4. Example of application of biobased packaging materials and edible films/coatings on meat products (Haugaard et al., 2001).

Product example	**Function of packaging**	**Value added function**	**Examples of materials**
BIOBASED PACKAGING			
Beef and chicken			Trays of virgin pulp, mixture of wood pulp and starch (Germany)
	Absorb moisture		Virgin paper with polyethylene top sheet
Ground Beef	Oxygen barrier		Starch polyethylene films containing corn starch
EDIBLE COATING			
Fresh meat	Moisture barrier	Antioxidants	Alginate, cellulose, soy protein
Cured meat	Oxygen barrier	Antimicrobial agents	
Cooked meat	Frying oil barrier		
Beef	Adhesion, mechanical protection, inhibit microbial growth		

The main objective of using edible films and coatings in seafood is to prevent the contamination with spoilage flora. The quality of seafood is quickly reduced during storage being chemical and enzymatic reactions the cause of the initial loss of freshness, while microbial spoilage produces the end of the shelf life. In many cases, the target inhibition is L. *monocytogenes* growth which constitutes the major risk in freshly processed cold smoked salmon (Datta et al., 2008).

Tab.1.5.Example of application of biobased packaging materials and edible films/coatings on seafood (Haugaard et al., 2001).

Product example	**Function of packaging**	**Value added function**	**Examples of materials**
EDIBLE COATING			
Fish	Oxygen barrier Moisture barrier	Antioxidants Time-dependent migration	Whey protein, acetylated monoglycerides
Frozen fish	Moisture barrier Oxygen barrier Mechanical protection	antioxidants antimicrobial agents batter adhesion	Caseins, whey proteins, lipids, alginate
Frozen crustacean (shrimp)	Moisture barrier Oxygen barrier Mechanical protection	Antioxidants Antimicrobial agents Batter adhesion	Caseins, whey proteins, lipids, alginate

On the other hand, another important objective is to avoid oxidative spoilage, in the case of fat specimens, by the use of antioxidant agents, and

also to prevent moisture loss (Tab.1.5) (Haugaard et al. 2001; Campos et al., 2011).

Milk, cream, fermented milk products and most cheeses require low oxygen permeability packaging to avoid oxidation and growth of undesirable microorganisms In the case of fresh and semi-hard cheese, microbial stability must be controlled. Edible films and coatings are used mainly to control microbial growth onthe surfaces and also to diminish the risk of post-processing contamination with L. *monocytogenes*. Also, the coating or film applied must be able to let gas exchange with the environment in order to maintain cheese quality (Tab.1.6)(Haugaard et al., 2001; Campos et al., 2011).Mainly during last decade, research concerning edible films and biobased packaging materials as supporters of antimicrobials has increased. (Campos et al., 2011).

Biobased packaging materials must comply with food and packaging legislation, and interactions between the food and packaging material must not compromise food quality or safety. Ideally, the material should biodegrade efficiently on disposal. Thus, environmental conditions conducive to biodegradation must be avoided during storage of the food product, whereas optimal conditions for biodegradation must exist after discarding (Petersen et al., 1999).

Tab.1.6. Example of application of biobased packaging materials and edible films/coatings on dairy products (Haugaard et al., 2001).

Product example	Function of packaging	Value added function	Examples of materials
BIOBASED PACKAGING			
Yogurt	Mechanical protection Moisture barrier Carbon dioxide barrier Oxygen barrier		Cardboard+unspecified biobased plastics, polyactic acid (Danone)
Butter/margarine	Moisture barrier Light barrier Grease barrier	Anti-fogging	Polylactic acid, co-polyester, co-polyamide, starch, colycaprolactone
Cheese		Formation of vacuum when the gas is adsorbed in the water phase (snug down)	Starch laminate
Soft cheese	Moisture barrier Gas barrier containment		Nitrocellulose coated cellophanes (Belgium)

Like conventional packaging, biobased packaging should provide consumers with mandatory product information as well as additional information such as cooking directions, recipes, etc. (Haugaard et al., 2001).

2.2 Chitosan and Methylcellulose films as carrier of Natamycin

Cellulose and chitosan are the most abundan tpolysaccharides on earth (Sangsuwan et al., 2009).

Chitosan is a weak cationic polysaccharide, formed mainly by (1,4)2-amino-2-deoxy-b-D-glucan. It is a natural carbohydrate polymer derived from deacetylation of chitin, which is a major component of the shells of crustaceans such as crab, shrimp, and crawfish (da Silva et al., 2013).

Shrimp by-products originate three phases (a solid, a liquid, and a lipid phase) by fermentation. The solid phase obtained from the fermentation produces chitin. It is mainly present in the exoskeletons of crustaceans but also in other arthropods and the fungi cell walls (Sanches-Silva et al., 2012).Basically, the process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin leaving behind a complete amino group (NH_2) and chitosan versatility depends mainly on this high degree chemically reactive amino group (Baskar and Sampath Kumar, 2009).The inherent antimicrobial properties and film forming ability of chitosan confer it ideal characteristics for its use as a biodegradable antimicrobial packaging material (No et al., 2007). Chitosan can also be used as an edible coating material to preserve quality and extend the shelf life of various food products. Quality parameters of chitosan, such as

purity, viscosity, deacetylation degree, molecular weight (Mw), and polymorphous structure, vary widely due to the many factors present in the manufacturing process conferring to the resulting chitosan different functional properties. The Mw of chitosan has a profound influence on the thermal, mechanical, and permeability properties of the resulting films. An increase in Mw of chitosan improves the final mechanical properties of films. Due to the fact that chitosan can be easily formed into films by a casting solvent evaporation technique, this is the conventional method currently being employed (Butler et al., 1996; Fernandez-Panet al., 2010).

Chitosan-based films have been proven to have adequate gas barrier properties, moderate mechanical properties but high WVP and water solubility because of their hydrophilic nature. To overcome these disadvantages, many researchers have investigated the polyelectrolyte complex (PEC) of chitosan with other polymers like sodium alginate, pectin, carrageenan and xanthan gum. These oppositely charged polysaccharides will form PECs that have interesting characteristics for controlled release applications (da Silva et al., 2013).

Chitosan is non-toxic, biodegradable, biofunctional, and biocompatible. It brings some advantages over other biomolecule-based active polymers used as packaging materials because of its antibacterial behavior and

bivalent minerals chelating ability (Fig. 1.9) (Aider, 2010; Abdollhai et al., 2012). Although the exact mechanism of the antimicrobial activity has not been elucidated, a feasible hypothesis is a change in cell integrity due to interactions between chitosan positively charged with negatively charged microbial cell membranes (Cè et al., 2012).

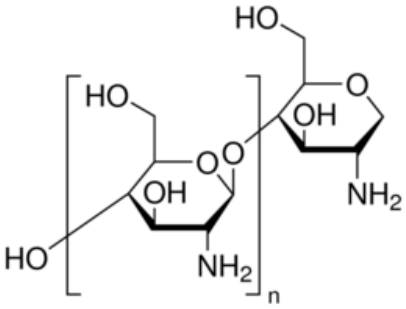


Fig. 1.9. Chitosan

Numerous informationhave been reported about chitosan potential to act as a food preservative, a functionthat was evaluated either on the basis of in vitro trials or through direct application of chitosan on real complex matrix foods (Ribeiro et al., 2007; Vásconez et al., 2009).Antimicrobial activity depends on the type of chitosan, degree of acetylation,molecular weight, the target microorganism, the pH of the medium, and presence of other additives or food components(Aider, 2010; Campos et al., 2011).

The effectiveness of chitosan depends on the application technique; in a coating solution it is more available to act as a preservative than when the preservative is forming the film (Vásconez et al., 2009). Taking into account the mentioned trend, it is frequent the addition of another antimicrobial agent such as potassium sorbate, nisin, and essential oils, to enhance chitosan antimicrobial action (Hosseini et al., 2009; Vásconez et al., 2009). Incorporation of other antimicrobials to chitosan films and coatings generally improved antimicrobial activity and also modified physical and mechanical properties of films (Campos et al., 2011).

Chitosan-based films have good mechanical properties and selective gas permeabilities (CO2 and O2), but, the high water vapor permeability limits their application (Campos et al., 2011). As for other biopolymers, many strategies have been explored to improve the barrier and mechanical properties of chitosan basedbiodegradable films. These include the addition of plasticizers and salt, chemical modification of hydroxyl groups, cross-linking of polysaccharides, the use of suitable solvent, the change of pH, the addition of different polysaccharides, and blending with other polymers (Bourtoom and Chinnan, 2008; Ghosh et al.,2010; Li et al., 2010; Gómez-Estaca et al., 2011; Abdollhai et al., 2012). The addition of different concentrations of oleic acid to chitosan film formulation led to a significant

decrease in the tensile strength, elongation at break, and elastic modulus of the composite films (Campos et al., 2011).

Chitosan was extensively used to protect, improve quality and extend the shelf-life of fresh and processed foods. Single chitosan coating was successfully applied on silver carp (Fan et al., 2009) and ready-to-eat roast beef (Beverlya et al., 2008). Chitosan coatings enriched with cinnamon oil retained the good quality characteristics and extended the shelf life during the refrigerated storage of rainbow trout (Ojagh et al., 2010). Modified atmosphere packaging in combination with chitosan edible coating maintained quality and enhanced phenolic content in carrot sticks(Simões et al., 2009) and coatings based on high molecular weight chitosan alone (Han et al., 2005) or combined with oleic acid extended strawberry shelf life (Vargas et al., 2006; Campos et al., 2011).

Also cellulose derivatives films are tough, flexible, totally transparent, and highly sensible to water presence but resistant to fats and oils (Vargas et al., 2008).Cellulose is the structural material of plant cell walls and it is composed of linear chains of (1→4)-β-D-glucopyranosyl units. Chemical substitution of some hydroxyl groups along the chain gives origin to ionic (carboxymethylcellulose) and nonionic cellulose ethers (methylcellulose;

hydroxypropylcellulose; hydroxypropyl methylcellulose) (Campos et al., 2011).

Cellulose-based films show good biodegradability and the polymer matrix allows adequate diffusion of incorporated compounds (dos Santos Pires et al., 2008).

Methylcellulose is one of the most important commercial cellulose ethers and it has been used in many industrial applications. Methylcellulose is usually synthesized by etherification of cellulose (reaction between cellulose, alkali, and chloromethane or iodomethane). Methylcellulose is the simplest cellulose derivative, where methyl groups (–CH3) substitute the hydroxyls at C-2, C-3 and/or C-6 positions of anhydro-D-glucose units (Fig.1.10) (Nasatto et al., 2015).

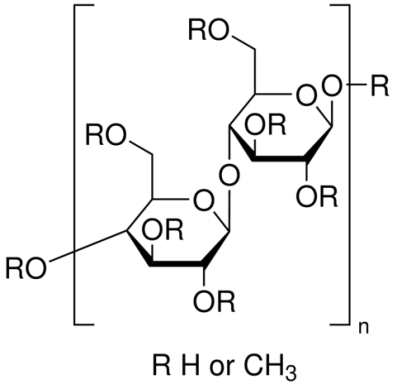


Fig.1.10. Methylcellulose

This cellulose derivative has amphiphilic properties and original physico–chemical properties.

Methylcellulose becomes water soluble or organo-soluble when the degree of substitution varies from 0 to 3.It shows a singular thermal behavior in which aqueous solution viscosity is constant or slightly decreasing when the temperature increases below a critical temperature point (29 ± 2 °C) (Nasatto et al., 2015). Methylcellulose films have flexible and transparent character. They also possess low oxygen and moisture vapor transmission rates when compared to other hydrophilic edible films (Ture et al., 2011). Ayranci and Tunc (2003) determined the water vapor and CO_2transmissions of methylcellulose-based edible films with varying amounts of the fatty acids, stearic acid, palmitic acid, and lauric acid; the results were compared with those obtained for a film without added fatty acid. In general, it was observed that WVP values decreased with increasing fatty acid content whereas CO_2 transmission parameters depended on the type of fatty acid incorporated (Campos et al., 2011). Antimicrobial cellulose-based films containing natamycin possess potential ability to inhibit the microorganisms on food products. Natamycin impregnated cellulose-based films showed inhibitory effect against P.

roquefortii present on the surface of Gorgonzola cheese (de Oliveira et al., 2007; Ture et al., 2011).

Natamycin (Fig.1.11), also known as pimaricin, is a fungicide of the polyene macrolide group, produced by Streptomyces *natalensis*. It has a molecular mass of 665.725 g/mol. The CAS Registry Number of natamycin is 7681-93-8 and the molecular formula is C33H47NO13 (EFSA, 2009).

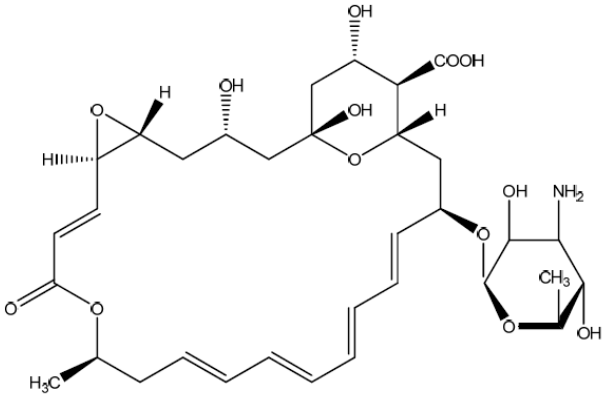


Fig.1.11. Natamycin (EFSA, 2009)

The primary structure of natamycin consists of a large lactone ring of 25 carbon atoms. The lactone ring is linked to a mycosamine moiety, m-amino-sugar, by a glycosidic linkage. Natamycin is classified as a polyene macrolide antibiotic and specifically as a tetraene antibiotic because of its

four conjugated double bonds. The mycosamine moiety (3-amino-3,6dideoxy-D- mannose) of natamycin at the C15 position is a six-membered pyranose ring. Natamycin forms a cylindrical structure due to the alignment of the hydroxyl groups of its amphipathic chain towards each other. The exterior of the cylinder is completely non-polar. The solubility of natamycin is 20-50 mg/L in water.

Natamycin is soluble in glacial acetic acid, methylpyrrolidone, dimethylformamide, dimethylsulfoxide, glycerol and propylene glycol. Natamycin is insoluble in higher alcohols, ethers, esters, aromatic or aliphatic hydrocarbons, chlorinated hydrocarbons, ketones, dioxane, cyclohexanol and various oils (EFSA, 2009).Natamycin shows good stability in foods provided that pH is in the range from 5 to 9 (Raab, 1972). It is less stable in foods outside this pH range (Stark, 2004). Natamycin is sensitive to inactivation byoxidants such as peroxides, chlorine and heavy metals (EFSA, 2009).Natamycinis commonly employed in dairy-based food products to prevent yeasts and molds contamination (Tab.1.7) (El-Diasty et al., 2008; Gallo &Jagus, 2007).

Tab.1.7. Example of yeast and moldthose are sensitive to natamycin (Lacroix, 2016).

Absidia	Bassochlymas fulva	P. commune	S. florentinus
Alternaria	Candida albicans	P. chysogenum	S. ludwigii
Aspergillus chevalieri	C. guillermondii	P. cyclopodium	S. rouxii
A. flavous	C. vinii	P. digitatum	S. sake
A. niger	Cladosporum cladosporiodes	P. islandicum	Sclerotina fructicola
A. ochraceus	Fusarium	P. notatum	Scopulariopsis saperula
A. oryzae	Gloesporum album	P. roquefortii	Toluropsis candida
A. penicilloides	Hansenula polymorpha	Rhizopus gracilis	T. lactis-condensi
A. roquefortii	Koeckera apiculata	Saccharomyces bailii	Wallensis sebii
A. versicolor	Mucor mucedo	S. bayanus	Zygosaccharomyces barkeri
Botrytis cinerea	M. raceous	S. cerevisiae	
Brettanomyces bruxellensis	Penicillum cambemberti	S. exiguus	

It has been approved as a food additive in over 40 countries and has-been considered as a GRAS product by the FDA (Koontz et al., 2003) and also indicated as a natural preservative by the European Union (EEC N_235) (Fig.1.12) (Ollé Resa et al., 2013).Following a request from the European Commission to EFSA, the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to provide a scientific opinion on

the safety in use of natamycin (E 235) as a food additive, and on the issue of antimicrobial resistance to natamycin (EFSA, 2009).

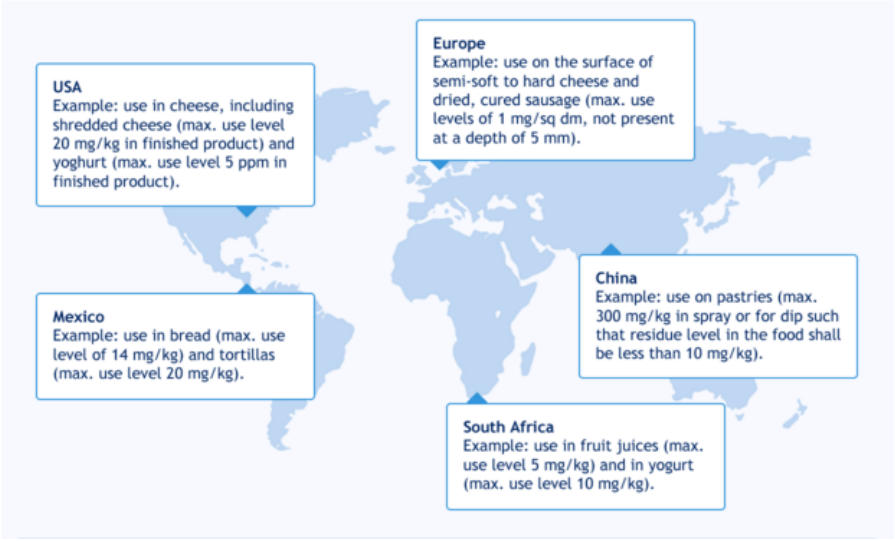


Fig.1.12. World diffusion of active packaging containing natamycin (www.natamycin.com/regulatory)

According to 95/2/EC directive and Regulation n. 1129/2011, natamycin may be used for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausage at a maximum level of 1 mg/dm^2 in the outer 5 mm of the surface, corresponding to 20 mg/kg (Lantano et al., 2014). The term 'cheese surface' is used for the outside layer of cheese or parts of cheese, even in the sliced, shredded or grated form. The term includes the outside

of whole cheese, disregarding whether a rind had been formed or not (Codex Stan, 2003).Mandatory labeling of the additive is necessary where it is not used on the rind, but directly on the cheese surface. In some countries outside Europe, *E* numbers are not used and natamycin can be labeled as “Natamycin (a natural mold inhibitor)” or as “Pimaricin (a natural preservative)” (www.natamycin.com/regulatory).

The Scientific Committee for Food (SCF) in 1979 did not establish an Acceptable Daily Intake (ADI) but considered that in relation to the uses of natamycin on cheese and sausages; the database was adequate and did not give rise to safety concern (EFSA, 2009).The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed the safety of natamycin in 2002 and assigned an ADI of 0.3 mg/kg body weight (bw)/day based on human data. The level causing no toxicological effects in man was estimated to be 200 mg/per/day, equivalent to 3 mg/kg bw/day and an uncertainty factor equal to 10 has been used to calculate the ADI. Because of the limitations in the database on natamycin (design of the animal studies, limited number of animals, lack of a carcinogenicity study) and in view of the inadequate description of the human data, the Panel considered that an ADI could not be established from these data (EFSA, 2009).

The highest potential exposure to natamycin was below 0.1 mg/kg bw/day for children at the 97.5th percentile (high level exposure estimates) and below 0.05 mg/kg bw/day for adults, derived from the high level consumption of cheese (assuming solely a rind treatment with natamycin) and dried, cured sausages. Given that natamycin is very poorly absorbed, the Panel considers that this conservative estimate would provide an adequate margin of safety from the effect level seen from the long-term animal studies and the human study used by JECFA to establish an ADI. Moreover, the Panel considered that the proposed use levels of natamycin are not of safety concern if it is only used for the surface treatment of the rind of semihard and semi-soft cheese and on the casings of certain sausages, and concluded that there was no concern for the induction of antimicrobial resistance (EFSA, 2009).

Natamycin had no adverse effect on the rind or the flavor of the cheese. It does not penetrate far into the cheese, therefore has less effect on the taste of the cheese and remains predominantly on the surface, where mold contamination occurs and need to prevent (Engel et al., 1983).

The depth of penetration of natamycin was found to depend on of the initial concentration, storage time and cheese type (Davidson et al., 2005).

Natamycin kills yeasts by specifically binding to ergosterol but without permeabilizing the plasma membrane. It inhibits vacuolar fusion through the specific interaction with ergosterol (teWelscher et al., 2008; 2010). Therefore, it is active against yeasts and moldsbut not against bacteria, viruses, and protozoa. Sterols are known to have an ordering effect on the membrane, it is thought that they reside in specific sterol-rich domains in membranes and they are also known to be involved in end ocytosis, exocytosis, vacuolar fusion (Wachtler & Balasubramanian, 2006), pheromone signaling (Jin et al., 2008), membrane compartmentalization (Klose et al., 2010), and the proper functioning of membrane proteins (Zhang et al., 2010). According to Athar and Winner (1971), structural modification and/or decreased expression of ergosterol via mutations in the sterol biosynthesis pathway substantially diminish fungal pathogenicity in vivo (Ollè Resa et al., 2014).

Antimicrobial agents can be applied by dipping, spraying, or brushing to food surfaces for controlling microbial growth (Ture et al., 2011) (Tab.1.8). However, these direct application techniques are laborious and have limited benefits (Ture et al., 2011), and the compound generally exhibits a rapid loss of activity due to a reduction of its active concentration resulting from interaction or reaction with food components.

When the additive diffuses to the bulk of the food, a phenomenon of dilution also acts against its effectiveness. Incorporation of antimicrobials in foods by means of the use of edible films where they are entrapped collaborates to a decrease of their diffusion rate from the surface to the bulk of the product, thus assisting in the maintenance of high concentrations of the active ingredient where it is required (Kristo et al. 2008; Ollè Resa et al., 2013).

Tab.1.8. Applications of Natamycin, with suggested dosage levels and methods of applications (Davidson et al., 2005).

Food Application	**Suggested Natamycin Dosage Levels, ppm**	**Method**
Hard/semi-hard cheese	1250-2000 500	Surface treatment by spray of immersion Direct addition to coating emulsion
Meat products: dry sausage	1250-2000	Surface treatment by spray of immersion
Yogurt	5-10	Direct addition to yogurt mix
Bakery products	1250-2000	Surface treatment by spray
Tomato purée/paste	7.5	Direct addition during mixing
Fruit juice	2.5-10	Direct addition
Wine	30-40 3-10	Direct addition to stop fermentation Added after bottling to prevent yeast/mold growth

According to Ture et al., (2011), the antimicrobials can exhibit a loss of activity due to the reduction of their active concentration resulting from interaction or reaction with other additives or components present in the food matrix. Incorporation of antimicrobials in food interfaces by means of the use of edible films where they are entrapped helps to decrease the rate of diffusion from the surface to the bulk of the product, thus assisting in the maintenance of high concentrations of the active ingredient where it is required (Kristo et al., 2008). It can also diminish the interaction with other additives and food components due to its presence being restricted mainly to the surface (Campos et al., 2011). As a consequence, edible matrices with antimicrobial activity constitute a promising form of antimicrobial delivery in the frame of food preservation (Dos Santos Pires et al., 2008; Fajardo et al., 2010; Ollé Resa et al., 2012, 2013; Ture et al., 2011).

Several studies reported that this active agent can be incorporated inside polymeric packaging by casting process (de Oliveira et al., 2007; Zactiti and Kieckbusch, 2009; Bierhalz et al., 2012; Bierhalz et al., 2013) or deposited onto plastic film surfaces (Hanušova et al., 2010; Lontano et al., 2014).Chitosan coating containing natamycin decreased mold/yeast population on Saloio cheese after 27 days of storage (Fajardo et al., 2010). The complex cheese composition along with environmental conditions

during handling and storage often promote extensive mold and bacteria development at cheese surface, which considerably reduces its quality. Fungal growth on dairy products not only causes economical losses but also results in health problems owing to mycotoxin production (Yildirim et al., 2006; Ture et al., 2011).Chitosan coatings were tested also on different types of cheese aiming at prolonging their shelf life, such as Mozzarella (Altieri et al., 2005), Emmental (Coma et al., 2002), Regional Saloio (Cerqueira et al., 2009), Apulia spreadable cheese (Gammariello et al., 2008).

Also natamycin-impregnated cellulose-based films showed inhibitory effects against Penicillium *roquefortii* present on the surface of Gorgonzola cheese (de Oliveira et al., 2007). Its combination with nisin included into cellulose film formulations improved the shelf life of sliced mozzarella cheese by6 days compared to control (dos Santos Pires et al., 2008). Ture et al. (2011) showed that methylcellulose and wheat gluten films containing natamycin could have potential to be used in the prevention and control of toxigenic molds on dairy products such as cheese samples, in combination with other preventive measures in a hurdle concept (Fajardo et al., 2010).

The use of packaging films based on antimicrobial polymers could prove more efficient, by maintaining high concentrations of the active substance on food surface while preventing its migration, thereby maintaining a critical concentration for an extended period of time (Ouattara et al., 2000). Then, it is interesting to quantify the release of natamycin from the film. Several approaches are reported on drug release modeling (Crank, 1975; Flores et al., 2007; Peppas et al., 2000; Siepmann and Siepmann, 2008). The benefit of mathematical modeling consists in the possibility of predicting parameters of the releasesystem aiming at providing information about the mechanisms that control drug release. Additionally, due to the low water solubility of natamycin their incorporation into a coating favors the good distribution in the cheese in order to protect cheese surface from mold growth (Fajardo et al., 2010).

The aim of the study was to (i) develop and evaluate chitosan and methylcellulose films incorporated natamycin, as antimicrobial agent, and (ii) determine the antimicrobial efficacy against yeast and molds present on cheese surface. The antimicrobial release behavior in food simulant was also investigated.

3. Materials and Methods

3.1 Development of Chitosan and Methylcellulose based films

The materials used to prepare the films and coatings were:Chitosan with a degree of deacetylation of approximately 75-85% (medium molecular weight, 200-800 mPa s viscosity, soluble in 1% Acetic acid aqueous solution) (Sigma chemicals, St-Louis, MO, USA); Glycerol (molecular biology grade,Calbiochem); Methylcellulose (3500-6000 mPa viscosity) (Sigma-Aldrich,Damstadt, Germany).Natamycin was provided by Sigma (Steinheim, Germany).Ethanol(analytical grade), Methanol (HPLC grade), Acetonitrile (HPLC grade) were provided by Merck(Darmstadt, Germany). Acetic acid solution (HPLC grade) was provided by Sigma-Aldrich (Germany).The water used to prepare all solutions was purified in aMilli-Q water purification system (Millipore) (Bedford, MA, USA).

A commercial semi-hard cheese was purchased from a local supermarket and stored in a refrigerator (4 °C) until use. The cheese chemical composition is showed in the Table1.9.

Tab.1.9. Cheese chemical composition.

Nutrition Facts	on 100 g
Calories	1120 kj/295 Kcal
Total Fat	23.0 g
Saturated Fat	15.5 g
Total carb.	<0.5 g
Sugars	<0.5 g
Protein	21.5 g
Sodium	1.8 g

3.1.1 Preparation of Chitosan and Methylcellulose basedfilms

Chitosan films (1.5% w/v) were prepared by dissolving chitosan in acetic acid aqueous solution 1% (v/v). Subsequently, the solution, containing glycerol as plasticizer (0.2 g⁄g biopolymer), was kept under stirring for 2 h at a constant temperature at 80°C and then 12 hat room temperature until the chitosan was fully dissolved.

Methylcellulose (3% w/v) was mixed with water-ethanol (50:50 v/v), and then homogenized for 5 minutes. After the addition of glycerol (0.4 g/g biopolymer), the solution was kept under stirring and heated to 80° C for 2 hours. To eliminate the adverse effect of the temperature, antimicrobial

agent was added into the film solutions at room temperature. The solutions were then casted in 8.5 cm polyacrylic plates and dried at 30°C for 12 hours (Fig.1.13).

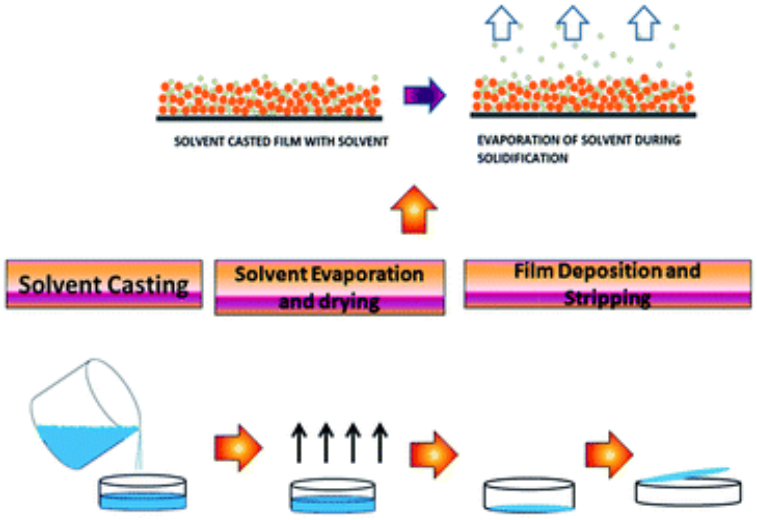


Fig.1.13. Mechanism of solvent casted film

(http://pubs.rsc.org/en/Content/ArticleHtml/2015/RA/c4ra11597j).

3.1.2 Measurement of thickness

The thickness of the samples was determined using a manual digital micrometer (0.001 mm, Mitutoyo, Mizonokuchi, Japan).Measurements were repeated in 5 different regions of each sample in order to determine the thickness homogeneity in the films.

3.2Migration of Natamaycin from Chitosan and Methylcellulose basedfilms

During migration tests, the films were fixed in glass tubes so that both sides of the tested films were in contact with food simulant. Chitosan and methylcellulose films were cut into pieces of 14.7 cm^2 area and immersedinto 20 mL of an ethanol 95% (v/v), at different temperatures: 10, 20 and 40°C (±0.2°C).

Aliquots (50µl) of this solution were taken out, at different time intervals. To determine the amount of natamycin released, aliquots of the food simulant were taken out from the tubes at preset times, filtered and injected by HPLC-DAD (high performance liquid chromatographic with diodearray detection). The analytical conditions were obtained by modification of a previously reported method (Paseiro-Cerrato et al., 2013).The preserving agent released from the film into simulant was determined as follows: the calculation of the final migration level included the correction for changing simulant volume, as well as for the amount of natamycin taken during the previous sampling. Then, to calculate the remaining amount in the film, a piece of film was introduced in a glass tube containing 20 mL

ofethanol80% (v/v) solution at 40°C for 24h. The experiment was performed in duplicate.

3.2.1 HPLC-DAD analysis

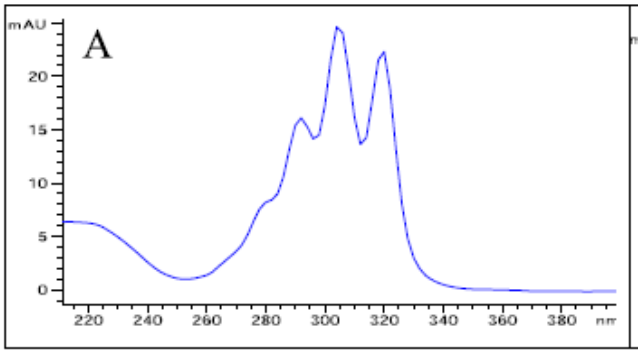
An HPLC HP1100 system (Hewlett Packard, Waldbronn, Germany) equipped with a quaternary pump, a degassing device, an autosampler, a column thermostating system, a diode-arraydetector (DAD), and Agilent Chem-Station for LC and LC/MS systems software, was used. Separation was performed on a KromasilODS (C18) (150 x 3.20 mm i.d., 5 mm particle size), and column thermostattedat 25 °C. Acetonitrile (A) and Milli-Q water (B) were used as mobile phase. Samples were eluted in gradient mode. The gradient elution program is shown in Table 1.10. Three selected wavelengths were set in DAD detector, 291, 304 and 319 nm, corresponding to the three absorption peaks of the characteristic natamycin spectrum (Fig. 1.14)(Paseiro-Cerrato et al., 2013). The injection volume was 20 µL.UV spectrum of natamycin shows three major absorption peaks in the range between 290 and 320 nm. The wavelength commonly used to quantify the antifungal is 304 nm (Koontz et al., 2003;Hanusová et al., 2010; Paseiro-Cerrato et al., 2013).

Tab.1.10. HPLC elution Natamycin program.

Time (min)	A%	B%	Flow (mL/min)
0	20	80	0.6
10	60	40	0.6
15	60	40	0.6
20	20	80	0.6

(A) Acetonitrile, (B) Milli-Q Water

Fig.1.14. UV spectrum of natamycin (Paseiro-Cerrato et al., 2013).



3.2.2 Calculation of diffusion coefficients and partition coefficients

The diffusion coefficients of natamycin from the active films into the food simulant 95% ethanol (v/v) were calculated by using a mathematical model based on Fick's Second Law.

$$\frac{\partial C_p}{\partial t} = D\frac{\partial^2 C_p}{\partial x^2}$$

where *Cp* is the concentration of the migrant in the film at time *t* and position *x*.

An analytical solution of this differential equation that describes the diffusion kinetics was proposed by Crank (1975); after a slight modification, this can be expressed by the following equation (Simoneau, 2010):

$$\frac{m_{F,t}}{A} = c_{P,0}\rho_P d_P\left(\frac{\alpha}{1+\alpha}\right)\times\left[1-\sum_{n=1}^{\alpha}\frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2}\exp\left(-D_P t\frac{q_n^2}{d_P^2}\right)\right]$$

$$\alpha = \frac{1}{K_{P/F}}\frac{V_F}{V_P}$$

Where:

$m_{F,t}$ is the mass of the migrant transferred from *P* into*F* after time *t*, (μg);

A is the area of *P* in contact with *F* (cm^2);

$C_{P,0}$ is the initial concentration of the migrant in *P* (mg/kg);

ρ_P is the density of *P* (g/cm^3);

t is the migration time (s);

d_p is the thickness of *P* (cm);

V_P is the volume of P (cm^3);

V_F is the volume of F (cm^3);

q_n is the positive root of the equation $\tan q_n = - \alpha \cdot q_n$;

D_P is the diffusion coefficient for the migrant in the polymer (cm^2/s);

$K_{P/F}$ is the partition coefficient for the migrant between P and F.

Partition coefficients between the film and food simulants were calculated according to the following equation:

$$K_{P/S} = \frac{C_P}{C_S}$$

where:

$K_{P/S}$is the partition coefficient between the film and the food simulant or substitute;

C_P is the concentration of substance in the film at equilibrium, in µg/g;

C_S is the concentration of substance in the simulant at equilibrium, in µg/g.

To measure the fit between the experimental and estimated data the root of mean-square error % (RMSE (%)) was calculated according the following equation:

$$RMSE(\%) = \frac{1}{M_{P,0}} \sqrt{\frac{1}{n} \sum_{i=1}^{n} ((M_{F,t})_{\exp,i} - (M_{F,t})_{pred,i})^2} x100$$

Where:

n is the number of experimental points per migration/release curve;

i is the number of observations;

$M_{P,0}$ is the initial amount of the migrant in the polymer (μg).

3.3 Microbiological analysis

To evaluate the feasibility of using antimicrobial films to improve the preservation of cheese during the storage, 15 pieces of cheese were randomly assigned to five treatments (Figg. 1.15, 1.16, 1.17):

1) coating with Polyethylene;

2) coating with chitosan film containing natamycin;

3) coating with methylcellulose film containing natamycin;

4) coating with chitosan film;

5) coating with methylcellulose film.

This procedure has been chosen instead of a direct measurement over agar plates because, due to the higher water content, it could change the properties of coatings and the natamycin release (Fajardo et al., 2010).

Cheese were completely covered with chitosan and methylcellulose films, sealed in polyethylene bags and finally stored at 20°C for 7 days. The

samples were analyzed for molds and yeasts using potato dextrose agar (PDA).

Fig.1.15. Cheese samples covered with Polyethylene.

Fig.1.16. Cheese samples covered with chitosan film (A) and chitosan whit natamycin film (B).

Fig.1.17. Cheese samples covered with methylcellulose film (A) and methylcellulose whit natamycin film (B).

At the times of bacterial enumeration, cheese were aseptically removed from their packaging and films were separated from cheese slices with sterile forceps. An aliquot (10 g) of cheese were aseptically collected and placed in 400 mL homogenizing bag along with 90 mL of 0,1% (w/v) of peptone water and massaged for 60 s at high speed in a Stomacher (AES Chemunex, Comburg, FR). Decimal dilutions were prepared from the initial homogenate. After that 0.1 mL were spread onto PDA plates and incubated at 25°C for 3 days, before counting colonies. Three samples of each treatment were analyzed.

3.4 Statistical analysis

Statistical analysis of data was performed with package SPSS 15.0 (SPSS Inc., Chicago). One-way ANOVA test was applied to determine significant differences ($P < 0.05$) among a)chitosan and methylcellulose film at the same temperature, methylcellulose films at different temperatures(according to the rate of release of natamycin), and b) mold/yeast counts.

4.Results and Discussion

4.1. Film appearance characterization

All of the films were transparent (Fig.1.18). The average thickness ranged between 38 to 51 µm for chitosan, and 56 to 76 µm for methylcellulose film.

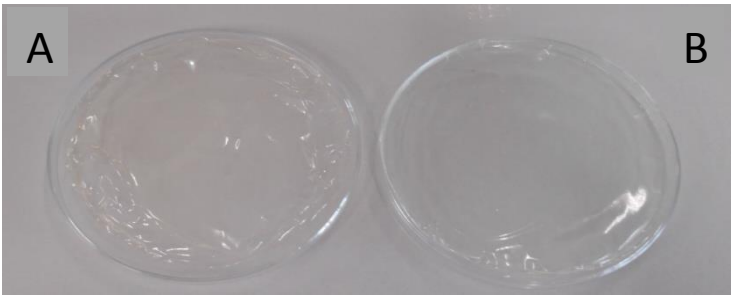


Fig.1.18. Chitosan (A) and methylcellulose (B) films.

The color of the packaging is an important factor in terms of general appearance and consumer acceptance (Bourtoom and Chinnan, 2008; Abdollahi et al., 2012). As natamycin addition affects mechanical properties and color of the films, it is advisable to use the lower natamycin concentration that allows the attainment of the goal pursued film application.

Natamycin was added at film solution at concentration of 0.006g per gram of chitosan and 0.003g per gram of methylcellulose. Above this concentration, films became opaque, brittle and showed a whitish

precipitate over their surface, making them not suitable for use (Ollè Resa et al., 2013).

4.2 Migration kinetics of Natamaycin from Chitosan and Methylcellulose based films

Figures 1.19- 22 illustrate the release profiles of natamycin from chitosan and methylcellulose films in food simulant (Ethanol 95% (v/v)) at different storage temperatures as a function of time.

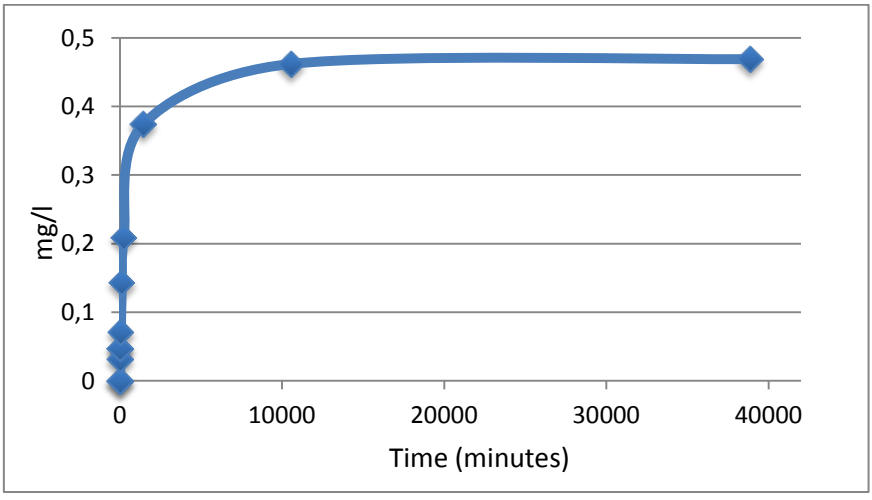


Fig.1.19.Release of Natamycin into liquid food simulant from chitosan films at 40 C°.

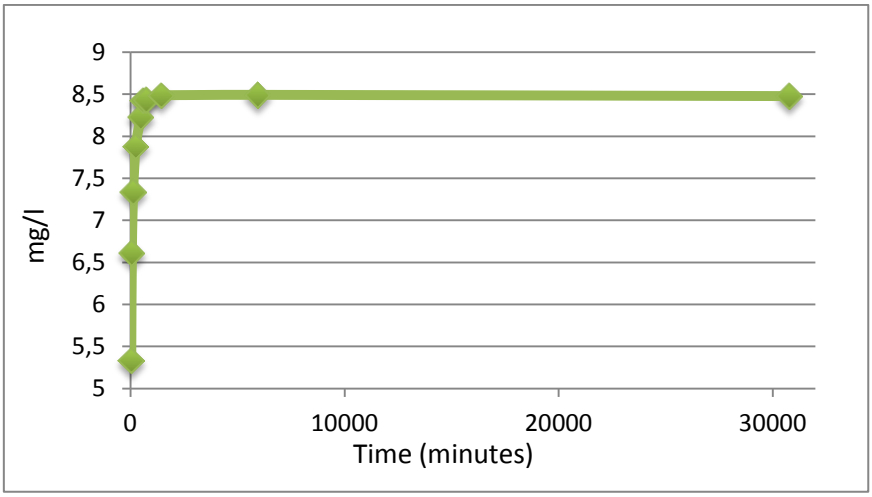


Fig.1.20.*Release of Natamycin into liquid food simulant from methylcellulose films at 10 C°.*

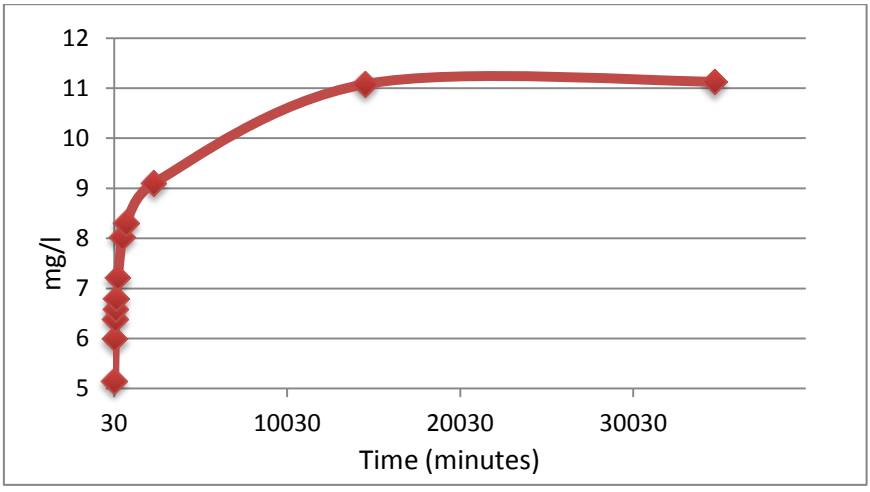


Fig.1.21.Release of Natamycin into liquid food simulant from methylcellulose films at 20 C°.

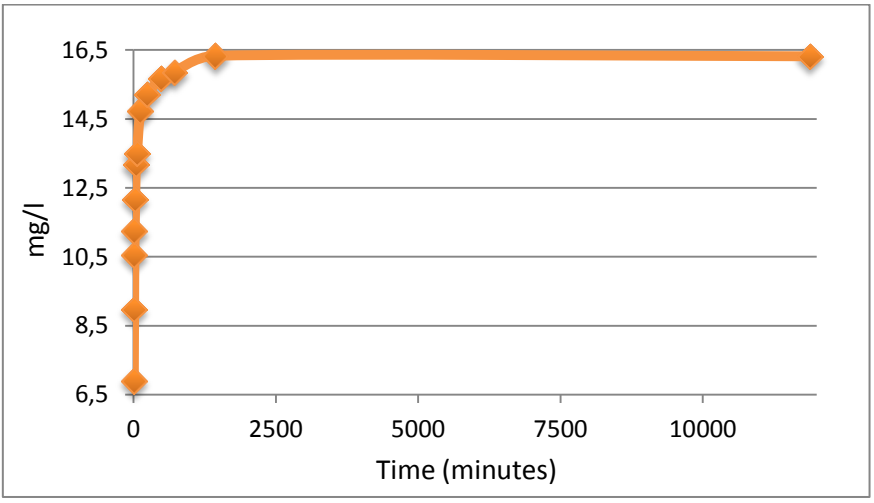


Fig.22.Release of Natamycin into liquid food simulant from methylcellulose films at 40 C°.

With respect to the migration kinetics the results showed that the release of natamycin from the chitosan based films was slower when compared with the methylcellulose films at the same temperature ($P<0.05$). After 3 days at 40°C, methylcellulose films were close to the equilibrium, releasing about 90% of natamycin; on the contrary, chitosan film reached the equilibrium conditions after 27 days, releasing less than 15% of the initial amount of natamycin in the film.

As the activity of antimicrobial films is based on the diffusion of the active agents from the film matrix to food surfaces, understanding the transport

mechanism of these substances is the most important factor in developing an antimicrobial food packaging. In many cases, the agents being carried are slowly released into the food surface and therefore remain at high concentrations for extended periods. Diffusivities can be used to quantify the release behavior of the antimicrobials, and also to obtain information about polymeric networks (Choi et al., 2005; da Silva et al., 2012).

For each temperature tested and for the same temperature (40°C) the diffusion coefficients in methylcellulose+ natamycin films were higher if compared to chitosan+natamycin films. Diffusion coefficients for natamycin from chitosan films were in the order of magnitude 10^{-13} cm^2/s, while diffusion coefficient values in the order of the range of magnitude of 10^{-8} to 10^{-10} cm^2/s were calculated for natamycin from methylcellulose films at different temperatures. As expected, the diffusion process of natamycin from methylcellulose films generally occurred faster at high temperatures ($P<0.05$).

In a study of the diffusion of nisin from different biobased films to water-ethanol solution, hydroxyl-propyl methylcellulose showed the highest D values while chitosan the lowest. Moreover, D values increased with temperature significantly for hydroxyl-propyl methylcellulose, but the

temperature variation resulted in negligible change for diffusion coefficient of nisin related chitosan film (Imran et al., 2014).

Controlled release of the active agent is desirable in order to maintain a concentration that inhibits deteriorating reactions in food surfaces, thus assuring safety and quality levels during storage (Franssen et al., 2004). A rapid release may cause migration of the active agent to internal parts of the food, reducing the protection in the surface (Appendini and Hotchkiss, 2002). A major advantage of slow release over direct addition of the antimicrobial into the food is continuous microbial inhibition obtained during an extended period (Chung et al., 2001). Alternatively, if the release rate is very slow, its inhibitory concentration cannot be reached (Bierhalz et al., 2012).

Diffusion coefficients of the order of magnitude of 10^{-10} and 10^{-12}cm^2/s have been reported for natamycin from chitosan films to phosphate buffered saline solution and cheese, respectively (Fajardo et al., 2010). Diffusion coefficient was higher in phosphate buffered saline solution because of swelling effect in the release phenomenon. The solution employed to test the release kinetics, most probably, led to overestimate the results when compared to the real amount that may migrate to a solid product as a cheese sample (Fajardo et al., 2010). Similar results were

obtained by Hanusova et al. (2010) with polyvinyl dichloride lacquer coatings also used as natamycin carrier in cheese packaging.

Water-ethanolsolution used in our study exhibits lower water activity that subsequently results in lower degradation, swelling and solubilization phenomena of biopolymers.

Diffusion coefficients for natamycin from alginate/chitosan film into the water in the order of the range of magnitude 10^{-11} to 10^{-12} have been reported also. These values are considered very low when compared with diffusivities of others antimicrobials incorporated in polymeric matrixes, suggesting a chemical interaction between natamycin and chitosan (da Silva et al., 2012).

Chen et al. (1996) attributed this fact to a possible electrostatic interaction between NH_3^+groups of chitosan and COO^-groups of antimicrobials. Methylcellulose carries no electrical charge (Nuijts, 1995) and the possibility of interactions between the polymer and the antimicrobial is lower.

Moreover, chitosan maintains its structure in a neutral environment but solubilized and degraded in acidic medium (Agnihotri et al., 2004). As water–ethanol solution used in the present study had neutral pH, chitosan film demonstrated higher stability with compact structure and thus lower

diffusion coefficients values (Imran et al., 2014). The increase in the ethanol concentration in the solution also limits the film hydration and weakening effect of the polymer network, which make the antimicrobial diffusion through the film matrix difficult (Sanchez Gonzalez et al., 2011).

The partition coefficient ($K_{P/F}$) indicates the ratio between the concentration of the active compound in the film and the concentration in the food simulant at equilibrium (Sanches Silva et al., 2007; Tehrany and Desobry, 2004).Values of $K_{P/F}>1$ means that the active compound is found in greater concentration in the film than in the food or food simulant. The partition coefficients for these systems correspond to the values predicted by the mathematical model used. Higher $K_{P/F}$ values are achieved in chithosan films, indicating high concentrations of the migrant in the film.

The lowest $K_{P/F}$ values were reached in methylcellulose film at 40°C. From food safety point of view, a large $K_{P/F}$ limits migration from packaging material to food; in contrast, a lower $K_{P/F}$ indicates that more migrant is absorbed into food from polymer. However, to minimize flavor loss in a package, a low K is preferred. Parameters such as temperature, pH, chemical structure of migrant, molecular size and structure, fat content of foods, and degrees of crystallinity influence partition coefficient (Tehrany and Desobry, 2004).

In order to measure the fit between the experimental and estimated data the root of mean-square error % (RMSE (%)) was calculated. Generally, acceptable values were obtained and the bestfit between the experimental and estimated data was found for chitosan films. Therefore this model can be used to predict the release of antimicrobial into food simulants (Rodriguez-Martinez et al., 2016).

4.3 Antimicrobial activity of Chitosan and Methylcellulose based films containing natamycin

The conditions used in this assay were drastic. The storage temperature used in this work is not adequate for storage under refrigeration, but this could be a reality commonly found for domestic consumption (Fajardo et al., 2010).

The mean values of the counts obtained for mold/yeast were 7.91 log (CFU/g) for chitosan containing natamycin. Values slightly higher for molds/yeasts counts (in the range of 8.24-8.95 log CFU/g) were observed in cheese coated with methylcellulose films cantaining natamycin, and chitosan, methylcellulose and Polyethylene films. Significant differences were observed between chitosan films containing natamycin and Polyethylene films, according tomold/yeast counts ($P<0.05$). It is important

to note that results were obtained under no inoculation conditions. This means that large differences were not to be expected once the counts were performed in whole cheese and not only at their surface (Fajardo et al., 2010).

Antimicrobial agents can be applied by dipping, spraying, or brushing to food surfaces for controlling microbial growth. However, these direct application techniques are laborious and have limited benefits (Ture et al., 2011). In addition, the antimicrobial compound generally exhibits a rapid loss of activity, resulting from interaction or reaction with food components. When the additive diffuses to the bulk of the food, a phenomenon of dilution also acts against its effectiveness (Kristo et al. 2008; Ollè Resa et al., 2013). The use of packaging films containing antimicrobial agents could prove more efficient, by slowing the migration of the agents away from the surface, thus helping maintain high concentrations where they are needed (Ouattara et al., 2000).Nevertheless, if the release rate is very slow, its inhibitory concentration cannot be reached (Bierhalz et al., 2012).

Several studies have reported that antimicrobial films containing natamycin possess potential ability to inhibit the microorganisms on food products. Natamycin protects the surface of cheese against the

development of mold. Chitosan coating containing natamycin decreased mold/yeast population on Saloio cheese (Fajardo et al. 2010). However, in some cases, films containing natamycin showed similar inhibition than those with chitosan alone (Cè et al., 2012). These results indicate, as mentioned above, a possible electrostatic interaction between natamycin molecules and chitosan polymeric chains, which could be hindering the release of the active substance (da Silva et al., 2013). On the other hand, the chemical stability of natamycin is favored in neutral pH and a possible degradation could take place at low pH values. The acid pH of the film forming solution of chitosan could suggest a chemical degradation of natamycin (da Silva et al., 2013).

The low antimicrobial activity of methylcellulose films could be explained by the rapid release that may cause migration of the active agent to internal parts of the food, reducing the protection in the surface (Appendini and Hotchkiss, 2002).

Moreover, natamycin concentration in our films was 0.3% in relation to methylcellulose weight. Above these concentrations, films became opaque making them not suitable for use (Ollè Resa et al., 2013). De Oliveira et al. (2007) tested films with different concentrations of natamycin in Gorgonzola cheese, but only films with 2-4% of natamycin presented

satisfactory results for fungus inhibition. The amount of the additive incorporated to food shall be limited to the lowest possible level necessary to accomplish the desired effect. In most works, the determination of natamycin's minimum inhibitory concentration (MIC) is performed absorbing natamycin on paper discs (Türe et al., 2011), or adding it into the film matrix (dos Santos Pires et al., 2008; Pintado et al., 2009). Later, MIC is calculated by direct measurement over agar plates spread with different indicator fungi. However, these methods present some problems: a) the antifungal properties of natamycin could be reduced due to the interaction with the coating film and also; b) agar has a high water content that could change the properties of the film; c) the release mechanism of active agents from film on cheese might differ (Fajardo et al., 2010).

In fact,MIC of natamycin against A. *niger* and *P. roquefortii*was determined as 2 mg /10 g film solution in vitro, but the value increased to 5 mg/10 g film solution for the cheese application (Ture at al., 2011).Antimicrobial films or coatings resulted more effective in terms of inhibiting target microorganisms if applied to nutrient media, than on real systems owing to complex structure of foods (Ture at al., 2011).

5. Conclusions

Antimicrobial packaging is gaining interest from researchers and industry due to its potential to provide quality and safety benefits. Antimicrobial packaging can play an important role in reducing the risk of pathogen contamination, as well as extending the shelf-life of foods. Active packaging should never substitute for excellent quality of raw materials, good manufacturing practices, and properly food process. It should be considered as a hurdle technology that in addition to other non-thermal mild technologies, such as pulsed light, high pressure, and irradiation, could reduce the risk of pathogen contamination and extend the shelf-life of perishable food products (Appendini and Hotchkiss, 2002).

Biobased packaging can be considered an additional factor for preserving food products, assuring its quality as well as a prolonged shelf life. When they aroused for supporting antimicrobials, the stability, concentration on the surface of the product, bioavailability, and gradual release are fundamental preservative characteristics related to their functionality (Weber et al., 2010).

Chitosan and methylcellulose films seem to show good physical properties, but further studies are needed to improve the effectiveness of these films on possible food systems. The good compatibility of natamycin with

chitosan, the low diffusion coefficient, and the antimicrobial properties indicate that the film has great potential to be used in antimicrobial packaging, such as in cheese wrappings to inhibit mold spoilage. Moreover, in future, ideal rate of antimicrobial release that offers the most effective inhibitory effect on mold/yeast may be achieved by blending two biopolymers with different nature (Imran et al., 2014). Alternatively, for chitosan film, the effective inhibitory effect may be obtained mixing with swelling agents, which expand the molecular structure of the film upon the exposure to moisture to promote the release of the bioactive compounds (Marsh et al., 2007; Sanchez-Gonzalez et al., 2011).

The challenge for the use of biobased films and coatings is their compatibility with other emergent stress factors like high pressures, electric fields, ultrasound, microwave radiation, and gamma radiation. Some modifications can be produced in biobased film structure helping to achieve target purposes (Siracusa et al., 2008,Campos et al., 2011). In this sense, the generation of a multilayer structure might contribute to minimal degradation and/or low diffusion and/or gradual release and/or adequate bioavailability of the antimicrobial. The evaluation of the rate of release together with the evaluation of antimicrobial activity trough the time could

help to optimize the development of films and coating for lengthening the shelf life of food products (Campos et al., 2011).

Biobased packaging, edible films and coatings could have an important impact on the agriculture sector by reducing the post-harvest losses and on industrialized food products makinglessexpensive packaging materials by the use of hydrocolloids that constitute underused materials (Campos et al., 2011).

Information nowadays about all these topics is abundant, but not systematic. A deeper insight concerning this subject is needed to elucidate the magnitude of the ratio benefit/cost and not only from a financial point of view (Debeaufort et al., 1998; Campos et al., 2011)

Great possibilities exist for packaging in biobased materials. However, further research within different areas of biopackaging, e.g. legislation, processing technology, and compatibility studies of foods and packaging are needed before biobased materials can be commonly used for primary food packaging.

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Chapter 2

Study on the effects of electrical stunning parameters for broilers on biochemical and histological markers of stress and meat quality

Mercogliano R, **Santonicola S**, Murru N, Paciello O, Pagano T B, Peruzy MF, Anastasio A, Cortesi ML (2016). Study on the effects of electrical stunning parameters for broilers on biochemical and histological markers of stress and meat quality. *Animal Production Science*.http://dx.doi.org/10.1071/AN15828

1. Introduction

In Europe pig meat production is the most significant (51 % of annual production of all meats), followed by poultry meat (30 %), bovine meat (17 %) and sheep and goats meat (2 %) (c.europa.eu/eurostat/statistics-explained/index.php/Meat_production_statistics). The European Union is one of the world's top producers of poultry meat and a net exporter of poultry products. Over the years, the market organization for poultry sector was improved to ensure the development of the sector, the quality of the products and consumers protection while harmonizing the entire market. The leading countries in poultry meat production are Poland (13.7 %), France (12.7 %), closely followed by UK (12.4 %), Germany (11.4 %), Spain (11.1 %) and Italy (9%). These six countries ensure 70% of the EU production of poultry meat (Fig.2.1) (http://ec.europa.eu/agriculture/poultry/index_en.htm).

The EU imports high value products, poultry breasts, and cooked preparations mainly from Brazil (60% of total EU poultry meat imports) and Thailand (30%).The EU-27 per capita consumption increased from 22 kg in 2006 to 24.3 kg in 2014.

With a stable or slightly decreasing population in the EU, the total consumption grew from 10.982 million tonnes to 12.088 million ton in 2014(http://ec.europa.eu/agriculture/poultry/index_en.htm).

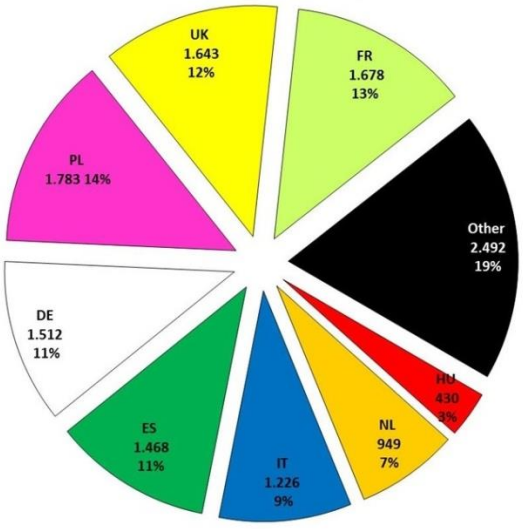


Fig. 2.1. Percentage of estimated poultry meat production in EU.

This trend is driven by the low production cost and price of poultry meat (relative to beef and pork), rising income and standard of living, changing consumer preferences on health (low in fat) or social (convenient, fast food) grounds. However, issues concerning the origin, safety, and quality of meat, bird welfare and environmental impact of poultry production and

processing are likely to play important roles in world trade (Duncan, 2001; Mead, 2004).

1.1 Poultry welfare issues

The World Organization for Animal Health has been actively involved in debating animal welfare within the context of animal health and consumer safety. Evidently, farm animal welfare, in general, has become an issue of increasing concern to consumers, producers, governments and nongovernmental organizations. Welfare issues that need to be addressed by the poultry industry are found in breeding, rearing, catching, transport and slaughter (Duncan, 2001; Mead, 2004).

In most developed countries, except the USA, animal welfare regulations require that poultry are stunned or stun/killed immediately prior to slaughter (neck cutting). Within Europe, the European Union Treaty of Amsterdam acknowledges that animals are sentient beings, rather than being agricultural products or commodities. Therefore, stunning before slaughter is mandatory and is performed to induce unconsciousness in the animals, so that death can occur through bleeding, without pain, suffering or distress (Mead, 2004).

Technical definitions of animal welfare put the individual animal more central and defining it as “the ability of an animal to cope physiologically, behaviourally, cognitively, and emotionally with its physiochemical and social life environment, including the animal’s subjective experience of its condition” (Sejian et al., 2010). For predominant animal production systems, broiler welfare concern has been discussed in relation to various farms and management practices (Bessei, 2006; Heleski et al., 2006; Robins and Phillips, 2011).At the slaughter plant, poultry can also be assessed for many conditions that are detrimental to animal welfare and are related to the transport and the farm. Some of these conditions are lameness, broken wings, death losses, poor body condition, animal cleanliness, serious injurious, and obvious neglected health problems (Grandin, 2010). Improving objective animal welfare levels in production systems and consumer perceptions may be achieved through the implementation of various adjustments to dominant production systems (de Jonge and van Trijp, 2013).

1.1.2 Poultry welfare at slaughterhouse

At the time the animals are put-up for slaughter within the abattoir, the pre-slaughter stress can influence the welfare of the poultry, as well as post-

mortem metabolism and quality meat (Gregory, 1994; Md Shwkat et al., 2008). The most universally accepted method to immobilize the poultry before slaughter is by electrical stunning (Fig.2.2) (Bilgili, 1999).

Fig.2.2. Electrical stunning equipment normally used in poultry slaughterhouse.

Council Regulation 1099/2009/EC recommended the use of a minimum current of 120 mA, which causes instantaneous and irreversible stunning, and a lack of consciousness and sensibility before, or at the same time, the animals are killed. In poultry sector, there are welfare concerns about the existing electrical stunning systems that need to be addressed (Poole and Fletcher, 1998; Raj, 1998). The intensity of electric current used for

stunning can vary among slaughterhouses. The time of unconsciousness increases with increasing stunning voltage, but the extent of carcass damage is aggravated, and incidence of ventricular fibrillation and death increase (Gregory and Wotton, 1990; EFSA, 2004; Kissel et al., 2015). High frequencies can increase the depth of unconsciousness, but the duration of unconsciousness decreases as the stunning frequency increases (Kannan et al., 1997; Mouchoniere et al., 1999; Huang et al., 2014).

The effects of electrical stunning on the final quality of meat are dependent on voltage, frequency, and duration of the treatment. Several studies have examined the relationship between electrical stunning and animal stress, with emphasis on the quality of meat (Lee et al., 1979; Craig and Fletcher, 1997; Northcutt et al., 1998; Bilgili, 1999; Alvarado and Sams, 2000; Gregory, 2005). The hanging operations, struggling on the shake line and exposure to heat can, in turn, lead to rapid glycolysis (pH drop), low ultimate pH, and muscle cytotoxicity (Van Hoff, 1979; Gregory and Bell, 1987; Kannan et al., 1997; Loschi et al., 2004; Rammouz et al., 2004; Debut et al., 2005; Petracci et al., 2010; Petracci and Cavani, 2012).

A potential indicator of animal welfare at the slaughterhouse is the absence of stress, but there is no standard definition of stress and no single biochemical assay system to measure stress (Velarde et al., 2010). In a

multidisciplinary approach to assess animal welfare-suffering and quality meat, as well as the effective stunning, measures easier to use practically at the poultry abattoir should be validated. As clinical markers, the return of eye reflexes and rhythmic breathing, escape behaviors, absence of the typical tonic/clonic muscle activity, resumption of rhythmic breathing, and righting attempts indicate an ineffective stunning (von Holleben et al., 2010). Elevated plasma corticosterone is an accepted biochemical indicator of stress in birds (McFarlane and Curtis, 1989; Xu et al., 2011), while Electrocardiogram (ECG) and Electroencephalogram (EEG) are considered reliable indicators of unconsciousness, insensibility in animals. Nevertheless, they may not be practical methods to ascertain, routinely, the effectiveness of a stun in the abattoir (EFSA, 2012).

Aim of the study was to investigate the effects of electrical stunning parameters for broiler chicken on physicochemical and histological markers of stress and meat quality.

2. Materials and Methods

2.1 Experiment

A number of 96 *Ross* commercial broilers (aged 56 days, with an average weight of 2.5 kg), was obtained from a conventional poultry farm (Fig.2.3). Birds were given *ad libitum* access to a standard diet throughout the period of growth (Council Directive 2007/43/EC).

Fig2.3. Example of a conventional poultry farm.

Before the slaughter, the animals were subjected to a 12 hour of feed and water withdrawal periodand then transported to an authorized slaughterhouse within 30 minutes. *The ante-mortem*inspection was carried out by an official veterinarian to evaluate the welfare conditions, health status and absence of stress in the birds (Council Regulations 1/2005/EC; 854/2004/EC).

The birds were randomly divided into 8 groups of 12 animals, with each lot of 12, sub-divided into 3 Lots of 4, and subjected to one of three treatments:

-slaughter without stunning (NS Lot or Control Lot);

-stunning at 200 mA; 53 V; 800 Hz (Mid-Volt Stunning or MV Lot);

-stunning at 200 mA; 67 V; 1000 Hz (High-Volt Stunning or HV Lot).

The broilers were individually shackled from head to feet. The duration of shackling before stunning was kept to 2 minutes, and a blue light intensity (50 lux) was used to calm the animals (Fig.2.4).

Fig.2.4. Pre-slaughter handling: broilers' unloading and shackling.

In a multi-bird water bath stunning (Cattaruzzi S02POL, Italy) the head of broilers of MV and HV Lots was brought in contact with an electric grid submerged in a saturated brine solution (Kannan et al., 1997; Velarde et al., 2010). The electrical stunning was conducted with a constant amperage, using sinusoidal alternating current (AC), in accordance with the minimum currents laid down (Reg. 1099/09/EC, Annex I, Chapter II, point 6.3), and the total stunning duration was 4 seconds.

According to the experimental design, the same operator, using a conventional unilateral neck cut, severing the carotid artery and jugular vein killed all the birds based on the following sequence (Table 2.1):

-birds of NS Lot;

-birds of MV Lot;

-birds of HV Lot.

Tab.2.1.Experimental design: slaughter and electrical stunning condition of NS (without stunning), MV(mid-voltage stunning), and HV (high-voltage stunning) Lots of broilers.

Lot	Slaughter	Stunning condition
NS Lot	Without stunning n. 32 broilers	Stunning Absence
MV Lot	Midvoltage stunning n. 32 broilers	800 Hz 200 mA 53 V
HV Lot	High voltage stunning n. 32 broilers	1000 Hz 200 mA 67 V

The pH of the Pectoralis *major* and Quadriceps *femoris* was measured immediately after the slaughter at 0.25 hours (pH_0), and after at 3 hours (pHi), and 24 hours (pHu) (Fig.2.5).

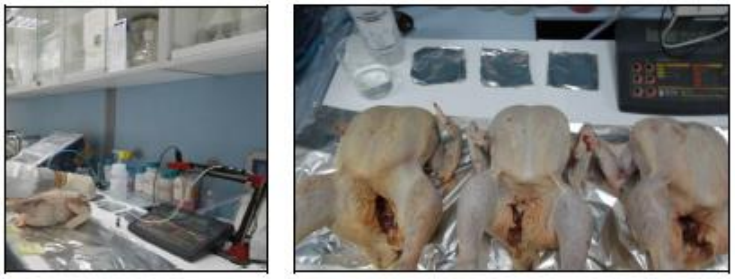


Fig.2.5. pH measurement of Pectoralis major and Quadriceps femoris.

For physicochemical analysis, a sample of carcass each from Pectoralis *major* and from Quadriceps *femoris* was chilled in a static ice, and then held packed. For histological analysis, the samples were frozen in isopentane, pre-cooled in liquid nitrogen and stored at a temperature of -80° C. The eviscerated birds were packed in polyethylene bags, placed in insulated boxes filled with ice until the transport to the laboratory within 3 hours from the slaughter, and refrigerated at 3°C to 5° C for 24 hours. The carcasses quality was assessed at 24 h, and appearance quality attributes

(skin and meat color, broken bones, appearance defects like bruises and hemorrhages) in each carcass were evaluated.

2.2 Physicochemical Analysis

The chemicals and solvents used in the study were obtained from Sigma-Aldrich, Germany. All solutions were prepared from reagent-grade chemicals. In each carcass, the pH of Pectoralis *major* and Quadriceps *femoris* was measured using a pH meter (Hanna pH211, Hanna Instruments, USA). As a marker of acute pre-slaughter stress in poultry, the pHi was particularly investigated, because it may correspond to the *start* of *shelf life of poultry meat*. Data about the pHiwere used for statistical analysis.

Muscle oxidation was evaluated according to Shantha and Decker (1994), homogenizing together a sample of Pectoralis *major* and Quadriceps *femoris* from each carcass. Superoxide free radical analysis was carried out following both the International Dairy Federation's (IDF 74A, 1991) and the Ferrous Oxidation-Xylenol orange's (FOX) methods based on the oxidation of iron (Fig.2.6).

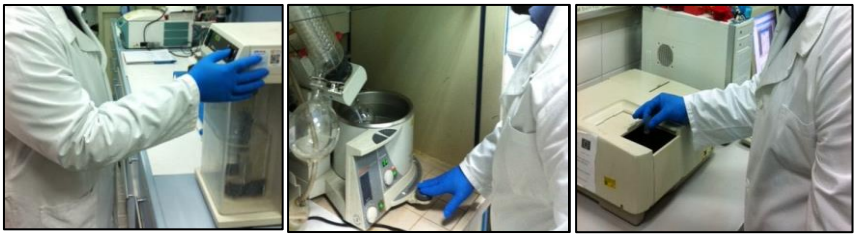


Fig.2.6. Application of IDF and FOX methods.

According to IDF method, the sample (≤0.01–0.30 g) was mixed in a disposable glass tube with 9.8 ml chloroform–methanol (7 + 3, v/v) in a vortex mixer for 2–4 seconds. Ammonium thiocyanate solution (50 µL) and iron (II) solution (50 µL) was added, and the sample was mixed in a vortex mixer for 2–4 seconds. After 5 minutes of incubation at room temperature, the absorbance of the sample was determined at 500 nm against a blank that contained all the reagents, except the sample, by using a spectrophotometer. The entire procedure was conducted in subdued light and completed within 10 minutes. The FOX was similar to the IDF method, except that, 0.01 mol/l xylenol orange sodium salt solution in water was used as the complexing dye, instead of ammonium thiocyanate (Shantha and Decker, 1994). Absorbance was determined at 560 nm after 5 minutes of incubation at room temperature. To construct the curve of Fe^{3+} concentration v/s absorbance, a standard solution of iron (III) chloride (10 µg Fe/ml) was prepared for both methods (Mehta et al., 2015).

2.3Histological Analysis

For each carcass, 10 µm thick cryostat sections of Pectoralis *major* and Quadriceps *femoris* were obtained. The sections were stained with the following panel of stains: a) Periodic Acid- Schiff (PAS) for the evaluation of glycogen reserve and glycosylated proteins; b) Hematoxylin and Eosin (H&E) and Engel Trichrome (ET) for basic morphological assessment; c) Cytochrome Oxidase (COX) and Succinic Dehydrogenase (SDH) for mitochondrial activity and distribution.

Two veterinary pathologists, by means of an optical microscope, examined the sections. Histo-pathological findings were scored according to Table 2.2.

Tab. 2.2. Scoring system of histo-pathological damages.

Variability of fiber size	**Degenerate fibres**	**Necrotic fibres with sarcoclastosis**	**PAS+ glycogen accumulation Pale fibres**
+mild	- absent	absent	+low
++moderate	+3-4 /section	+1/section	++medium
+++severe	++>3-4/section	++2/section	+++high
_	_	+++>3/section	_

2.4Carcass quality

To evaluate the carcass quality, the skin on the breast area was removed and several incisions were made along the breast, thus, the muscles were examined for superficial and internal (deep) defects. The carcasses were scored from zero to 2 for all conditions: a score of 0 indicated no defects; 1 slight to moderate defects; 2 severe defects. The percentage of carcasses with defects was calculated, using the ratio between the number of birds, showing specific defect, to the total number of birds examined.

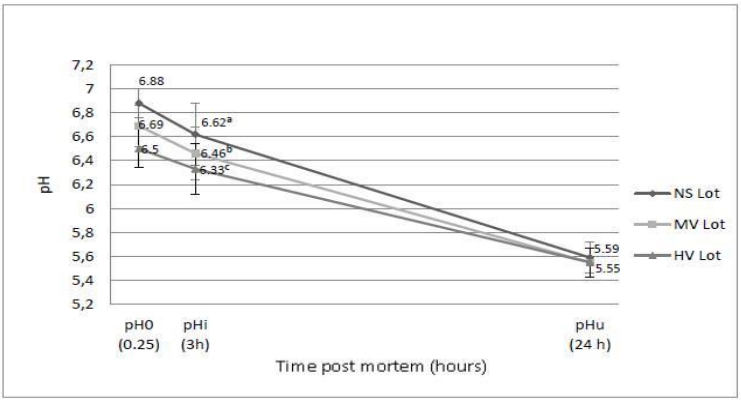
2.5Statistical Analysis

Analysis of Variance (one way ANOVA), with a model that included the effects of three different slaughters, was performed to detect significant differences ($P < 0.05$) among the groups (according to pHi Pectoralis *major* and Quadriceps *femoris*, Superoxides free radicals values, histological data) with the statistical package SPSS 15.0 (SPSS Inc.,Chicago).

3.Results and Discussion

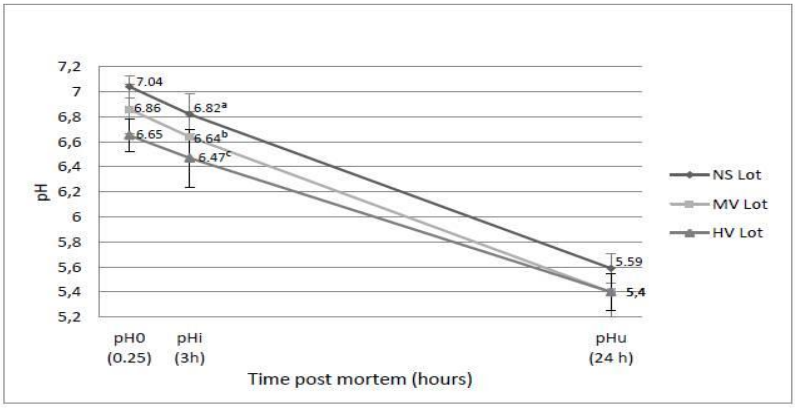
3.1Physicochemical Analysis

The Figures 2.7- 8, and Table 2.3 reported the pH values and statistical data on physicochemical parameters (pHi and Superoxides free radical values). At the end of the slaughter stress induced by bird agitation leads increases the concentration of lactate, and leads to a rapid pH drop in poultry muscle (Gregory and Bell, 1987; Petracci *et al*., 2010).The use of high current stunning (100mA-200 mA) increases significantly the pH values of Pectoralis *major* at 15 minutes, and unstunned birds had the fastest pH decline if compared to stunned broilers.



ANOVA test, different letters mean significant differences for P<0.05 (a, b, c)

Fig 2.7.Poultry slaughter - pH values (mean) of Pectoralis major at Time 0.25hours, 3hours, and 24 hours of NS (without stunning), MV (mid-voltage stunning) and HV (high-voltage stunning). The numbers associated to superscript letters showed significant differences.



ANOVA test, different letters mean significant differences for P<0.05 (a, b, c)

Fig.2.8.Poultry slaughter - pH values (mean) of Quadriceps femoris at Time 0.25hours, 3hours, and 24 hours of NS (without stunning), MV (mid-voltage stunning) and HV Lot (high-voltage stunning). The numbers associated to superscript letters showed significant differences.

Moreover, after 6 hours post-mortem, no difference is observed between stunned and unstunned birds (Papinaho and Fletcher, 1995; Papinaho and Fletcher, 1996; Craig and Fletcher, 1997).

According to literature, when Ross broilers were stunned at 200 mA and high frequencies (800 and 1,000 Hz)both Pectoralis *major* and Quadriceps *femoris* of MV and HV carcasses had the fastest pH decline 15 minutes post-mortem up to 3 hours post mortem. After 24 hours no differences were observed between the stunned and unstunned birds. According to Sante et al. (2000) and Xu et al. (2011) the glycogen concentration of

Pectoralis *major* decreased showing a fast exhaustion of glycogen at the early postmortem examination in MV and HV carcasses. Results confirm that high frequencies lead to major glycogen depletion and lower pH values in HV and MV than NV carcasses. At abattoir, the monitoring of pHi might be considered as a feasible marker of pre-slaughter stress in poultry, showing high-volt stunned carcasses with a more adequate muscle acidification and quality meat at 3 hours after slaughter.

Tab. 2.3.Poultry slaughter – Superoxide free radicals values (mean) (±SE) for measures of free radicals for NS (without stunning), MV (mid-voltage stunning) and HV Lot (high-voltage stunning) carcasses. Different letters within columns in ANOVA test mean significant differences for P<0.05 (a, b, c).

LOT	**superoxide free radicals values** Pectoralis *major* Quadriceps *femoris*	
	IDF Method	FOX Method
NS Lot (N=32)	0.57 [a] ± 0.20	0.32 [a] ± 0.22
MV Lot (N=32)	0.35 [b] ± 0.11	0.24 [b] ± 0.16
HV Lot (N=32)	0.25 [c] ± 0.09	0.16 [c] ± 0.09

Pre-slaughter heat stress can lead to muscle oxidative activity. Superoxide free radicals concentration provides useful data on the degree of peroxidation and is considered a marker of pre-slaughter stress in the muscle of poultry (Gregory, 1994; Shantha and Decker, 1994; Mujahidet al., 2005).

In the study, the statistical data showed a reduction of the superoxide free radicals in HV Lot, while the peroxides production gradually increased under low voltage conditions (MV Lot) and in absence of stunning (NS Lot). MV and NS carcasses clearly showed a more rapid and intense muscle oxidation as sign of pre-slaughter stress.

3.2Histological Analysis

The histological findings (PAS) showed a large number of pale fibers and rare positive fibers in all sections of NS, MV, and HV Lots. Morphological stains H&E and ET scoring showed the increase of variability in fiber size, presence of degenerated fibers, and angular atrophy (Fig.2.9). Damages as necrosis and sarcoclastosis were the most prominent findings, while COX and SDH showed a normal mitochondrial pattern in all the sections observed.

The pre-slaughter stress of poultry can be related to histological damages (Petracci et al., 2010). Nevertheless, changes of the glycogen reserve and muscle histological damages did not show relevant differences in the analyzed Lots.

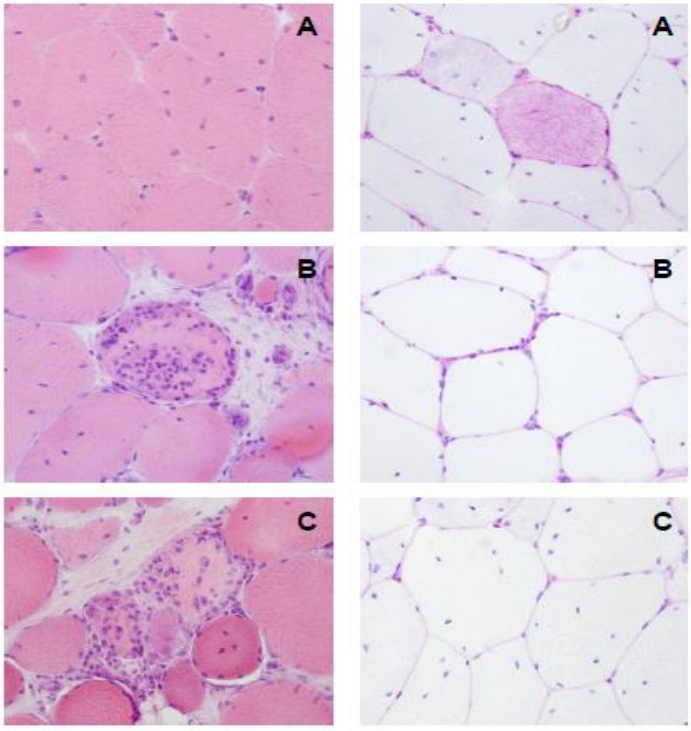


Fig.2.9. Histological findings H&E and PAS in muscle sections of NS (A), MV (B), and HV (C) Lots.

In addition, the necrosis and sarcoclastosis might be related both to acute pre-slaughter stress and to the slaughter itself. Therefore the histological

cellular damages cannot be considered as pre-slaughter stress markers in analyzed Lots (Table 2.4).

Table 4. Poultry slaughter –Prevalence of histo-pathological damages in NS (without stunning), MV (mid-voltage stunning) and HV Lot (high-voltage stunning) carcasses. Results are expressed as prevalence (+) (++) (+++) of alterations according to the scoring system of Table 2.

	Variability of fiber size			**Degenerate fibres**			**Necrotic fibers Sarcoclastosis**			**Pale fibres**		
	+++	++	+	+++	++	+	+++	++	+	+++	++	+
NS Lot (N=32)	0 [a]	28 [b]	4 [c]	0 [a]	27 [b]	5 [c]	18 [a]	11 [b]	3 [c]	21 [a]	9 [b]	2 [c]
MVLot (N=32)	0 [a]	29 [b]	3 [c]	0 [a]	28 [b]	4 [c]	20 [a]	9 [b]	3 [c]	22 [a]	9 [b]	1 [c]
HVLot (N=32)	1 [a]	29 [b]	2 [c]	1 [a]	28 [b]	3 [c]	22 [a]	9 [b]	1 [c]	22 [a]	10 [b]	0 [c]

3.3Carcass quality

In our study, the evaluation of carcass quality showed that the incidence of birds with carcass defects sufficient to cause downgrading or rejection was generally low.

High stunning frequencies may improve meat quality without aggregating stress, when the current is not too low, with a considerable commercial advantage (Wilkins et al., 1999; Xu et al., 2011). The HV Lot showed a lower incidence of defects (87.5% scored 0; 12.5 % scored 1) than MV Lot (81.25% scored 0; 12.5% scored 1; 6.25% scored 2). The incidence of defects was limited in the majority of NS carcasses (90.62% scored 0; 9.38% scored 1). According to the literature, stunning frequencies above 1,000 Hz resulted in significant reduction of breast muscle hemorrhaging and broken bones, and current stunning conditions above 60V produced the best carcass quality, if compared to low or high voltage treatments (Gregory and Wilkins, 1989; Gregory and Wotton, 1990; Ali *et al*., 2007).

4.Conclusions

Animal welfare is a complex concept that needs to be approached from different perspectives. The Brambell report (1965) has been very influential and still serves as an important benchmark for legal definitions such as the EU minimum standards and the Treaty of Lisbon (article 13), whichexplicitly recognizes farm animals as sentient beings (de Jonge and van Trijp, 2013). Most developed countries have slaughter laws for food animals which are designed to ensure that animals are killed quickly,

painlessly, and without suffering in other ways. However, the last Report FVO (2014) highlighted the need of welfare monitoring, through indicators, in poultry slaughterhouse to evaluate the efficiency of the procedures under practical conditions. The welfare indicators can support decision-making on the acceptable conditions for animals, and be used to underpin monitoring and control programmer, implemented at slaughterhouse, to guarantee standards of animal welfare. In addition, they can help poultry industries to obtain good quality products (FVO Report, 2014).

In broiler chickens processed without stunning or slaughtered by using sinusoidal alternating current and different frequencies, the high frequencies stunning (1,000Hz; 200 mA; 67V) increased lactate production, induced a gradual pH decline, and reduced the muscle oxidative activity and incidence of carcass defects. Therefore, to assess animal welfare and quality poultry meat pH monitoring and measurement of superoxide radical production, as additional and feasible parameters, might be markers easier to use under practical conditions at slaughterhouse.

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Chapter 3

Biogenic Amines as Quality Index in ozone treated poultry carcasses

Mercogliano R., De Felice A., Murru N., **Santonicola S.,** Cortesi M.L. (2014).Ozone Decontamination ofPoultry Meat and Biogenic Amines as QualityIndex. *J Food Process Technol*, 5:3.

1. Introduction

The worldwide poultry meat production has increased rapidly and, per capita consumption of poultry meat in many parts of the world will continue to grow. Competitive price, the absence of cultural and religious obstacles, and dietary and nutritional properties are the main factors explaining the poultry meat's attractiveness for consumers (Petracci and Cavani, 2012).

Hygiene intervention in the poultry meat processes alone does not lead to safe products, owing to the constant flow of bacteria entering the processing plant and unavoidable cross- contamination. Eradication of pathogens in the livestock, or the rearing of animals that are "Specified Pathogen Free" (SPF), might make relevant contributions (Bolder, 1997).

Food-borne diseases have a major health impact in industrialized countries. Zoonotic pathogens are of special concern in foodof animal origin and have to be controlled by a feed-to-food system. In recent years, healthy food animals were recognized as carriers of pathogens responsible for human illness (Nørrung and Buncic, 2008). To counter this threat, the focus is currently on preventive systems in accordance with the hazard

analysis and critical control point (HACCP) principles (Ropkins and Beck, 2000; Loretz et al., 2010).

1.1 Decontamination treatments of poultry carcasses

Decontamination of meat and poultry carcasses can help to reduce human food borne infections and seems to be the only possibility to assure food safety. So many decontamination treatments of poultry carcasses have been described, which can roughly be divided into three types: chemical, physical and combinations of the two (Table 3.1).

The problems are that not all of the treatments are applicable in the meat industry. Physical or chemical treatments of poultry carcasses, approved in the USA, are not allowed in Europe according to EU regulations. However, the decontamination of poultry carcasses has recently re-attracted attention in Europe, because poultry meat is often implicated as a risk factor in human campylobacteriosis and food-borne diseases (EFSA, 2009; Loretz et al., 2010).In foodindustry, the presence of bacteria biofilms is a significant problem, because of the environmental persistence and their resistance to desiccation, UV radiation, and other antimicrobial treatments. Since the processes used for the cleaning and disinfection of equipment and poultry surfaces can reduce, but not always eliminate, bacteria and

pathogens contamination, in Europe there is an increased interest in developing decontamination methods applicable to meat (EFSA, 2009).

Tab.3.1. Chemical and physical decontamination treatments

Chemical treatments*	**Physical treatments***
Chlorine (hypochlorite, ClO_2)	Water (rinse, spray, steam)
Organic acids (lactic acid, acetic acid, etc.)	Ultrahigh pressure
Inorganic phosphates (trisodium phosphate)	Irradiation
Organic preservatives (benzoates, propionates)	Pulsed-field electricity
Bacteriocins (nisin)	Ultrasonic energy
Oxidizers (hydrogen peroxide, ozone)	UV light

*Combinations of the above chemical and physical applications can also be used

1.1.1 Ozone decontamination treatment

Ozone (O_3) is a tri-atomic gaseous molecule consisting of three oxygen atoms (Fig.3.1). It is an allotrope of oxygen much less stable than the diatomic allotrope (O_2) characterized by strong oxidizing nature that makes it a useful tool for the inactivation of bacteria, fungi, and viruses (Moore et al., 2000).

Ozone first attacks the bacterial membrane at the glycoproteins, glycol lipids, or at certain amino acids such as tryptophan, and then also acts on

the sulphydryl groups. Bacterial death is rapid and often attributed to changes in cell permeability followed by lysis. The bactericidal effect depends on several factors, such as temperatures, relative humidity, pH values and presence of organic matter. Ozone was effective against Gram-positive (including spore-formers) and Gram-negative bacteria (Moore et al., 2000).

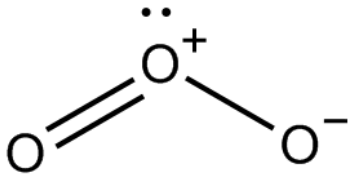

Fig.3.1. Chemical structure of Ozone

The U.S. FDA -Department of Health and Human Services that has considered ozone as food additives and GRAS substance has approved decontamination treatment with ozone. The FDA has approved its use, as antimicrobial agent, in gas phase and water, during the storage and processing of food, according to the Good Practices Manufacturing (GMP) (Code of Federal Regulation-CFR document 21).Ozone treatments for the food decontamination are also permitted in Canada, but the gas concentrations do not exceed the minimum levels technologically

necessary. Ozone represents a public health hazard above concentrations of 100 µg/kg, especially when it is applied in production areas, because of high oxidizing potential, that can cause damage in mucus and respiratory tissues, but the modern ozone generators can be better controlled the gas emissions in habitat (Bodmer et al., 1999).

European Regulations n. 852/2004/EC and853/2004/EC authorize only the use of ozonized potable water, for pathogens control in meat production. Furthermore, the experts of Italian National Committee for Food Security (CNSA, 27/10/2011) have expressed a positive opinion regarding gaseous ozone treatment of chambers of seasoning or storage of cheese in the absence of food.

In foodindustry, ozone is used as sanitizing agent capable of killing numerous microorganisms by oxidizing their cell membranes (Moore et al., 2000; Guzel-Seydim et al., 2004).There are numerous application areas of ozone in the industry such as food surface hygiene, sanitation of food plant equipment, reuse of waste water, treatment and lowering biological oxygen demand and chemical oxygen demand of food plant waste. The antimicrobial efficacy can be enhanced considerably when ozonation is combined with other chemical (e.g., hydrogen peroxide) or physical (e.g., ultraviolet radiation) treatments (Guzel-Seydim et al., 2004).Sometimes

ozone generators are used in food storage rooms to control the growth of microorganism and the shelf life of products. It has been proposed as chemical decontamination treatment of poultry carcasses because it is capable of experimentally extending the shelf life of perishable foods by reducing microbial activity (Bolder, 1997; Rice et al., 2001).

1.2 Biogenic Amines as quality index

Biogenic Amines (BAs) (Fig.3.2.) are organic, basic nitrogenous compounds of low molecular weight that are present in large number of foods, including meat and meat products (Bodmer et al., 1999). They are classified in three categories according to their chemical structures: aromatic biogenic amines, aliphatic diamines, and natural polyamines. Biogenic amines are produced mainly by decarboxylation of precursor amino acids by specific microbial enzymes. This group includes aromatic amines (tyramine, phenylalanine, histamine, and tryptamine) and aliphatic amines (putrescine, cadaverine, agmantine). The physiologically natural polyamine, spermine, and spermidine, are not associated with microbial activity and their biosyntesisfollows a more complex process (Bardocz, 1995).

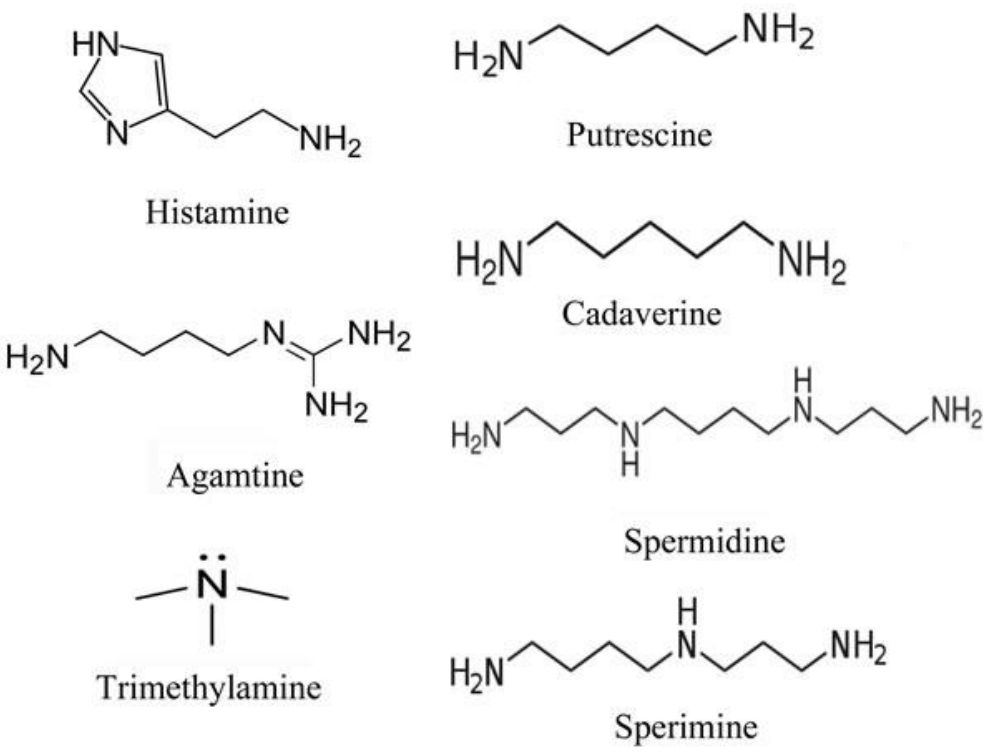

Fig.3.2. Chemical structuresof Biogenic Amines.

The presence of specific BAs depends on the decarboxylase enzyme, that catalysis the decarboxylation of amino acids, and a transporter responsible for aminoacid–BA interchange (Linares et al., 2011). Decarboxylation increases bacterial survival under stress conditions via the consumption of protons and the excretion of amines and CO_2, helping to restore the

external pH (Rhee et al., 2005). BAs production may also offer a way of obtaining energy in the bacteria metabolism (Durlu-Özkaya et al., 2001; Bunkova et al., 2010).

The formation of BAs is primarily a consequence of the enzymatic decarboxylation of specific aminoacids due to microbial enzymes or tissue activity, and their production depends on the availability of free amino acids and conditions that favour decarboxylase enzymatic processes, such as temperature, pH and saline and oxygen concentrations (Vinci and Antonelli, 2002). The most important BAs in food are putrescine,cadaverine, tyramine, histamine, 2 -phenyl ethylamine and agmatine, produced by decarboxylation of the amino acids ornithine, lysine, tyrosine, histidine, phenylalanine, and arginine, respectively (Fig.3.3).

BAs may have a role as indicators of quality and/ or acceptability in some foods (Veciana-Nogues et al., 1995; Mietz and Karmas, 1997; Carou, 1999). The estimation of BAs in foods is also an important toxicological point of view (Halasz et al., 1994). Knowledge of BA levels in food is important for assessing health hazards; for example, they can cause some neurotransmission disorders due to their action as false neurotransmitters

(Silla Santos, 1996). Moreover, the presence of BAs can cause headaches, nausea, and palpitations (Arlorio et al., 1998).

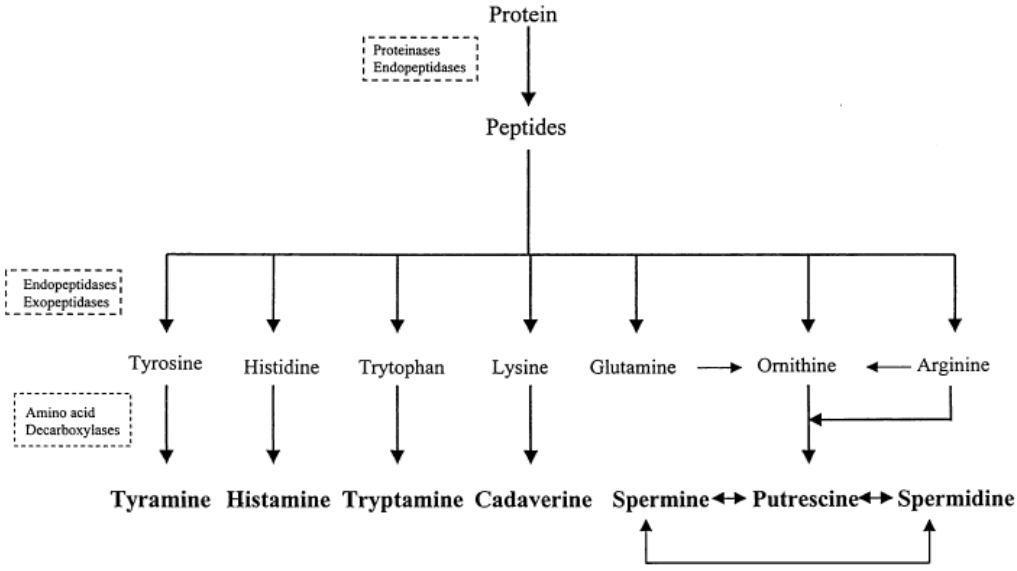

Fig.3.3. Formation of biogenic amines (Ruiz-Capillas and Jimenez-Colmenero, 2004)

In particular, tyramine excess could cause hypertension while serotonin is a vasoconstrictor (Vinci and Antonelli, 2002). Biogenic amines constitute a potential health risk, especially when coupled with additional factors, such as monoamine oxidase inhibitor drugs, alcohol and gastrointestinal diseases (Stratton et al., 1991; Silva et al., 2002).

The concentration of some BAs (tyramine, putrescine, and cadaverine) normally increases during the processing and storage of meat and meat

products, whereas other (spermine and spermidine) decrease or remain constant (Ten Brink et al., 1990; Bardocz, 1995; Ruiz-Capillas and Moral, 2001). In meat and cooked meat product, BAs have been used as indicators of unwanted microbial activity(Halasz et al., 1994). A combination of putrescine (PUT) and cadaverine (CAD) has been suggested as an index of acceptability in fresh meat because their concentrations increase prior to spoilage and are well correlate with themicrobial load (Slemr and Beyermann, 1985; Leuschner et al., 1998). High cadaverine concentrations are considered clearly indicative of spoilage (Edwards et al., 1985).

1.2.1 Occurrence of Biogenic Amines in poultry meat

Poultry surface and meat provide a condition suitable especially for the growth of microorganisms and thus also for production of BAs (Wortberg and Woller, 1992, Tamin and Doerr, 2003). An increase of BAs formation in poultry muscle can occur even during freezing (Moreira et al., 2008). BAs can indicate the quality of meat, and more specifically hygienic conditions under which it was processed and stored (Bunkova et al., 2010). It is reported that putrescine, cadaverine, and tyramine, could serve as quality indicators of modified atmosphere packaging-stored broiler chicken (Rokka et al., 2004).

In particular, BA levels in white meat could be used as valuable indicators of spoilage because of their susceptiblity to proteins degradation, which takes place under appropriate conditions (Vinci and Antonelli, 2002). In fact, in chicken muscles, there are shorter fibers than in bovine muscles, which can be easily attached by proteolytic enzymes. This can favor the availability of free amino acids, which represent amine precursors. In white meat, quite all BAs were normally found but CAD is produced in the greatest quantity, probably due to the high amount of the precursor lysine (Vinci and Antonelli, 2002). Moreover, the increasing of PUT levels are often associated to spoilage conditions. Consequently, BAs determination in meat is suitable for detecting incipient spoilage and certain amines levels can be related to the freshness of the poultry meat. As a quality index of freshness in white meat, the determination of CAD and PUT concentrations could be used to monitor spoilage (Vinci and Antonelli, 2002, Rokka et al., 2004).

Aim of this study was to evaluate the effects of an experimental ozone treatment during the storage of chilled poultry carcasses, and the correlation of BAs (CAD and PUT) production as quality index in poultry meat.

2. Materials and Methods

Immediately after slaughter and chilling, 50 broilers, weight 2.5 kg, were collected in an EU authorized slaughterhouse. Lot control C (25 carcasses) was stored in refrigerated cell equipped with thermograph set to 0-1°C (Fig.3.4).

Fig.3.4. Poultry carcasses stored in refrigerated cell.

To obtain a major decontamination effect, according to literature, Lot A (25 carcasses) was previously washed by ozonized water, before the gaseous sanitizing treatment (Gorman et al., 1995; Bolder, 1997; Balamatsia et al., 2006). Then the carcasses of Lot A were stored in a refrigerated cell, equipped with video thermograph set to 0-1°C and a generator of gaseous ozone (OZONET of OXITECH Srl). To deliver the ozone distribution for an interval 60 minutes every 4 hours, on the basis of

preliminary bacterial reduction tests on carcasses surfaces, a timer was installed (Kim et al., 1999; Murray et al., 2008).

Dimensions of both refrigerated cells were: height 3.60 cm, inches deep 3.24 cm and length 1.32 cm. Temperature values and ozone air levels inside the cells was monitored using a probe and a display, positioned on the outside of the cell, along the time of the experiment. At 0th, 4th, 6th, 8th, 11th, 14th and 20th days of storage three carcasses of each batch were collected. The breast muscle (predominantly white fibers) and limbs (prevalence of red fibers), of each carcass, were separately subjected to the determination of the pH values. Then sensory analysis ofBAs were carried out on a pool of the two aliquots combined.

2.1 Physicochemical Analysis

The pH evaluation was carried out by incision of the muscles of the breast and limb regions. Determination of pH was carried out with a pH meter (Microprocessor pH Meter Hanna Instruments).

2.2 Sensorial analysis

For the evaluation of sensory parameters a panel of 5 people, that have consistently followed the analysis, completed a questionnaire using a score

between 0 and 4. Abnormal coloration and discolored surface were considered.

2.3 Analysis of biogenic amines

Solvents and reagents were of analytical or High-Performance Liquid Chromatography (HPLC) grade. The biogenic amine standard of putrescine dansyl-hydrochloride (PUT) and Cadaverine Dansyl-Dihydrochloride (CAD) were obtained from Sigma (St. Louis, MO).

Analyses were carried out using an HPLC system (JASCO), equipped with a quaternary pump (JASCO 2089 plus), and a 20 µL loop, combined with a Jasco integrator, and an 821-Fp fluorescence detector. Detection of amines was accomplished at an excitation wavelength of 350 nm and an emission wavelength of 520 nm. Separation was performed on a reversed- phase C18 Luna column 5 µm 518 (Phenomenex Inc.,Torrance CA) (250 mm length x 4.6 mm internal diameter, and 5 µm particle size) with a 3 X 4 mm Security Guardcartridge guard column (Phenomenex Inc., Torrance CA).

2.3.1Sample preparation

Perchloric acid 0.2 M was added and mixed to homogenized chicken meat (da Silva et al., 1998).The extract was centrifuged at 4000 rpm for 20 min

in three steps. The surfactant was collected, divided into portions of 2 mL and then centrifuged at 12.000rpm for 10 min. Finally, amine extract was reacted with dansyl- chloride overnight at 25°C and extracted with 2 mL diethyl ether. The extract was concentrated to dryness, solved in 100 µL of methanol. Twenty microlitres of methanol were injected into the HPLC system.

2.3.2Chromatography

The mobile phase was water (A) and methanol (B). Before the use, the solvents were filtered through 0.1 µm and degasser under vacuum. Amines were eluted with a flow rate of 2 mL/min by the gradient: 35%water and 65% methanol for 7 min, and then 100% methanol for 15 min. The linearity and the quantification of the instrumental response were checked by injecting five different concentrations ranging from 0.6 up to 200 µg/L of a PUT and CAD standard mixture.

3. Results

Ozone concentrations inside the cells ranged from 0.82 ppm (minimum value) to 1.2 ppm (maximum value). Therefore, under these conditions, the results showed the following considerations.

3.1Physicochemical and Sensory Analysis

In poultry meat of Lots A and C, pH values, as a function of storage time, were reported in Figures 3.5 and 3.6.

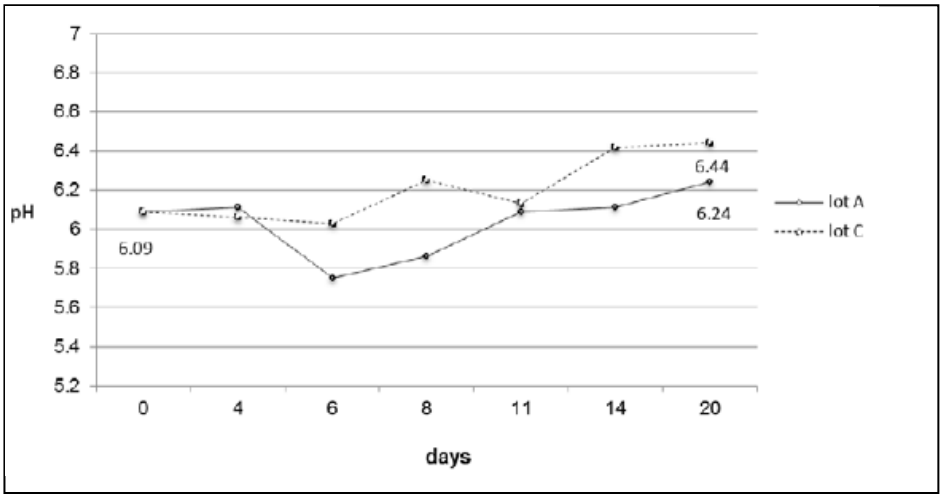

Fig. 3.5. Values of pH (breast) during the storage of ozone treated poultry carcasses of Lot A and Lot control C.

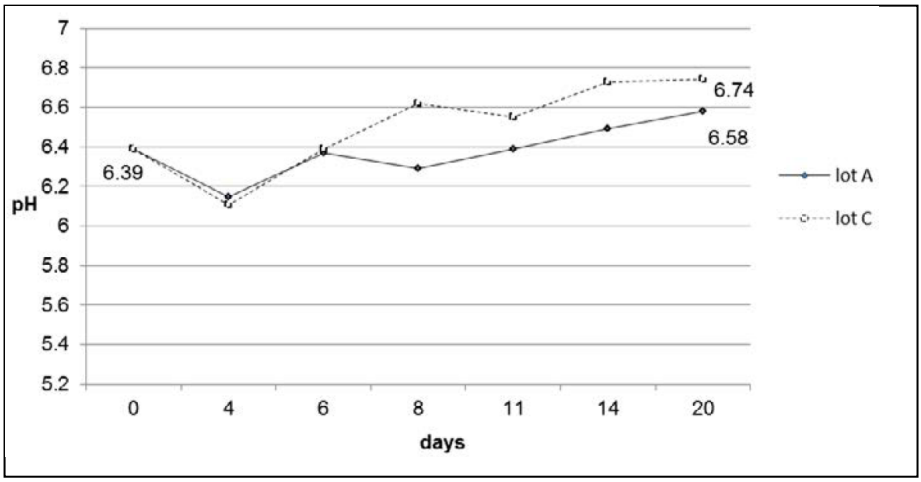

Fig. 3.6. Values of pH (leg) during the storage of ozone treated poultry carcasses of Lot A and Lot control C.

At 20th day of storage both in carcasses of Lot C and A, pH results showed values more acid (6.44 Lot C and 6.24 Lot A) in chicken breast than in the leg, probably because of the prevailing anaerobic metabolism of white fibers. During the storage, a gradual increase of pH values in meatof simply chilled carcasses was found, and it could be related to BAs production (Hernandez-Jover et al., 1997; Tamim et al., 2002; Balamatsia et al., 2006).

Furthermore, in ozone treated meat of Lot A the shelf life was 6 days longer than those of Lot C. It is possible that more acid pH values have better controlled the microbiological quality of poultry treated carcasses. In

particular, carcasses of Lot control showed a surface discoloration, while in the treated carcasses of Lot A an acceptable sensory quality occurred until to 20th day (Table 3.2).

Tab.3.2. Results of the sensory evaluation at 14th and 20th day.

Defects	Score	14th day		20th day	
		LOT C N.	LOT A N.	LOT C N.	LOT A N.
General coloration	0	1	4	0	2
	1	2	15	0	12
	2	2	5	3	8
	3	11	1	12	3
	4	9	0	10	0
Discoloration %	0	0	8	0	3
	1	1	11	0	10
	2	4	3	2	9
	3	12	1	13	3
	4	8	0	10	0

3.2 Analysis of Biogenic Amines

In poultry meat of Lots A and C, BAs values, as a function of storage time, were reported in Figures 3.7-9. BAs are considered indicators of quality of raw materials and process hygiene in meat and their levels depend on the specific micro flora and the bacterial count of food (Mercogliano et al., 2010).According to Balamatsia et al. (2006) higher levels of PUT (53.63 mg/kg) and CAD (175.20 mg/kg), were found in simply chilled poultry

meat of LotC, characterized by a growing trend and significant increases to 15th days.

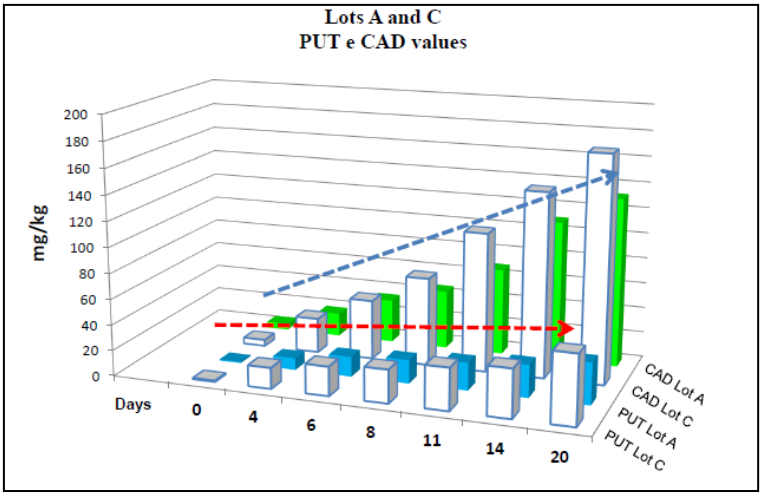

Fig.3.7. Individual levels of BAs during the storage of Lot A treated poultry carcasses and Lot control C.

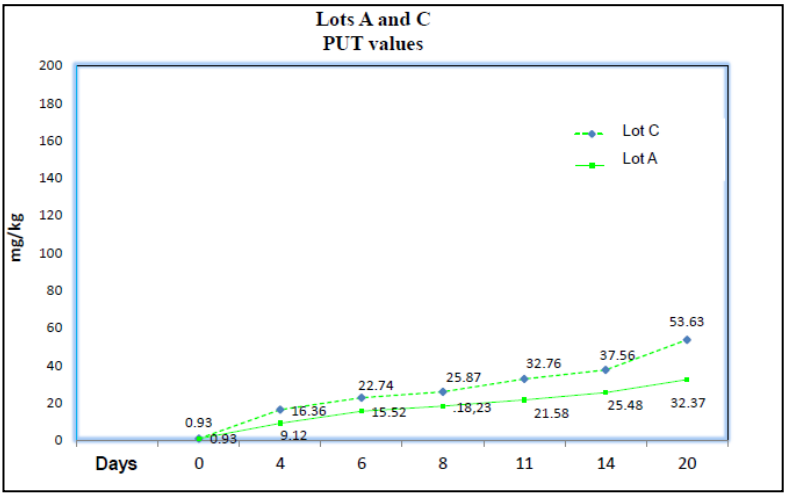

Fig.3.8. Mean levels of PUT (mg/kg) during the storage of Lot A treated poultry carcasses and Lot control C.

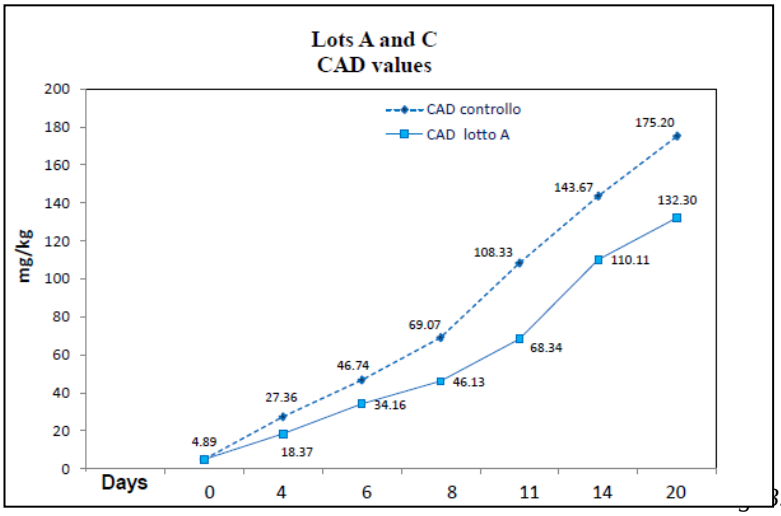

Fig. 3.9. Mean levels of CAD (mg/kg) during the storage of Lot A treated poultry carcasses and Lot control C.

The decontamination effect of ozone associated with chill temperatures, resulted in a lower level of PUT (32.7 mg/kg) and CAD (132.30 mg/kg) in ozone treated poultry meat.In3creasing the ranges of gaseous O_3 injection on the chambers might cause a further reduction of BA levels.

4. Conclusions

Decontamination ozone treatments can limit the surface contamination of poultry carcasses and can be a valuable method in poultry slaughterhouses if an integrated approach to the problem “decontamination” is considered.

In fact, the ozone treatment of chilled poultry carcasses may show positive effects on the bacterial decontamination and on shelf life of poultry meat (Bolder, 1997). Actually, European Regulations authorize only the use of ozonized potable water, for pathogens control in meat production (Reg. 852/2004/EC; Reg. 853/2004/EC).

Several indicators have been proposed for the evaluation of meat quality (volatile bases, nucleotides break-down, volatile acidity), but all are limited (Nakamura et al., 1979; Slemr and Beyermann, 1985; Yano et al., 1995; Mietz and Karmas, 1997; Veciana-Nogues et al., 1997; Lima and Gloria, 1999; Min et al., 2007).Quality sensory analysis is undoubtedly a subjective method to detect quality of fresh meat (Mietz and Karmas, 1997).Furthermore, it would be desirable to identify parameters most effective to evaluate meat's shelf life, before the effects of the spoilage and changes of sensory quality (Durlu-Ozkaya et al., 2001). In particular, European expert group BIOHAZrecognized a strong correlation between the BAs presence in food and the quality of raw materials. Biogenic amines, histamine, putrescine, tyramine, tryptamine, 2pheniylethylamine and cadaverine, could beconsidered useful indicators of freshness of meat (Wortberg and Woller, 1992; Mietz and Karmas, 1997; da Silva et al., 1998; Gloria et al., 1999; Silva et al., 2002). Levels of all amines increase

with increasing putrefaction except spermidine and spermine, which decrease during the storage of poultry carcasses. (Wortberg and Woller, 1992). PUT and CAD also exhibited a common response pattern, and in poultry carcasses, these amines reached their plateau at 48 h (Wortberg and Woller, 1992).

PUT and CAD levels, as indicators of freshness/spoilage, were correlate to microbial quality of poultry meat in treated and no-treated carcasses (Wortberg and Woller, 1992).Therefore the detection of PUT and CAD, as BAs indicators, may be a valuable and sufficiently rapid method for monitoring the effectiveness of a decontamination treatment.

Results showed a reduction of microbial contamination as effect of the ozone treatment during the storage of chilled poultry carcasses. In fact, the shelf life of treated carcasses was 6 days longer than those of untreated carcasses. Moreover, Lot A carcasses showed lower levels of Bas. Evaluation of PUT and CAD levels was useful to highlight the loss of meat freshness before sensorial changes and the effectiveness of the ozone treatment.

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