UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

Tesi di Dottorato di Ricerca in

Scienze e Tecnologie delle Produzioni Agro-Alimentari

XX ciclo

DEVELOPMENT AND APPLICATION OF ANTIMICROBIAL FOOD PACKAGING

Il Tutor

Prof. Gianluigi Mauriello

Il Dottorando

Dott. Antonietta La Storia

Coordinatore

Prof. Salvatore Spagna Musso

INDEX

Preface

Packaging has a significant role in the food supply chain and it is an integral part both of the food processes and the whole food supply chain. Food packaging has to perform several tasks as well as fulfilling many demands and requirements. Traditionally, a food package makes distribution easier. It has protected food from environmental conditions, such as light, oxygen, moisture, microbes, mechanical stresses and dust. Other basic tasks have been to ensure adequate labelling for providing information e.g., to the customer, and a proper convenience to the consumer, e.g., easy opening, reclosable lids and a suitable dosing mechanism. Basic requirements are good marketing properties, reasonable price, technical feasibility (e.g., suitability for automatic packaging machines, sealability), suitability for food contact, low environmental stress and suitability for recycling or refilling. A package has to satisfy all these various requirements effectively and economically. For a long time packaging has also had an active role in processing, preservation and in retaining quality of foods. Changes in the way food products are produced, distributed, stored and retailed, reflecting the continuing increase in consumer demand for improved safety, quality and extended shelf-life for packaged foods, are placing greater demands on the performance of food packaging. Consumers want to be assured that the packaging is fulfilling its function of protecting the quality, freshness and safety of foods. The trend to ensure the quality and safety of food without, or at least fewer, additives and preservatives means that packaging has a more significant role in the preservation of food and in ensuring the safety of food in order to avoid wastage and food poisoning and to reduce allergies.

According to the definitions of the ACTIPAK-FAIR CT98-4170 project “Active packaging changes the condition of the packed food to extend shelf life or to improve safety or sensory properties, while maintaining the quality of the packaged food”.

Food condition in the definition of active packaging includes various aspects that may play a role in determining the shelf-life of packaged foods, such as physiological processes (e.g., respiration of fresh fruit and vegetables), chemical processes (e.g., lipid oxidation), physical processes (e.g., staling of bread, dehydration), microbiological aspects (e.g., spoilage by microorganisms) and infestation (e.g., by insects). Through the application of appropriate active packaging systems, these conditions can be regulated in numerous ways and, depending on the requirements of the packaged food, food deterioration can be significantly reduced. As reported in Table 1, active packaging techniques for preservation and improving quality and safety of foods can be divided into three categories; absorbers (i.e. scavengers), releasing systems and other systems. Absorbing (scavenging) systems remove undesired compounds such as oxygen, carbon dioxide, ethylene, excessive water, taints and other specific compounds. Releasing systems actively add or emit compounds to the packaged food or into the head-space of the package such as carbon dioxide, antioxidants and preservatives. Depending on the physical form of active packaging systems, absorbers and releasers can be a sachet, label or film type. Sachets are placed freely in the head-space of the package. Labels are attached into the lid of the package. Direct contact with food should be avoided because it impairs the function of the system and, on the other hand, may cause migration problems.

Table 1 - Examples of sachet, label and film type absorbing (scavenging) active packaging systems for preservation and shelf-life extension of foods or improving their quality and usability for consumers.

Packaging type	Examples of working principle/ mechanism/reagents	Purpose	Examples of possible applications
Oxygen absorbers (sachets, labels, films, corks)	Ferro-compounds, ascorbic acid, metal salts, glucose oxidases, alcohol oxidase	Reduction/preventing of mould, yeast and aerobic bacteria growth Prevention of oxidation of fats, oils, vitamins, colours. Prevention of damage by worms, insects and insect eggs	Cheese, meat products, ready-to-eat products, bakery products, coffee, tea, nuts, milk powder
Carbon dioxide Absorbers (sachets)	Calcium hydroxide and sodium hydroxide orpotassium hydroxide Calcium oxide and silica gel	Removing of carbon dioxide formed during storage in order to prevent bursting of a package	Roasted coffee Beef jerkey Dehydrated poultry products
Lactose remover	Immobilised lactase in the packaging material	Serving milk products to the people suffering lactose intolerance	Milk and other dairy products
Humidity absorbers (dripabsorbent sheets, films, sachets)	Polyacrylates (sheets) Propylene glycol (film) Silica gel (sachet) Clays (sachet)	Control of excess moisture in packed food Reduction of water activity on the surface of food in order to prevent the growth of moulds, yeast and spoilage bacteria	Meat, fish, poultry, bakery products, cuts of fruits and vegetables
Absorbers of off flavours, amines and aldehydes (films, sachets)	Cellulose acetate film containing naringinase enzyme. Ferrous salt and citric or ascorbic acid (sachet) Specially treated polymers	Reduction of bitterness in grapefruit juice Improving the flavour of fish and oilcontaining food	Fruit juices Fish Oil-containing foods such as potato chips, biscuits and cereal products Beer
UV-lightabsorbers	Polyolefins like polyethylene and polypropylene doped the material with a UV-absorbent agent Crystallinity modification of nylon UV stabiliser in polyester bottles	Restricting lightinduced oxidation	Light-sensitive foods such as ham Drinks

Table 1 *(continued)*

Packaging type	Examples of working principle/ mechanism/reagents	Purpose	Examples of possible applications
Cholesterol remover	Immobilised cholesterol reductase in the packaging material	Improving the healthiness of milk products	Milk and other dairy products
Carbon dioxide emitters (sachets)	Ascorbic acid Sodium hydrogen carbonate and ascorbate	Growth inhibition of gram-negative bacteria and moulds	Vegetables and fruits, fish, meat, poultry
Ethanol emitters (sachets)	Ethanol/water mixture absorbed onto silicon dioxide powder generating ethanol vapour	Growth inhibition of moulds and yeast	Bakery products (preferably heated before consumption) Dry fish
Flavouring emitters (films)	Various flavours in polymers Minimisation of flavour scalping	Masking off-odours Improving the flavour of food	Miscellaneous
Antimicrobial preservative releasers (films)	Organic acids, e.g. sorbic acid Silver zeolite Spice and herb Extracts Alylisothiocyanate Enzymes, e.g. Lyzozyme bacteriocins	Growth inhibition of spoilage and pathogenic bacteria	Meat, poultry, fish, bread, cheese, fruit and vegetables

Taken from: Han J.H. (2000) Antimicrobial Food Packaging. *Food Technology* **54**, 56-65.

1. ANTIMICROBIAL FOOD PACKAGING

1.1 Introduction

Antimicrobial packaging is one of many applications of active packaging. Antimicrobial packaging is the packaging system that is able to kill or inhibit spoilage and pathogenic microorganisms that are contaminating foods. The new antimicrobial function can be achieved by adding antimicrobial agents in the packaging system and/or using antimicrobial polymers that satisfy conventional packaging requirements. Antimicrobial packaging materials have to extend the lag phase and reduce the growth rate of microorganisms to prolong the shelf life and maintain food quality and safety (Han, 2000).

1.1.1. Developing the antimicrobial packaging systems

Most food packaging systems represent either a package/food system or a package/headspace/food system (Fig. 1.1). A package/food system is a solid food product in contact with the packaging material, or a low-viscosity or liquid food without headspace. Diffusion between the packaging material and the food and partitioning at the interface are the main migration phenomena involved in this system. Antimicrobial agents may be incorporated into the packaging materials initially and migrate into the food through diffusion and partitioning (Han, 2000). Package/headspace/food systems are represented by foods packed in flexible packages, cups, and cartons. Evaporation or equilibrated distribution of a substance among the headspace, packaging material and/or food has to be considered as a part of main migration mechanisms to estimate the interfacial distribution of the substance. A volatile active substance can be used in these systems, as it can migrate through the headspace and air gaps between the package and the food.

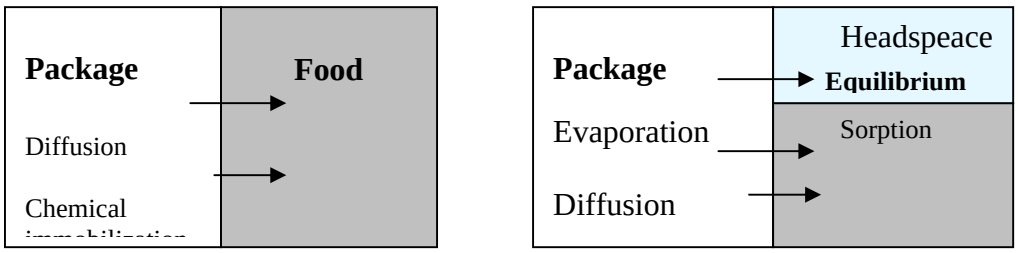


Fig. 1.1 - Food packaging systems and relative behaviour of active substances (Han, 2000).

Antimicrobial packaging can take several forms including:

- ◊ Addition of sachets/pads containing volatile antimicrobial agents into packages
- ◊ Incorporation of volatile and non volatile antimicrobial agents directly into polymers
- ◊ Coating and adsorbing antimicrobials onto polymers surfaces
- ◊ Immobilization of antimicrobials to polymers by ion or covalent linkages
- ◊ Use of polymers that are inherently antimicrobial (Appendini *et al.*, 2002).

Besides diffusion and equilibrated sorption, some antimicrobial packaging uses covalently immobilized antibiotics or fungicides, or active moieties such as amine groups. This case utilizes surface inhibition of microbial growth by immobilization of the non-food grade antimicrobial substance without diffusion mass transfer. Figure 1.2 shows the mass transfer phenomena of an active substance incorporated into a film or coating, with different applications. The incorporation of an antimicrobial substance into a food packaging system can take several approaches. One is to put the antimicrobial into the film by adding it in the extruder when the film or the co-extruded film is produced. The disadvantage of doing so is poor cost effectiveness since antimicrobial material not exposed to the surface of the film is generally not totally available to antimicrobial activity. An alternative to extrusion is to apply the antimicrobial additive in a controlled matter where the material is needed and not lost; for

example, it can be incorporated into the food-contact layer (usually also serving as the inner heat-seal layer) of a multilayer packaging material.

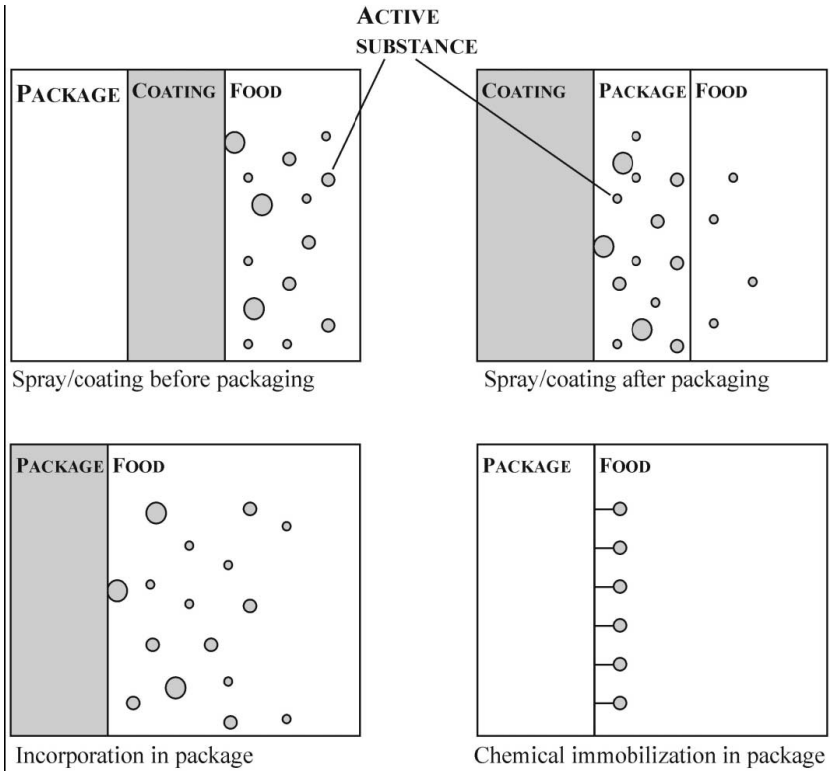


Fig. 1.2 - Migration of active substance in different applications of antimicrobial packaging systems (Han, 2000)

1.1.2 Modelling of the antimicrobial film or package

According to Han (2000), several factors must be taken into account in the design or modelling of the antimicrobial film or package:

- *Chemical nature of films/coatings, casting process conditions and residual antimicrobial activity*. The choice of the antimicrobial is often limited by the heat lability of the component during extrusion or by the incompatibility of the component with the packaging material. For example, 1% potassium sorbate in a LDPE film inhibited the growth of yeast on agar plates.
- *Characteristics of antimicrobial substances and foods.* Food components significantly affect the effectiveness of the antimicrobial substances and their release. Physico-chemical characteristics of food could alter the activity of antimicrobial substances.

For example, the pH of food influences the ionisation (dissociation/association) of most active chemicals, and could change the antimicrobial activity of organic acids and their salts. The antimicrobial activity and chemical stability of incorporated active substances could be influenced also by the water activity of food. Moreover, each food has its own characteristic microflora. The release kinetics of antimicrobial agents has to be designed to maintain the concentration above the critical inhibitory concentration with respect to the contaminating microorganisms that are likely to be present.

- *Storage temperature*. Storage temperature can affect the antimicrobial activity of chemical preservatives. Generally, increased storage temperature can accelerate the migration of the active agents in the film/coating layers, while refrigeration slows down the migration rate. The temperature conditions during production and distribution have to be predicted to determine their effect on the residual antimicrobial activity of the active compounds.
- *Mass transfer coefficients*. The simplest system is the diffusional release of active substances from the package into the food. A multilayer design has the advantage that the antimicrobial can be added in one thin-layer and its migration and release controlled by the thickness of the film layer or coating. In practice, a matrix of several layers is used to control the rate of release of the active substance. Control of the release rates and migration amounts of antimicrobial substances from food packaging is very important. Han (2000) summarized traditional mass transfer models and his own proposed models that may be used to describe the migration of active agents through food packaging systems consisting of single, double, or triple layers.
- *Physical properties of packaging materials.* When antimicrobial activity is added to packaging materials to reduce microbial growth, it may affect the general physical properties of the packaging materials. Han and Floros (1997) found that the

transparency of the plastic film under study decreased with the addition of the active agent. The performance of the packaging materials must be maintained after the addition of the active substances, even though the materials contain more heterogeneous formulations.

1.2 Applications of antimicrobial food packaging

In table 1.1 reviews some typically compounds that have been proposed and tested for antimicrobial activity in food packaging including organic acids such as sorbate, propionate and benzoate or their respective acid anhydrides bacteriocins e.g. nisin and pediocin or enzymes such as lysozym. All antimicrobial agents have different activities which affect microorganisms differently. There is no ‘Magic Bullet’ antimicrobial agent effectively working against all spoilage and pathogenic microorganisms. This is due to the characteristic antimicrobial mechanisms and due to the various physiologies of the microorganisms (Han, 2000).

Simple categorisation of microorganisms may be very helpful to select specific antimicrobial agents. Such categories may consist of oxygen requirement (aerobes and anaerobes), cell wall composition (Gram positive and Gram negative), growth-stage (spores and vegetative cells), optimal growth temperature (thermophilic, mesophilic and psychrotropic) and acid/osmosis resistance.

Table 1.1 - Applications of antimicrobial food packaging. Incorporation of organic acids and their salts in plastic or edible films (Han, 2000).

Antimicrobial agents	Packaging materials[a]	Food	References
Acetic and propionic acid	CHITOSAN	water	Ouattara *et al.*. (2000b)
	CHITOSAN	Bologna, cooked ham, pastram	Ouattara *et al.* (2000a)
		Colture media	Weng and Chen (1997)
Benzoic acid	PE and PE-co-MA	Colture media	Weng and Chen (1997)
Sorbic acid	WPI	Colture media	Cagri *et al.*. (2001)
p-aminobenzoico	WPI	Colture media	Cagri, *et al.* (2001)
Lactic acid	ALGINATE	Lean beef muscle	Siragusa and Dickinson (1992)
Lauric acid	Corn zein film	Colture media	Padgett, *et al.* (2000)
	Corn zein film	Colture media	Hoffman, *et al.* (2001)
benzoic anhydride	LDPE	Colture media	Weng and Hotchkiss (1993)
sorbic anhydride	PE	Colture media	Weng and Chen (1997)
sodium benzoate	MC/CHITOSAN	Colture media	Chen, *et al.* (1996)
potassium sorbate	MC/HPMC/FATTY ACID MC/palmitic acid Starch/glycerol MC/CHITOSAN LDPE LDPE	Colture media Colture media Chicken breast Colture media Cheese Colture media	Vojdani and Torres (1990) Rico-Pena and Torres (1991) Baron and Summer (1993) Chen *et al.* (1996) Han (1996) Han and Floros (1997)
Glucose-oxidase	ALGINATE	Fish	Field *et al.* (1986)
Lysozyme	PVOH, NYLON, CELLULOSE ACETATE SPI film, corn zein film	Colture media	Appendini and Hotchkiss (1996) Padgett *et al.* (1998)
Pediocin	CELLULOSE	Cooked meats	Ming *et al.* (1997)
Nisin	Silicon coating SPI, corn zein film PE Corn zein film PVC, LDPE, nylon PE HPMC SPI, WPI, WG, EA Corn zein film	Beef tissue Culture media Broiler drumstick skin Culture media Culture media Phospate buffer Culture media Phospate buffer Culture media	Daeschel, *et al.* (1992) Padgett *et al.* (1998) Siragusa, et al (1999) Padgett *et al.* (2000) Natrajan and Sheldon (2000) Cutter, et al (2001) Coma, *et al.* (2001) Ko, Janes, *et al.* (2001) Hoffman *et al.* (2001)

[a] :LDPE, low-density polyethylene; MC, methyl cellulose; HPMC, hydroxypropyl MC; CMC, carboxyl MC; PE, polyethylene; MA, methacrylic; PVOH, polyvinyl alcohol; PVC, polyvinyl chloride; SPI, soy protein isolate; WPI, whey protein isolate; WG, wheat gluten; EA, egg albumen.

Besides the microbial characteristics, the characteristic antimicrobial function of the antimicrobial agent is also important to understand the efficacy as well as the limits of the activity. Some antimicrobial agents inhibit essential metabolic (or reproductive genetic) pathways of microorganisms while some others alter cell membrane/wall structure. For example, lysozyme destroys cell walls without the inhibition of metabolic pathways and results in physical cleavages of cell wall, while lactoferrin and EDTA act as coupling agents of essential cationic ions and charged polymers.

1.3 Bacteriocin

Bacteriocins are antimicrobial peptides or small proteins which inhibit, by a bactericidal or bacteriostatic mode of action, micro-organisms that are usually closely related to the producer strain (De Vuyst and Vandamme 1994b; Schillinger and Holzapfel 1996). Bacteriocins produced by lactic acid bacteria are of great interest to the food fermentation industry as natural preservatives because of their ability to inhibit the growth of many food spoilage and pathogenic bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum* (Bredholt *et al*. 2001).

These fermentation products include nisin, lacticins, pediocin, diolococcin, and propionicins (Daeschul, 1992; Han, 2002). The bacteriocins were first characterized in Gram-negative bacteria. The colicins of *E. coli* are the most studied (Lazdunski, 1988). The colicins constitute a diverse group of antibacterial proteins, which kill closely related bacteria by various mechanisms such as inhibiting cell wall synthesis, permeabilizing the target cell membrane, or by inhibiting RNase or DNase activity. Among the Gram-positive bacteria, the lactic acid bacteria have been comprehensively exploited as a reservoir for antimicrobial peptides with food applications.

Bacteriocins from lactic acid bacteria (LAB) have been classified, initially by Klaenhammer (1993), in four classes on the basis of common, mainly structural, characteristics. In a later

review, Nes *et al.* (1996) restricted the LAB-produced bacteriocins to three classes because the existence of a fourth class proposed by Klaenhammer (1993), i.e. complex compounds needing a carbohydrate or lipid moiety for activity, had been based on unpurified, and therefore ill-defined, compounds.

Class I are small, heat-stable peptides containing other amino acids, like lanthionine, and are for this reason named lantibiotics.

Class II are small, hydrophobic, heat-stable, non-modified bacteriocins consisting of either a single peptide with antilisterial activity (class IIa) or two polypeptide chains (class IIb), and also include other peptide bacteriocins (class IIc).

Class III consist of large, hydrophilic, heat-labile proteins (Klaenhammer 1993; Vaughan *et al.* 2001; Eijsink *et al.* 2002; Messens and De Vuyst 2002).

Even if chemical, enzymatic or physical characteristics of the food, food processing, or the physiological state of the bacteriocin producing micro-organism can limit the bacteriocin activity in situ (Eckner 1992; Messens and De Vuyst 2002), it has been claimed that micro-organisms producing bacteriocins possess a competitive advantage over other organisms living in the same natural environment (Vaughan *et al.* 2001).

The bacteriocin nisin, discovered in England in 1928, is produced by strains of *Lactococcus lactis* subsp. *lactis.* Structurally, it is a 34-aminoacid polypeptide, a cationic molecule due to combination of three lysine residues and one or more histidine residues (Cleveland *et al*., 2001). Purified nisin has been evaluated for toxicological effect and found harmless or at least with very low toxicity using rat and guinea pig models (Frazer and others 1962; Shtenberg and Ignatev 1970). Its use is approved as a food additive in over 50 countries. It is probably safe to say that in most of these countries, nisin is the only bacteriocin authorized for use as a food preservative. International acceptance of nisin was given in 1969 by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on

Food Additives (WHO 1969). Nisin is believed to bind to the outer membrane receptors by conjugation with other cell components (i.e., phospholipids), or by aggregation with other proteins (i.e., glycoproteins). Such binding creates ion channels in the cytoplasmic membrane, rendering the cell permeable (Delves-Broughton, 1990). Nisin is generally protected by food ingredients to which it is added, and does not lose its activity by processes such as pasteurization or sterilization (Henning *et al*., 1986). The binding of antimicrobial agents directly to polymeric packaging is an exciting development, which allows industry to combine the preservative functions of antimicrobials with the protective functions of the pre-existing packaging concepts.

1.3.1 Bacteriocins in packaging film

Incorporation of bacteriocins into packaging films to control food spoilage and pathogenic organisms has been an area of active research for the last decade. Antimicrobial packaging film prevents microbial growth on food surface by direct contact of the package with the surface of foods, such as meats and cheese. For this reason, for it to work, the antimicrobial packaging film must contact the surface of the food so that bacteriocins can diffuse to the surface. The gradual release of bacteriocins from a packaging film to the food surface may have an advantage over dipping and spraying foods with bacteriocins. In the latter processes, antimicrobial activity may be lost or reduced due to inactivation of the bacteriocins by food components or dilution below active concentration due to migration into the foods (Appendini and Hotchkiss 2002). Two methods have been commonly used to prepare packaging films with bacteriocins (Appendini and Hotchkiss 2002). One is to incorporate bacteriocins directly into polymers. Examples include incorporation of nisin into biodegradable protein films (Padgett *et al*. 1998). Two packaging film-forming methods, heat press and casting, were used to incorporate nisin into films made from soy protein and corn zein in this study. Both cast

and heat press films formed excellent films and inhibited the growth of *L. plantarum*. Compared to the heat press films, the cast films exhibited larger inhibitory zones when the same levels of nisin were incorporated. Incorporation of EDTA into the films increased the inhibitory effect of nisin against *E. coli*. Siragusa and others (1999) incorporated nisin into a polyethylene based plastic film that was used to vacuum-pack beef carcasses. Nisin retained activity against *Lactobacillus helveticus* and *B. thermosphacta* inoculated in carcass surface tissue sections. An initial reduction of 2 log cycles of *B. thermosphacta* was observed with nisin impregnated packaged beef within the first 2 days of storage at 4°C. After 20 days of refrigerated storage at 4 or 12°C (to simulate temperature abuse), *B.thermosphacta* populations from nisin coated plastic wrapped samples were significantly less than control (without nisin). Coma *et al*. (2001) incorporated nisin into edible cellulosic films made with hydroxypropyl methyl cellulose by adding nisin to the film forming solution. Inhibitory effect could be demonstrated against *L. innocua* and *S. aureus*, but film additives such as stearic acid, used to improve the water vapour barrier properties of the film, significantly reduced inhibitory activity. It was noted that desorption from the film and diffusion into the food required further optimization for nisin to function more effectively as a preservative agent in the packaged food. Another method to incorporate bacteriocins into packaging films is to coat or adsorb bacteriocins to polymer surfaces. Examples include nisin/methylcellulose coatings for polyethylene films and nisin coatings for poultry, adsorption of nisin on polyethylene, ethylene vinyl acetate, polypropylene, polyamide, polyester, acrylics, and polyvinyl chloride (Appendini and Hotchkiss 2002). Bower *et al* (1995) demonstrated that nisin adsorbed onto silanized silica surfaces inhibited the growth of *L. monocytogenes*. Nisin films were exposed to medium containing *L. monocytogenes* and the contacting surfaces were evaluated at 4 h intervals for 12 h. Cells on surfaces that had been in contact with a high concentration of nisin (40000 IU/ml) exhibited no signs of growth and many displayed evidence of cellular

deterioration. Surfaces contacted with a lower concentration of nisin (4000 IU/ ml) had a smaller degree of inhibition. In contrast, surfaces contacted with films of heat inactivated nisin allowed *L. monocytogenes* to grow. *L. innocua* and *S. aureus* (along with *L. lactis* subsp. *lactis*) were also used in a study by Scannell and others (2000) of cellulose based bioactive inserts and antimicrobial polyethylene/polyamide pouches. Lacticin 3147 and nisin were the tested bacteriocins. Although lacticin 3147 adhered to plastic film and was active for 3 months with or without refrigeration. Bacterial reductions of up to 2 log cycles in vacuum packed cheese were seen in combination with modified atmosphere packaging (MAP) with storage at refrigeration temperatures. Cellulose based bioactive inserts were placed between sliced products of cheese and ham under MAP. Inserts with immobilized nisin reduced *L. innocua* (starting inocula of 2 to 4 x 10^5 CFU/g) by 3 log in cheese after 5 d at 4°C, and by approximately 1.5 log in sliced ham after 12 days, while *S. aureus* (starting inocula of 2 to 4 x 10^5 CFU/g) was reduced by 1.5 and 2.8 log in cheese and ham, respectively. The efficacy of bacteriocins coatings on the inhibition of pathogens has also been demonstrated in other studies. For example, coating of pediocin onto cellulose casings and plastic bags has been found to completely inhibit growth of inoculated *L. monocytogenes* in meats and poultry through 12 week storage at 4°C (Ming *et al.* 1997). Coating of solutions containing nisin, citric acid, EDTA, and Tween 80 onto polyvinyl chloride, linear low density polyethylene, and nylon films reduced the counts of *Salmonella typhimurium* in fresh broiler drumstick skin by 0.4 to 2.1 log cycles after incubation at 4°C for 24 h (Natrajan and Sheldon 2000). Although shelf life was extended in food products as populations of food spoilage organisms were reduced, the primary thrust was towards control of specific anticipated pathogens in the product. In this regard, Rhodia, is developing a casing to be used in hot dog manufacture and other cooked meats. The film in combination of bacteriocins, enzymes, and botanicals. The components have received regulatory clearance. The approach is to cook the meat product

while tightly contained within the bioactive casing or wrapper. The target is *L. monocytogenes* and results are described as very promising. The added cost is considered economically sound given the large product recalls experienced by major meat brands as the result of product contaminated with *L. monocytogenes*.

1.4 Allowing the introduction of “active” food packaging

Approved on 27th October 2004 by the European Parliament and the Council of the European Union, Rules n° 1935/2004 “concerning materials and objects destined to come into contact with food products” abrogates the previous Directives 590/1980/EEC and 109/1989/EEC. Note that these rules are already in force: in fact, unlike “directives”, “rules” do not have to be ratified by national parliaments to have legal effect. Rules n° 1935/2004 disciplines the materials and the objects destined to contain food, both "those which reasonably foresee that they will come into contact with food products, or that they transfer their own components to the food products during normal or foreseeable conditions of use". It also defines the role of EFSA, the European Food Safety Authority (based in Parma) set up by the so-called "Food Law" n° 178/2002. Moreover, it foresees that materials and objects that have not yet come into contact with the food product at the moment of their entering the market must be marked with the wording "suitable for contact with food", with a specific indication as to their use or an appropriate symbol. Finally it requires that all materials and objects that come into contact with food must be traceable, in order to facilitate the control and withdrawal of defective products (this last obligation, as everyone knows, only comes into effect on 27th October 2006).

One of the innovations concerns the so called active and intelligent packaging. Unless there will be last minute changes, the indication is to proceed with targeted authorisation for each case: the green light, after favourable evaluation by EFSA and the decision of the

Commission, will regard not so much the "substance used", a very generic concept, but the single commercial product, accompanied with explicit conditions of use.

The main principles are that active packaging must protect the food and not mislead the consumer; therefore solutions that hide the state of deterioration of a food will never be authorised. Another much desired intervention (meeting Italian demands) concerns the "Declaration of Conformity" foreseen under 1935/2004: this must, in fact, be supported by scientific documentations, available to the competent authority upon request.

1.5 REFERENCES

- **Appendini, P., and Hotchkiss, J. H.** (1996) Immobilization of lysozyme on synthetic polymers for the application to food packages. Book of Abstracts (1996 IFT Annual Meeting, June 22-26, 1996. New Orleans, LA), Institute of Food Technologists. Chicago, 177.
- **Appendini, P., Hotchkiss, J.H.** (2002) Review of antimicrobial food packaging. *Innovative Food Science & Emerging Technologies* **3**, 113-126.
- **Baron, J. K., and Summer, S.** (1993) Antimicrobial containing edible films as an inhibitory system to control microbial growth on meat products. *Journal of Food Protection* **56**, 916.
- **Chen, M.C., Yeh, H.C., and Chiang B.H.** (1996) Antimicrobial and physicochemical properties of methylcellulose and chitosan films containing a preservative. *Journal of Food Processing and Preservation* **20**, 379–90.
- **Cleveland, J., Montville, T.J., Nes, I.F. & Chikindas, M.L.** (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology* **71**, 1–20.
- **Coma, V., Sebti, I., Pardon, P., Deschamps, A., and Pichavant, F.H.** (2001) Antimicrobial edible packaging based on cellulosic ethers, fatty acids, and nisin incorporation to inhibit *Listeria innocua* and *Staphylococcus aureus. Journal of Food Protection* **64**, 470–475.

- **Cutter, C. N., Willett, J. L., and Siragusa, G. R.** (2001) Improved antimicrobial activity of nisin-incorporated polymer films by formulation change and addition of food grade chelator. *Letters in Applied Microbiology* **33**, 325–328.
- **Daeschel, M.A. and Mc Guire, J.** (1992) Antimicrobial activity of nisin adsorbed to hydrophilic and hydrophobic silicon surfaces**.** *Journal of Food Protection* **55,** 731-735**.**
- **de Vuyst, L., Vandamme, E.,** (1994) Nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis*: properties, biosynthesis and applications. In: de Vuyst, L., Vandamme, E. ŽEds., Bacteriocins of Lactic Acid Bacteria. Microbiology, Genetics and Applications, Blackie Academic and Professional, London, 151–221.
- **Ehrmann, M.A., Remiger, A., Eijsink, V.G., Vogel, R.F.** (2000) A gene cluster encoding plantaricin 1.25beta and other bacteriocin- like peptides in *Lactobacillus plantarum* TMW1.25. *Biochim. Biophys. Acta* **90**, 355–361.
- **Fields, C., Pivarnick, L. F., Barnett, S. M., and Rand, A.** (1986) Utilization of glucose oxidase for extending the shelflife of fish. *Journal of Food Science* **51**, 66–70.
- **Han, J. H.** (1996) Modelling the Inhibition Kinetics and the Mass Transfer of Controlled Releasing Potassium Sorbate to Develop an Antimicrobial Polymer for Food Packaging (Ph.D. dissertation), Purdue University, West Lafayette, IN.
- **Han, J. H.** (2000) Antimicrobial food packaging. *Food Technology* **54**, 56-65.
- **Han, J. H.** (2002) Protein-based edible films and coatings carrying antimicrobial agents. in Gennadios A, Protein-based Films and Coatings, Boca Raton, FL, CRC Press, 485–99.
- **Han, J. H., and Floros, J. D.** (1997) Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. *Journal of Plastic Film and Sheeting* **13**, 287–298.
- **Hoffman, K. L., Han, I. Y., and Dawson, P. L.** (2001) Antimicrobial effects of corn zein films impregnated with nisin, lauric acid, and EDTA. *Journal of Food Protection* **64**, 885–889.
- **Klaenhammer, T.R.,** (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Rev.* **12**, 39–85.
- **Ko, S., Janes, M. E., Hettiarachchy, N. S., & Johnson, M. G.** (2001) Physical and chemical properties of edible films containing nisin and their action against *Listeria monocytogenes. Journal of Food Science* **66**, 1006–1011.

- **Ming, X., Weber, G. H., Ayres, J. W., and Sandienew, E**. (1997) Bacteriocins applied to food packaging materials to inhibit *Listeria monocytogenes* on meats. *Journal Food Science* **62**, 413–415.
- **Nes, I.F., Diep, D.B., Havarstein, L.S., Brurberg, M.B., Eijsink, V., Holo, H.** (1996) Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie van Leeuwenhoek* **70**, 113–128.
- **Natrajan, N. and Sheldon, B.W.** (2000) Efficacy of nisin-coated polymer films to inactivate Salmonella typhimurium on fresh broiler skin. *Journal of Food Protection* **63**, 1189-1196.
- **Ouattara, B., Simard, R. E., Piette, G., Begin, A., and Holley, R. A**. (2000a) Diffusion of acetic and propionic acids from chitosan-based antimicrobial packaging films. *Journal Food Science* **65**, 768–773.
- **Ouattara, B., Simard, R. E., Piette, G., Begin, A., and Holley, R. A.** (2000b) Inhibition of surface spoilage bacteria in processes meats by application of antimicrobial films prepared with chitosan. *International Journal Food Microbiology* **62**, 139–148.
- **Padgett, T., Han, I. Y., and Dawson, P. L.** (1998) Incorporation of food-grade antimicrobial compounds into biodegradable packaging films. *Journal of Food Protection* **61**, 1330–1335.
- **Rico-Pena, D. C., and Torres, J. A.** (1991) Sorbic acid and potassium sorbate permeability of an edible methylcellulose-palmitic acid film: water activity and pH effects. *Journal of Food Science* **56**, 497–499.
- **Scannel, A. G. M., Hill, C., Ross, R.P., Marx, S., Hartmeier, W., and Arendt, E.K.,** (2000) Development of bioactive food packaging materials using immobilised bacteriocins Lacticin 3147 and Nisaplin®. *International Journal of Food Microbiology*, **60**, 241-249.
- **Siragusa, G. R., Cutter, C. N., and Willett, J. L.** (1999) Incorporation of bacteriocin in plastic retains activity and inhibits surface growth of bacteria on meat. *Food Microbiology* **16**, 229–235.
- **Vojdana, F., and Torres, J. A.** (1990) Potassium sorbate permeability of methylcellulose and hydroxypropyl methylcellulose coatings: Effect of fatty acid. *Journal of Food Science* **55**, 841–846.

- **Weng, Y. M., and Chen, M. J.** (1997) Sorbic anhydride as antimycotic additive in polyethylene food packaging films. *Lebensm Wissu Technol*ogy **30**, 485–487.
- **Cagri, A., Ustunol, Z., & Ryser, E. T.** (2001) Antimicrobial, mechanical, and moisture barrier properties of low pH whey protein-based edible films containing p-aminobenzoic or sorbic acids. *Journal Food Science* **66**, 865–870.
- **Weng, Y.M., and Hotchkiss, J. H.** (1993) Anhydrides as antimycotic agents added to polyethylene films for food packaging. *Packaging Technology and Science* **6**, 123–128.

2. BACTERIOCIN PRODUCING LACTIC ACID BACTERIA AND BACTERIOCIN CHARACTERIZATION

2.1 INTRODUCTION

In recent years there is an increasing demand of minimally processed foods with fresh like quality; moreover, modern distribution systems require an adequate way to extend the shelf life of foods. The use of bacteriocins and other biologically derived antimicrobials in packaging material is attracting increasing interest recently, and patents have been filed in the area (Wilhoit 1996, 1997; Ming *et al.*, 1997; Siragusa *et al.*, 1999). The bacteriocin-producing bacteria are isolated from foods that normally contain lactic acid bacteria, such as meat and dairy products. The aim of this first activity of PhD thesis project was to screen a range of meat products, raw cow milk and dairy products for the presence of bacteriocin-producing strains.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of bacteriocin-producing bacteria from foods

One hundred and ninety six samples of a variety of food products obtained from different Italian manufacturers were analysed. They included:

- fresh meat, cooked, matured, dried or fermented meat products;
- raw milk, yogurt, dairy products, sheep's milk cheese, goat's milk cheese, soft cheese, aged cheese, processed cheese;
- olives in brine.

After purchase all the samples were stored at 4ºC for up to a maximum of 24 h before analysis. A 25 g portion of each sample was aseptically weighed and homogenized in 225 ml

of quarter-strength Ringer's solution (Oxoid) for 2 min in a stomacher (LAB Blender 400, PBI, Italy) at room temperature. Decimal dilutions were prepared and aliquots of 0.1 ml of the appropriate dilutions were spread on MRS agar (Oxoid) for LAB isolation. The plates were incubated at 30°C for 48 h. After the incubation period, for each sample, ten colonies were picked from the plates containing up to 10^2 CFU/g. The cultures were purified on MRS agar plates and then cultivated in MRS broth at 30ºC for 18 h and used for all the experiments.

2.2.2 Evaluation of the antimicrobial activity of isolates

All bacterial colonies picked were screened for bacteriocin production. *Listeria monocytogenes V7* was cultured in TSB broth (Oxoid) at 30°C for 24h and used as indicator strain. For detection of antimicrobial activity of the microorganisms and to verify the release of antimicrobial substance into the medium, an agar spot test and a well diffusion agar test were performed, respectively.

- *Agar spot test.* Each strain was cultured in 5 ml of MRS broth at 30°C for 16 h. Aliquots (10 µl) of the culture were spotted onto agar plates containing 10 ml of MRS medium. After 18 h at 30°C, the plates were overlaid with 5 ml of the appropriate soft agar (0.75% agar) inoculated with the cell suspension of the indicator strain at a final concentration of 10^5 CFU/ml. The plates were incubated for 24 h and the appearance of inhibitory zones was observed and measured. Inhibition was scored positive if the zone was wider than 2 mm.
- *Well diffusion agar test.* The indicator strain suspension was used to inoculate 20 ml of a melted (47°C) TSB soft agar (0.75% agar) medium at a final concentration of 10^5 CFU/ml. After homogenizing, the agar was poured in Petri dishes (90 mm diameter), cooled at 25°C for 30 min and wells of 6 mm diameter were made with a sterile cork borer. Bacterial strains were grown in 5 ml of MRS broth and then were centrifuged

at 18000 *g* at 4°C for 15 min. After pH measuring, the supernatants were adjusted to pH 6.5 with NaOH 0.5 M and sterilized by filtration (0.22 µm). A 50 µl aliquot of supernatant was placed into each well of the seeded plates. After pre-diffusion at 25°C for 20 min, the plates were incubated at 30°C. The antimicrobial activity, expressed in cm, was determined by measuring the diameter of the inhibition zone around the wells.

The purified strains producing bacteriocin-like substances were examined by Gram staining and catalase production, assayed according to Harrigan and McCance (1976).

2.2.3 Assays for bacteriocin activity

The antimicrobial activity of bacteriocin raw extract solutions was expressed in arbitrary units per ml (AU/ml) and it was determined by an agar diffusion assay as described by Villani *et al.* (1993). Briefly, a serial two-fold dilution in phosphate buffer solution 50 mmol/l pH 7.0 of bacteriocin was prepared, and 10 µl of each dilution were spotted onto a TSB agar soft plate seeded with about 10^5 CFU/ml *Listeria monocytogenes* V7.

The AU/ml was calculated as:

$$\mathbf{UA/ml} = \frac{\mathbf{1000}}{\mathbf{A}} \mathbf{\cdot D}$$

where:

- A is the volume of bacteriocin aliquot spotted on agar plate (10 µl in this case);

- D is the reciprocal of the highest dilution showing a clear inhibition of the indicator strain.

In the table 2.1 is reported the calculation of AU/ml of each dilution and in figure 2.1 is showed a the title of a bacteriocin produced by *Lactobacillus curvatus* 32Y isolated from Naples type salami.

Table 2.1 - Calculation of AU/ ml of each dilution.

N° spot	Dilutions	AU/ml
1°	1	100
2°	1/2	200
3°	1/4	400
4°	1/8	800
5°	1/16	1600
6°	1/32	3200
7°	1/64	6400
8°	1/128	12800
9°	1/256	25600
10°	1/512	51200
11°	1/1024	102400
12°	1/2048	204800
13°	1/4096	409600
14°	1/8192	819200

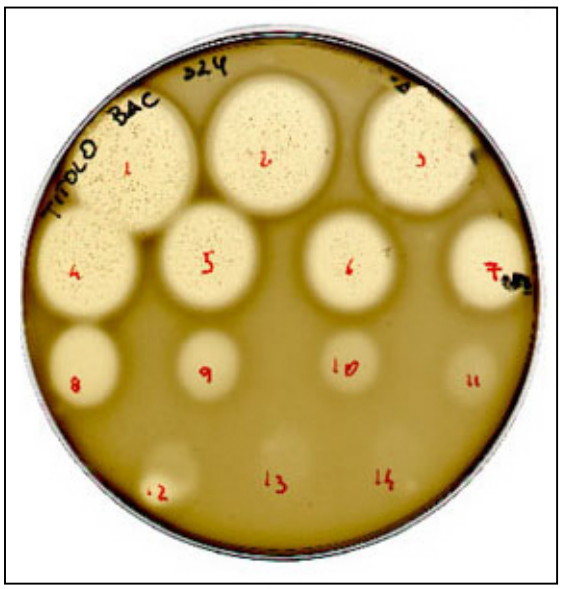


Fig. 2.1 – Title of a bacteriocin produced by *Lactobacillus curvatus* 32Y

2.2.4 Genotypic identification of lactobacilli

Unidentified Gram-positive and catalase-negative rods showing positive results after the well diffusion assay were subjected to genotypic identification. Two bacteriocin-producer strains were also identified by 16S rDNA gene sequencing. Genomic DNA of the isolates was extracted from isolated bacteria colony of overnight cultures grown on MRS agar by the method of InstaGene matrix. Briefly, the colonies were picked and resuspended in sterile water in microfuge tube and centrifuged for 1 min at 10000 rpm. The pellets were added of the 200 µl InstaGene matrix and incubated at 56°C for 15-30 minutes and after vortex at high speed for 10 seconds the tubes were treated for 8 minutes at 100°C. Finally, the mixtures were centrifuged at 10000 rpm for 2-3 min and 20 µl of the resulting supernatants were used for 50 µl PCR reaction. PCR was carried out using Taq PCR (Qiagen, Hilden, Germany).

The 50 µl PCR mixtures contained 0.5 µl of Taq PCR, 0.1 µl of each primer, 5 µl of template DNA, 5 µl of buffer (PCR-$MgCl_2$), 2.5 µl of $MgCl_2$ and 0.5 µl of Mix of dNTPmix nucleotide 25 mmol/l and sterile distilled H_2O. PCR amplification of the 16S rDNA was performed using the PCR Cycler (Biorad). The primers utilized are fD1 5'AGAGTTTGATCCTGGCTCAG3' and rD1 5'AAGGAGGTGATCCAGCC 3'.

The PCR program comprised an initial template denaturation step for 3 min at 95°C followed by 30 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 55°C and extension for 1 min at 72°C. The final extension step was for 5 min at 72°C.

2.2.5 Preparation of bacteriocin solution

A partial purification of the bacteriocins produced by cultures isolated was performed with two methods with Amberlite resin and ammonium sulphate precipitation.

- *Purification by Amberlite resin.* The preparation of bacteriocin solution with Amberlite resin was performed as described in Villani *et al*, (2001). Briefly, overnight

MRS broth cultures of the strains were centrifuged at 19000 x g for 30 min, the supernatant was mixed with 5% (w/v) of Amberlite XAD 16 (Sigma) and the mixture was stirred at room temperature for 30 min. The mixture of supernatant broth and Amberlite resin was then used to pack a low pressure chromatographic column (1.5 x 20 cm). After repeated column washings with deionized water the bacteriocin was eluted by using 1/10 the initial volume of a solution of 70% isopropanol and 30% 10 mmol/l acetic acid. The partially purified bacteriocin solution was stored at 4°C prior to use.

- *Precipitation by ammonium sulphate*. Each strains producing bacteriocin-like substance were propagated in 1 L of MRS broth and after 16 h of incubation the cultures were centrifuged at 19000 x g for 10 min. The cell free solution was precipitated with ammonium sulphate (55% saturation). The mixture was stirred for 24 h at 4°C and latter centrifuged at 19000 x g for 45 min at 4°C. The precipitates were resuspended in 20 ml of potassium phosphate buffer (50 mmol/l pH 7.0) and exhaustively dialysed *overnight* through 1000 molecular weigh-cut-off-dialysed membrane against the same buffer apposite membrane. The dialysed was then filtered (Millex®GV , 0.22 µm) and stored at 4°C until use.

2.2.6 Characterization of bacteriocin-like substances

The partially purified bacteriocins were characterized for: sensitivity to enzymes, spectrum of inhibitory activity and molecular weight. Detection and measurement of bacteriocin activity expressed in arbitrary units per ml (AU/ml), as well as proteinaceous nature of the substances were carried out as previously described in Villani *et al.*1994.

- *Sensitivity of bacteriocin-like substance to enzymes.* The sensitivity to proteolytic enzymes of each bacteriocin-like substances containing antimicrobial activity was

tested by treatment with protease, trypsin, pronase and pepsin. The enzymes (all from Sigma, St Louis, MO USA) were used at a final concentration of 1 mg/ml in phosphate buffer (pH 7.0). Aliquots (10µl) of the cell free solution were spotted onto agar plates containing 20 ml of the appropriate MRS soft agar (0.75% agar) inoculated with the cell suspension of *Listeria monocytogenes* at a final concentration of 10^5 CFU/ml. Then on the plats, near the spot of supernatants were spotted 6 µl of each enzyme solution. The plates were incubated for 24h and the appearance of inhibitory zones was observed. Proteinaceous nature of the substances was scored positive if the halo of inhibition was shaped like as half-moon.

- *Spectrum of inhibitory activity.* Bacteriocin-producing cultures isolated were also tested against the strains of spoilage and pathogen bacteria. Food spoilage and foodborne pathogenic bacterial strains used as indicator organisms for assays of antagonistic activity and respective incubation conditions are reported in Table 2.2. The well-diffusion assay was used as described before. The bacteriocins that presented a broad spectrum of activity were also assayed using the critical dilution assay.
- *Detection of molecular weight.* The molecular mass of bacteriocins was estimated in a SDS-PAGE system as described by Schägger H. & von Jagow G. (1987), using 16.5%, 10% and 4% acrylamide in the separation, spacer and stacking gel respectively. Electrophoresis was performed in vertical gels in a Mini-Protean II cell (Bio-Rad Laboratories, Richmond, CA, USA) at 200V for 45 min. After electrophoresis, the gel was cut in two vertical parts. One part was fixed and stained with Coomassie brilliant blue R-250 (1 g/L) in 50% methanol and 10% acetic acid. The other part was assayed for antimicrobial activity according to Bhunia *et al.* (1987). Briefly, the gel was fixed for 30 min (25% isopropanol, 10% acetic acid),

rinsed with distilled water (1 h initial rinse followed by two washes of 5 min), and overlaid with 25 ml of soft TSB (1% agar) seeded with 10^5 CFU/ml of listeria. After incubation at 30°C for 24 h the gel was examined for the presence of an inhibitory zone. Molecular Mass Markers for Peptides (Sigma) were used for mass standards.

Table 2.2 - Food spoilage and pathogenic bacterial strains used as indicator organisms

Indicator strains	**Source**	**Growth conditions**
Staphylococcus aureus DSM 20231	DSM	[a]TSB 24h at 37°C
Listeria monocytogenes V7	Carminati	TSB 24h at 30°C
Listeria monocytogenes CAL	Carminati	TSB 24h at 30°C
Listeria monocytogenes ATCC 7644	ATCC	TSB 24h at 30°C
Listeria welshmani 3Z	Meat	TSB 24h at 30°C
Escherichia coli O157:H7 25	Meat	TSB 24h at 37°C
Salmonella enterica serovar Thompson	Poultry	TSB 24h at 37°C
Brochrothrix thermosphacta 7R2	Meat	TSB 24h at 20°C
Pseudomonas sp. 6P2	Meat	TSB 24h at 30°C
Lactobacillus sp. 3A	Meat	[b]MRS 24h at 30°C
Enterococcus faecalis 227	NWC	TSB 24h at 30°C

[a]TSB: Tryptone Soya Broth (Oxoid, Milan - Italy) supplemented with 0.5% yeast extract

2.3 RESULTS AND DISCUSSION

2.3.1 Bacterial isolation and antimicrobial properties

Of the 2960 isolates, only 13 produced inhibition zones against *L. monocytogenes* V7. In Table 2.3 are reported foods analysed and the diameter of inhibition of strains producing bacteriocin-like substance showing positive results after agar spot test against indicator strain. In Figure 2.2 is reported the plate with agar spot test against listeria. The diameters of inhibition are included between 1.5 and 1.9 cm. The biggest diameter of 1.4 cm inhibition is obtained with the bacteriocin producer strain A2. These bacteriocin producing strains were all rods, Gram-positive and catalase negative. Only the strain A2 was Gram-positive and catalase positive. The strains 162W and AM09 were subjected to rDNA sequencing and were thus classified as *Lb. curvatus* and *Lb. plantarum*, respectively. Others strains remained unclassified. Work is in progress to further classify this strains.

Table 2.3 - Food analysed and strains producing bacteriocin-like substances showing positive results after agar spot test against *L. monocytogenes* V7

Food	**Producer strains**	**Diameter of inhibition (cm)**
Minced chicken and pork	145 Z	1.6
Soppressata from Picerno (PZ) EMMEDUE	162 W	1.8
Sousage pork	149 X	1.5
Salsiccia dolce ‘Sorrentino’	8 A	1.7
Salsiccia fresca from beef	A2	1.9
Soppressata from Picerno(PZ) EMMEDUE	123 T	1.6
Salame tipo Napoli	26 E. 27 E. 28 E	1.5-1.5-1.7
Provolone del Monaco from Sorrento 1	060. 131	1.6-1.7
Provolone del Monaco from Sorrento 2	5SD	1.7
Artisanal fermented sausages	AM 09	1.8

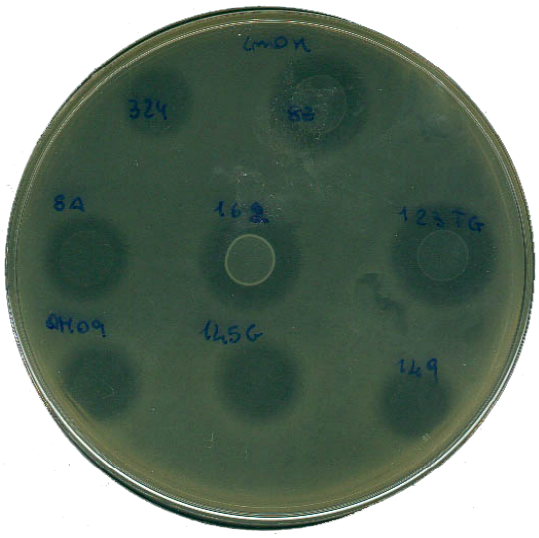


Fig 2.2 - Antimicrobial activity of strains producing bacteriocin-like substance against *L. monocytogenes* V7

Subsequently, the cell free supernatants from the 13 strains were neutralized, sterilized by filtration and tested by the well diffusion assay against listeria. The measure of diameter of inhibition expressed in cm and the results as arbitrary units per millilitre (AU/ml) against listeria for all seven strains are reported in Table 2.4.

Only the supernatants of seven strains called 8A, AM09, 123TG, 162W, 145 ZG, 149X and A2 were found to maintain the antimicrobial activity against the *L. monocytogenes* V7 showing a measurable clear zone around the well (Table 2.4). On the other hand, remaining strains did not show antimicrobial activity of supernatants, probably due to absorption of antimicrobial substances on the cells. The biggest diameter of 1.4 cm inhibition is obtained by the strain A2. The supernatants of strains 123TG, 162W, AM09 and 149X showed the best activity against *L. monocytogenes* V7. The diameters of inhibition are included between 1.1 and 1.4 cm.

Table 2.4 - Inhibitory activity of supernatants of selected bacteriocin-producing strains determined by well diffusion assay against *L. monocytogenes* V7

		Antimicrobial activity of supernatants	
Strains	**pH**	Diameter of inhibition (cm)	UA/ml
8A	4.24	1.1	1600
AM09	4.52	1.3	6400
123 TG	4.33	1.2	3200
162 W	4.28	1.3	6400
145 ZG	4.37	1.3	1600
149 X	4.49	1.2	3200
A2	4.75	1.4	1600

2.3.2 Antimicrobial activity of partial purified bacteriocins

The antimicrobial activity of partial purified bacteriocins against the indicator strain *Listeria monocytogenes* V7 is reported in Table 2.5. The two extraction methods showed different results. In fact, the extracts obtained by ammonium sulphate exhibit higher activity than the bacteriocins prepared by Amberlite resin. The bacteriocins 162W and 123TG showed the best activity against *L. monocytogenes* V7 of 25600 UA/ml.

Table 2.5 - Antimicrobial activity of bacteriocins purified against *L. monocytogenes* V7

Antimicrobial activity of bacteriocins purified (AU/ml)		
Bacteriocins (Bac)	Amberlite resin	Ammonium Sulphate
162W	12800	102400
123TG	3200	25600
8A	6400	12800
A2	nd	6400
AM09	6400	56200

2.3.3 Sensitivity of bacteriocin-like substance to enzymes

The bacteriocins from the five strains producing antimicrobial substances were assayed for sensitivity to hydrolytic enzymes. All antibacterial compounds produced by those strains were inactivated by all proteolytic enzymes (protease, trypsin and pronase) except pepsin (Table 2.6) indicating that the inhibitory compounds are of proteinaceous nature, a general characteristic of the bacteriocins.

Table 2.6 - Effect of enzymes on inhibitory activity lactobacilli determined by well diffusion assay

	Enzymes			
Supernatants	*Protease*	*Pepsin*	*Trypsin*	*Pronase*
123 TG	+	-	+	+
162 W	+	-	+	+
8A	+	-	+	+
AM09	+	-	+	+
A2	+	-	+	+

2.3.4 Antimicrobial spectrum of bacteriocins

The inhibitory spectra of antimicrobial substances evaluated against a range of Gram-positive and Gram-negative bacteria are showed in Table 2.7. Each bacteriocin was able to inhibit all *Listeria monocytogenes* strains tested. In addition, Bac162W was also active against the strains of *Brochrothrix thermosphacta* and *Lactobacillus* sp. 3A and *Enterococcus faecalis* 227. The bacteriocins tested are only active against Gram-positive bacteria. The target of bacteriocins is the cytoplasmic membrane, so due to the protective barrier provided by the LPS of the outer membrane of Gram-negative bacteria. The bacteriocins are generally only active against Gram-positive cells (Stevens, K.A. *et al.,* 1991).

Table 2.7 - Antimicrobial spectrum of bacteriocins purified by ammonium sulphate against foodborne pathogenic and spoilage microorganisms.

Microorganisms	Partial purified bacteriocins (AU/ml)				
	123TG	**162W**	**8A**	**AM09**	**A2**
Foodborne pathogenic					
Staphylococcus aureus DSM 20231	0	0	0	0	0
Listeria monocytogenes V7	25600	102400	12800	56200	6400
Listeria monocytogenes CAL	1600	12800	800	12800	100
Listeria monocytogenes ATCC 7644	3200	12800	3200	12800	800
Listeria welshmani 3Z	6400	12800	6400	25600	800
Clostridium perfringens	0	0	0	0	0
Escherichia coli O157:H7 25	0	0	0	0	0
Salmonella enterica serovar Thompson	0	0	0	0	0
Spoilage					
Brochrothrix thermosphacta 1R2	0	12800	0	0	0
Brochrothrix thermosphacta 3R2	0	12800	0	0	0
Brochrothrix thermosphacta 7R1	0	12800	0	0	0
Pseudomonas 6P2	0	0	0	0	0
Lactobacillus spp. 3A	0	12800	0	6400	0
Enterococcus 227	100	6400	0	0	0

2.3.5 Molecular mass determination of bacteriocins

Electrophoretic analysis performed with bacteriocins extracted by ammonium sulphate showed a wide band, vertically occupying most of the gel. When the gel was overlaid with the indicator bacteria as described by Bhunia test a clear inhibition band in the indicator lawn

were found (Fig. 2.3). In table 2.8 are reported the molecular mass expressed in kDa of bacteriocins purified. The bacteriocins showed molecular mass included between 2.5 and 15.5 kDa. The bacteriocin AM09 showed biggest molecular weight (15.5 kDa); on the contrary the smaller molecular weight was showed by the bacteriocin A2.

Table 2.8: Molecular mass of bacteriocins

Bacteriocins	Weight (kDa)
123TG	15.5
162W	8.1
8A	6.2
AM09	15.5
A2	2.5

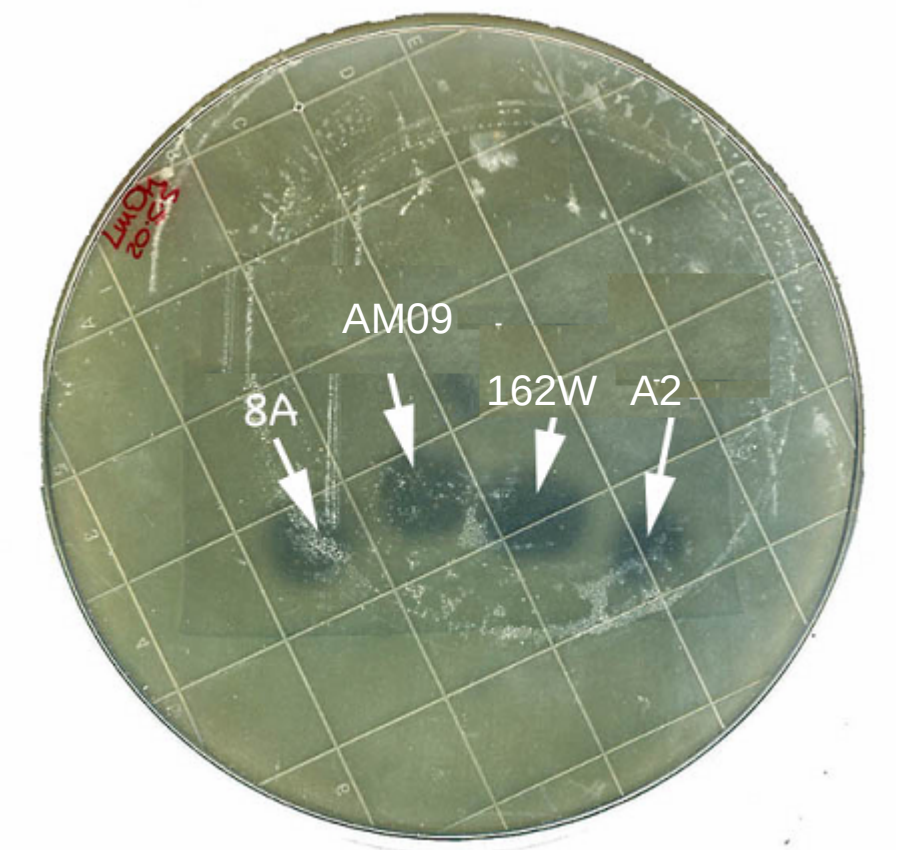


Fig. 2.3 - Elettrophoretic Gel overlaid with TSB soft agar containing *Listeria monocytogenes* V7. Inhibition bands in the indicator lawn of bacteriocins purified: 8A, AM09, 162W and A2.

2.4 REFERENCES

- **Buhnia, A. K., Johnson, M. C., Ray, B.,** (1987) Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal Ind. Microbiology* **2**, 319-322.
- **Corsetti, A., Settanni, L., Van Sinderen D.** (2004) Characterization of bacteriocin-like inhibitory substances (BLIS) from sourdough lactic acid bacteria and evaluation of their in vitro and in situ activity. *Journal of Applied Microbiology* **96**, 521–534.
- **Harrigan, W.F., McCance, M.E.** (1976) Basic methods. *In:* Harrigan, W.F.; McCance, M.E. (eds.). *Laboratory methods in food and dairy Microbiology*. Academic Press, London, 111-115.
- **Jack, R.W., Tagg, J.R. and Ray, B.** (1995) Bacteriocins of Gram-positive bacteria. *Microbiological Reviews* **59**, 171–200.
- **McAuliffe, O., Ross, R.P. and Colin, H.** (2001) Lantabiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Rev* **25**, 285–308.
- **Ming, X., Weber, G. H., Ayres, J. W., & Sandine, W. E.** (1997). Bacteriocins applied to food packaging materials to inhibit *Listeria monocytogenes* on meats. *Journal of Food Science* **62**, 413–415.
- **Nes, I.F., Diep, B.D., Havarstein, L.S., Brurberg, M.B., Eijsink, V. and Holo, H.** (1996) Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie van Leeuwenhoek* **70**, 113–128.
- **Siragusa, G.R., Cutter, C.N., Willett, J.L.** (1999) Incorporation of bacteriocin in plastic retains activity and inhibits surface growth of bacteria on meat. *Food Microbiology* **16**, 229-235.
- **Stevens, K.A., Sheldon, B.W., Klapes, N.A., Klaenhammer, T.R.** (1991) Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. *Applied Environmental Microbiology* **57,** 3613-3615.
- **Tagg, J.R., Dajani, A.S. and Wannamaker, L.W.,** (1976) Bacteriocins of gram positive bacteria. *Bacteriology Rev* **40**, 722-756.
- **Villani, F., Aponte, M., Blaiotta, G., Mauriello, G., Pepe, O. and Moschetti, G.** (2001) Detection and characterization of a bacteriocin, garviecin L1-5, produced by *Lactococcus garvieae* isolated from raw cow's milk. *Journal of Applied Microbiology* **90**, 430-439.

- **Villani, F., Pepe, O., Mauriello, G., Salzano, G., Moschetti, G. and Coppola, S.** (1994) Antimicrobial activity of *Staphylococcus xylosus* from Italian sausage against *Listeria monocytogenes*. *Letters in Applied Microbiology* **18**, 159-161.
- **Villani, F., Salzano, G., Sorrentino, E., Pepe, O., Marino, P. and Coppola, S.** (1993) Enterocin 226NWC, a bacteriocin produced by *Enterococcus faecalis* 226, active against *Listeria monocytogenes*. *Journal Applied Bacteriology* **74**, 380-387.
- **Wilhoit, D.L.** (1996) Film and method for surface treatment of foodstuffs with antimicrobial compositions. US patent No.**5**, 573-797.
- **Wilhoit, D.L.** (1997) Antimicrobial compositions, film and method for surface treatment of foodstuffs. EU patent No. EP0 750 853 A2.

3. DEVELOPMENT OF POLYETHYLENE FILMS FOR FOOD PACKAGING ACTIVATED WITH AN ANTILISTERIAL BACTERIOCIN FROM *LACTOBACILLUS CURVATUS* 32Y

3.1 INTRODUCTION

Numerous types of food packaging, in combination with different storage techniques can be used in order to extend the shelf life of meat. One of the key technological measures needed during storage is the preservation of the meat from microbial spoilage and contamination/proliferation of pathogenic microorganisms. *Listeria* species have been found in meat and meat products (Johnson, *et al.,* 1990) Foodborne transmission of *L. monocytogenes* has been implicated in human outbreaks of listeriosis involving the consumption of various foods. There is a further need to implement antimicrobial packaging systems that can prevent the growth of pathogenic bacteria**.**

The aims of the second activities of PhD thesis were:

- to use a bacteriocin produced by *Lactobacillus curvatus* 32Y active against *Listeria monocytogenes* to activate polyethylene films by different methods,
- to implement a large-scale process for antilisterial polyethylene films production
- to verify the efficacy of the developed films in inhibiting the growth of *Listeria monocytogenes* during the storage of meat products.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and growth conditions

Lactobacillus curvatus 32Y (Mauriello *et al.,* 1999) and *Listeria monocytogenes* V7 were maintained in storage at –20°C in 30% glycerol. *L. curvatus* 32Y was grown in MRS broth

(Oxoid) at 30°C and *L. monocytogenes* V7 in TSB (Oxoid) at 37°C prior to their use in the experiments.

3.2.2 Preparation of the bacteriocin 32Y solution

The partially purified bacteriocin 32Y solution (PPBAC) was prepared with Ambelite Xad 16 resin as described in Materials and Methods of chapter 2 (see paragraph 2.2.5). The PPBAC showing an activity of 102400 AU/ml against *L. monocytogenes* V7 and was stored at 4°C prior to use. The concentration of the bacteriocin solution, expressed in arbitrary units (AU) per ml, was determined by an agar diffusion assay as described in chapter 2 (see paragraph 2.2.3).

3.2.3 Procedures for antimicrobial polyethylene films preparation

Four methods of film treatment with bacteriocin 32Y were tested.

- *Soaking.* Samples of coupled polyethylene-oriented polyamide (PE-OPA) films (2 x 2 cm^2) were soaked in PPBAC diluted in phosphate buffer 50 mmol/l pH 7.00 at concentration of 0, 6400, 12800 and 25600 AU ml/l for 10 min and 1, 6 and 8 hours. After the soaking, the films were air dried and assayed for antimicrobial activity against *L. monocytogenes* V7 as described below.
- *Spray-coating.* PPBAC diluted in 70% iso-propanol at a concentration of 6400 AU/ml was sprayed onto PE-OPA film samples (20 x 20 cm^2). The films were then treated with warm air in order to let the solution dry and promoting a homogenous distribution of the bacteriocin onto the surface of the plastic film. After the treatment the films were assayed for antimicrobial activity against *Listeria monocytogenes* V7 as described below.

ü *Industrial production of bacteriocin coated PE-OPA films.* The coating procedure was also used to produce bacteriocin coated PE-OPA films in an industrial plant patent right of Flex Packaging AL spa from Cava de' Tirreni (Salerno). A solution of PPBAC in 70% isopropanol was prepared with an activity of 6400 AU/ml against *L. monocytogenes* V7 and used in a large scale coating plant; a scheme is depicted in Figure 3.1. A 46 cm wide PE-OPA film (300 m in length) was employed for the activation. Briefly, PPBAC solution was spread on the PE-OPA film in thin layer by a spreading roll that dipped in a vessel containing the antimicrobial solution. The isopropanol fraction of the PPBAC solution was immediately evaporated in a warm air tunnel at 70°C. Eventually, the activated film was cooled at room temperature and collected in a reel. The antimicrobial activity of the activated PE-OPA films was tested, as described below, soon after the treatment and after 24 h, 1, 2, and 3 weeks and 1, 2 and 3 months during which the film reel was kept at room temperature.

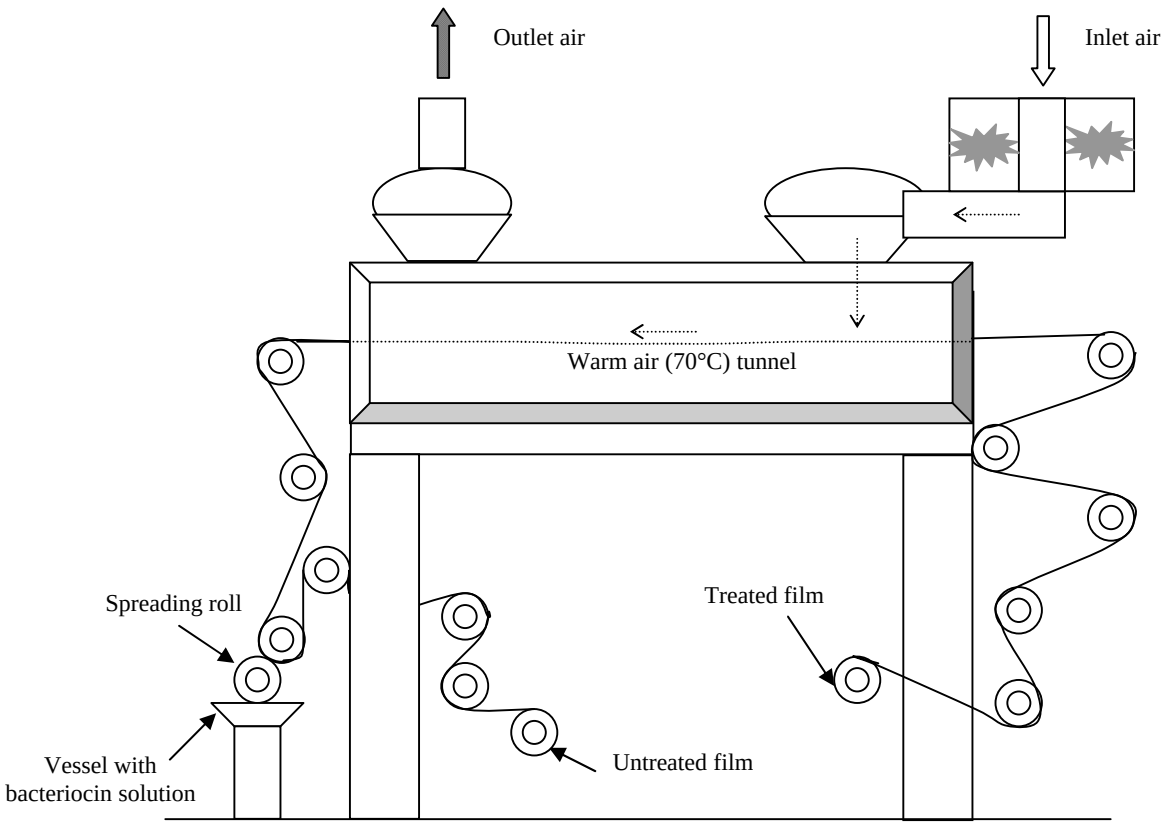


Fig. 3.1 - Industrial plant for PE-OPA film treatment

- *Incorporation of lyophilized BAC32Y during plastic film extrusion.* The PPBAC 32Y solution at concentration of 6400 AU/ml, determined against *L. monocytogenes* V7, was freeze dried. One hundred grams of powdered low density polyethylene (0.9 g/cm^3 and *melt flow index* at 190°C=5.5 g/min) was blended with 0.05 – 0.1 – 0.2 – 0.4 e 0.8 g of lyophilized PPBAC 32Y. Film was produced at Dipartimento di Ingegneria Chimica Alimentare from Università degli Studi in Salerno, using a pilot plant extruder equipped with a plain head and a single screw. The temperature profile was 190/180/175/184°C, from the first barrel zone to the dye; screw speed was 75 rpm. The plastic films produced under these conditions were 50 µm thickness and 10 cm wide. Control film was produced with no addition of bacteriocin.

3.2.4 Antimicrobial activity assay of the developed PE-OPA films

After the above treatments of activation with the bacteriocin 32Y the PE-OPA films were assayed for antimicrobial activity against the indicator *Listeria monocytogenes* V7. Samples (2 x 2 cm^2) of the treated PE-OPA films were located onto the surface of a TSB (Oxoid) soft (0.75%) agar plates seeded with 2.5% of an overnight culture of *Listeria monocytogenes* V7. The treated face of the film was in contact with the agar, untreated films were also assayed as negative controls. The plates were incubated at 37°C for 16 h and the antagonistic activity was evaluated by observing a clear zone of growth inhibition in correspondence of the active PE-OPA film.

In order to resemble a superficial development of listeria on the surface of food products and the antimicrobial effect of the developed films on superficial growth of listeria, a further inhibition assay was performed. 0.1 ml of a suspension containing about 1.0 x 10^5 UFC/ml of *Listeria monocytogenes* V7 were spread plated on TSB agar plates, the active face of the bacteriocin treated PE-OPA film was located in contact with the agar surface and the Petri

dish was incubated at 37°C for 16 h. After the incubation, the antimicrobial activity was revealed by the absence of growth of the indicator *Listeria* in the part of the plate in contact with the activated PE-OPA film.

3.2.5 Adsorption and release of the Bac 32Y from the PE-OPA films

For adsorption assays, 20 µl of PPBAC were spotted on the surface of samples of untreated PE-OPA films, removed after 1, 2, 3, 5 and 10 min and tested for antimicrobial activity on TSB agar plates inoculated with *Listeria monocytogenes* V7 as above described. For bacteriocin release assays, the simulating solution chosen was water, as recommended by analytic method defined in DM 21 Marzo 1973, which indicates water as simulating solution to be used for release experiments regarding meat products. Therefore, 20 µl of sterile deionized water were spotted onto the surface of industrially developed PE-OPA films and removed for every 5 min for 1 h. Afterwards, both film and water spots were assayed for antimicrobial activity against *Listeria monocytogenes* V7.

3.2.6 Antilisterial activity of bacteriocin 32Y coated films during the storage of meat products: pork steaks and minced beef

The industrially developed antilisterial PE-OPA film in challenge tests of control of *L. monocytogenes* growth during the storage of meat products. Pork steaks were superficially spiked with a 2 ml suspension of *Listeria monocytogenes* V7 at 1.0 x 10^6 CFU/ml, the steaks were then packed with the active PE-OPA films and stored at 4°C. Steaks packed with untreated films, and unpacked steaks were included in the analysis as controls. After 0, 24, 48 and 72 h of storage, selective viable counts of listeria on Oxford agar (Oxoid) were performed on 4 pieces (3.8 cm^2 each) of pork steak ten-fold diluted in a quarter Ringer solution (Oxoid). The experiments were performed in triplicate and the results were expressed as CFU/cm^2.

Similarly, minced beef was contaminated at 1% with a suspension of *L. monocytogenes* V7 at 1.0 x 10^6 CFU/ml and 45 g hamburgers were prepared 1 cm thick. The hamburgers were packed on both faces with the antilisterial PE-OPA films and stored at 4°C. Hamburgers unpacked and packed with untreated films were included in the analysis as controls. After 0, 24, 48 and 72 h of storage, selective viable counts of *Listeria* on Oxford agar were performed and the results were expressed as CFU/g.

3.3 RESULTS

3.3.1 Antimicrobial activity of polyethylene coated with bacteriocin 32Y

The activation of PE-OPA films with the bacteriocin 32Y was performed by different methods. The soaking procedure yielded positive results; in fact, after immersion for different times of the PE-OPA films into bacteriocin 32Y solutions at different concentrations, the films showed to be always active against *L. monocytogenes* V7 in agar inhibition assays. In all the cases untreated films and the polyethylene films obtained after extrusion did not show any antimicrobial activity. An example of the detection of antimicrobial activity of the bacteriocin-soaked PE-OPA films is shown in Figure 3.2 (Panels A and B). There was no difference in inhibition intensity when films were treated for 10 minutes (Fig. 3.2, panel A) and 1 h (Fig. 3.2, panel B). Moreover, the growth inhibition area was not confined to the film area but irregularly spread across the plate (Fig. 3.2, panel A).

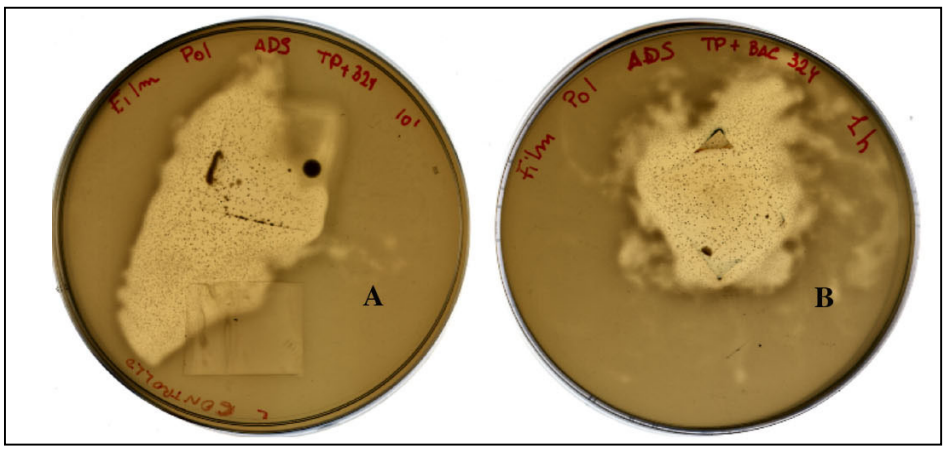


Fig. 3.2 - Antimicrobial activity of bacteriocin-soaked PE-OPA films against the indicator strain. **A**, antimicrobial activity after 10 min soaking; **B**, antimicrobial activity after 1 h.

In order to assess whether the bacteriocin was actually absorbed by the surface of the PE-OPA film or migrated from the cut margins into the film, another experiment was carried out. Aliquots of the PPBAC 32Y solution at 6400 AU/ml were spotted on the surface of the film for different contact times and the antimicrobial activity of the films was then tested. As shown in Figure 3.3, the antimicrobial activity was observed in correspondence to the spot area and the intensity of the activity was the same, regardless of the bacteriocin solution contact time.

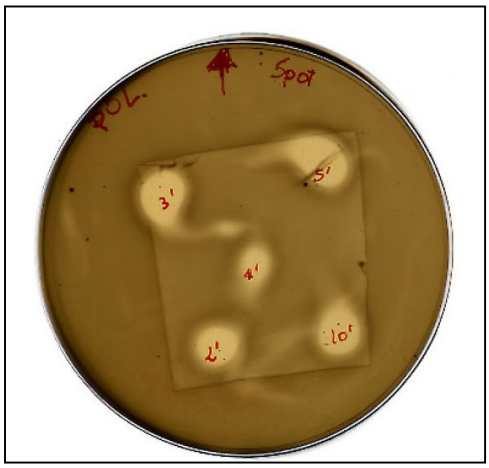


Fig. 3.3 - Antimicrobial activity of PE-OPA films spotted with PPBAC 32Y solution at 6400 AU/ml. The inhibition zones correspond to 2, 3, 4, 5 and 10 min of PPBAC 32Y contact with the film.

The same PPBAC 32Y solution at 6400 AU/ml was sprayed on the surface of the plastic films and the results of the antimicrobial activity are shown in Figure 3.4. Also in this case the spray-activated films showed antilisterial activity and it could be noted that the bacteriocin diffused from the plastic film as the inhibition zone was not confined to the film area.

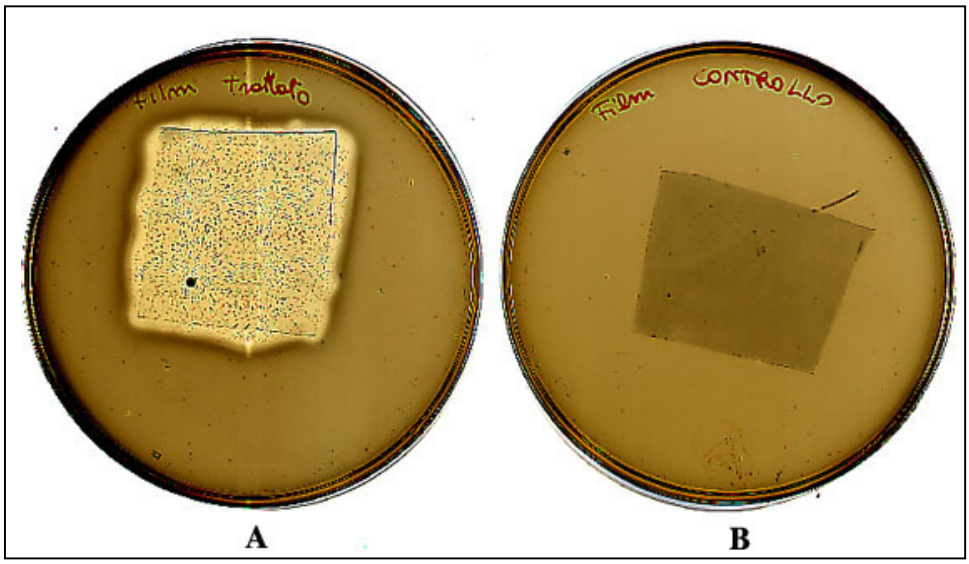


Fig. 3.4 - Antimicrobial activity of the PE-OPA film spray-activated with PPBAC 32Y solution at 6400 AU/ml against the indicator strain *Listeria monocytogenes* V7. **A**, treated film; **B**, untreated film.

The spray-coated PE-OPA films were also assayed for activity against superficial growth of *Listeria monocytogenes* V7 on agar plates. As shown in Figure 3.5, the superficial growth of indicator strain was limited to the area surrounding the activated film that could clearly inhibit the development of the pathogen; in contrast, the pathogen could grew homogeneously on the surface of the plate and underneath the untreated PE-OPA film used as control (Fig. 3.5, panel B).

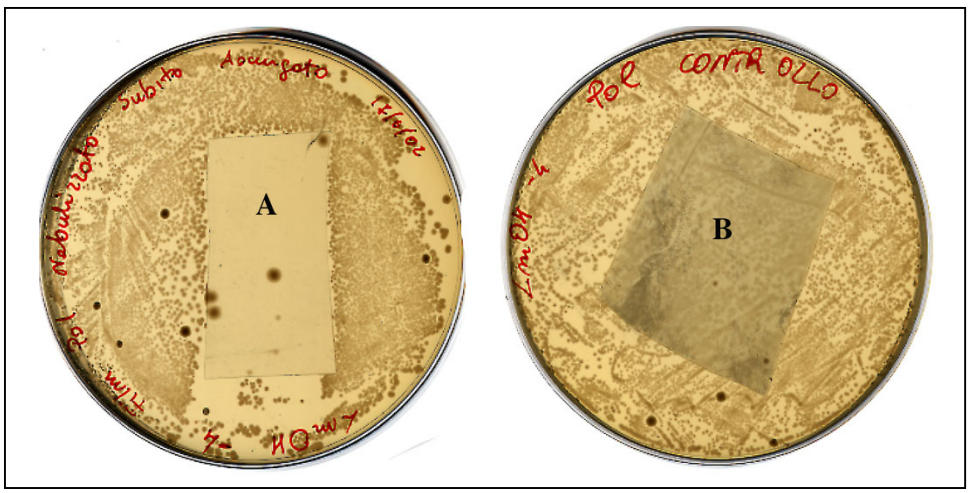


Fig. 3.5 - Antimicrobial activity of the PE-OPA film spray-activated with PPBAC 32Y solution at 6400 AU/ml against the indicator strain *Listeria monocytogenes* V7 spread-inoculated on TSA plates. **A**, treated film; **B**, untreated film.

Since the bacteriocin 32Y showed good potential for the development of antimicrobial PE-OPA films, an industrial procedure of coating was developed in order to produce 32Y activated PE-OPA films in an industrial plant. The activation of the PE-OPA films did not alter their mechanical properties nor influenced its transparency and appearance. Moreover, the activated films did not show solvent release as detected by head space gas chromatography analysis (Figure 3.6).

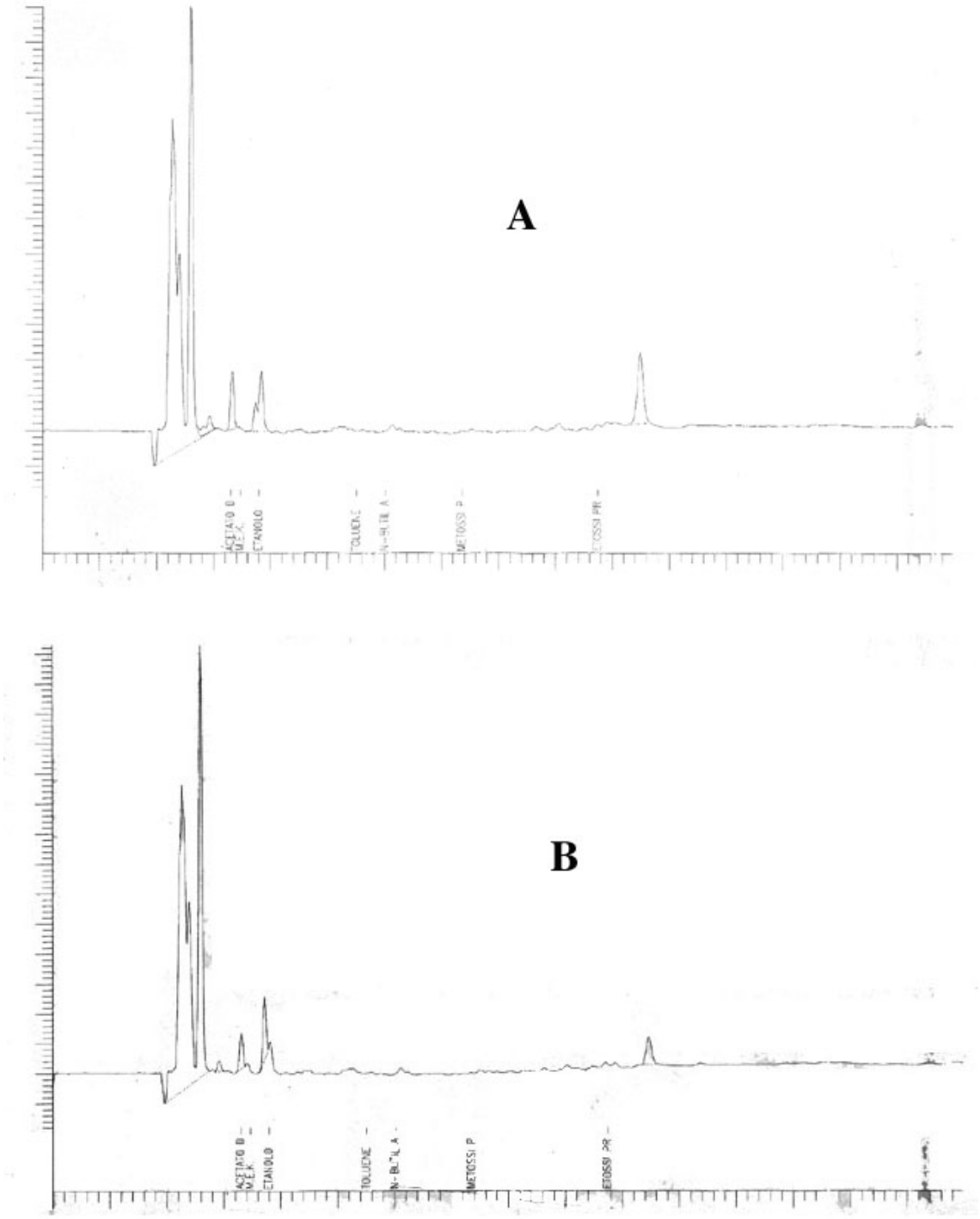


Fig. 3.6 - Results of head space gas chromatography analysis **A:** treated film **B:** untreated film

Interestingly, the coated films displayed a clear and stable antilisterial activity during 3 months after the treatment. As shown in Figure 3.7, the antimicrobial activity of the bacteriocin coated PE-OPA films was tightly confined to the area of the film and was not diffused across the plate as did the antimicrobial activity of soaked and sprayed films.

Fig. 3.7 - Antimicrobial activity of the PE-OPA film coated in the industrial plant with PPBAC 32Y solution at 6400 AU/ml against the indicator strain *Listeria monocytogenes* V7. **A**, treated film; **B** untreated film.

The coated films were also subjected to experiments release of the bacteriocin as consequence of prolonged contact of the film with water spots. The water spots collected after different times (every five minutes for 1 h) did not show any antimicrobial activity in agar plate assays. However, the bacteriocin-coated PE-OPA films, after being assayed for bacteriocin release in water spots, displayed loss of antilisterial activity in correspondence of the zones where the water spots were left.

3.3.2 Effect of antimicrobial packaging in meat products

The industrially developed antimicrobial films were also used in challenge tests of storage of meat products artificially contaminated by *Listeria monocytogenes* V7. The results of the viable counts of listeria in meat during the storage of meat in antimicrobial and control plastic films are reported in Table 3.1.

Table 3.1 - Viable counts of *Listeria monocytogenes* V7 in pork steak and in hamburgers during storage at 4°C for 72 h.

Time (hours)	Viable count of *Listeria monocytogenes* V7.				
	Pork steak UFC/cm^2		Hamburgers UFC/g		
	LF	LFB	L	LF	LFB
0	208 ± 79[a]	208 ± 79	16233 ± 4966	16233 ± 4966	16233 ± 4966
24	105 ± 9	89 ± 13	13350 ± 1202	15100 ± 2687	2200 ± 163
48	98 ± 0	59 ± 37	11550 ± 3464	10050 ± 70	3800 ± 320
72	180 ± 144	120 ± 27	9150 ± 3747	10100 ± 3535	5233 ± 980

L: meat contaminated with listeria
LF: meat storage untreated film
LFB: meat storage in antimicrobial film
[a]: average of the three replicates ± standard deviations

The results of the viable counts of *Listeria* on pork steaks at different times of storage at 4°C are reported in Figure 3.8. The film was washed prior to viable counts and the washing liquid was plated on Oxford agar plates yielding only occasionally viable listeria counts after 48 h at 37°C. The trend of the *Listeria* population appeared to be the same during the storage of the pork steaks in PE-OPA films both with and without bacteriocin treatment. However, the viable counts of *Listeria* were lower during the whole period of storage when the pork steaks were packed with bacteriocin activated films (Fig. 3.8). Good results were also obtained by storing hamburgers in bacteriocin activated films. The initial load of *Listeria* was reduced of almost 1 log during the first 24 h of storage (Fig. 3.9). Moreover, the *Listeria* viable counts after 24 h of storage in activated films were kept lower than the counts of hamburgers packed with untreated films or unpacked hamburgers (Fig. 3.9).

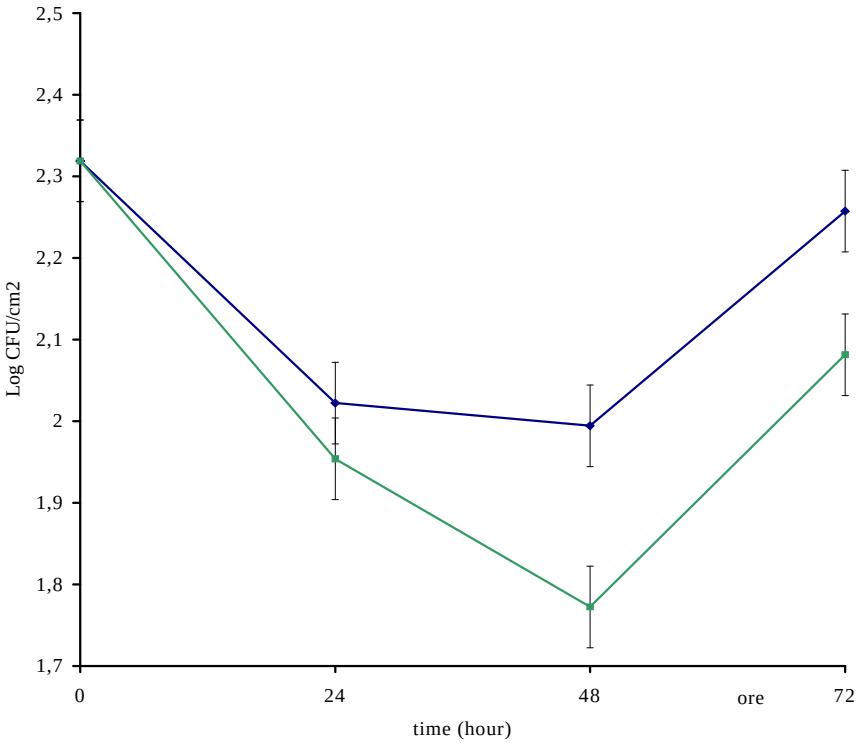


Fig. 3.8 - Viable counts of listeria population during the storage at 4°C of pork steaks spiked with *Listeria monocytogenes* V7.
(n) Steaks packed with industrially developed bacteriocin activated PE-OPA film; (n) Steaks packed with untreated film.

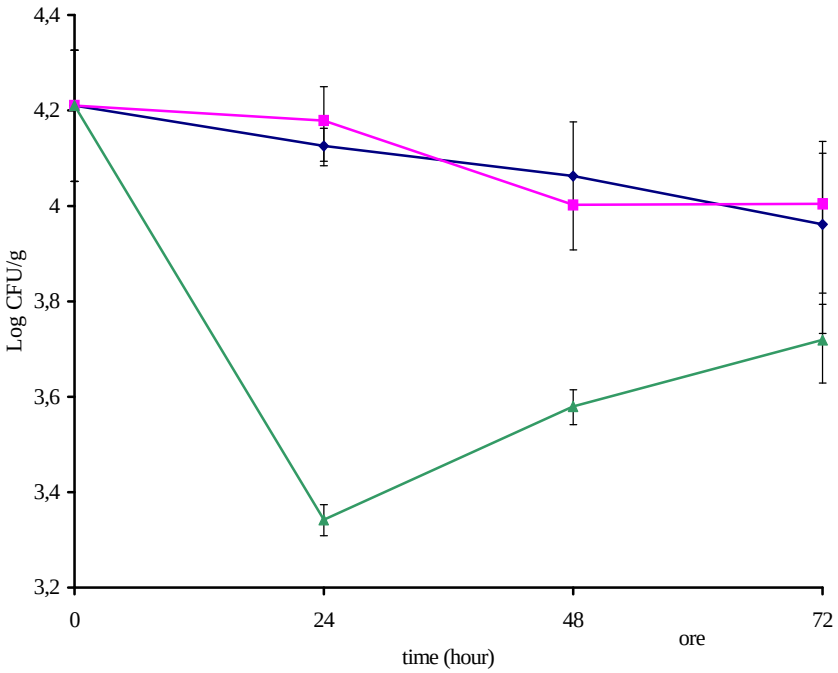


Fig. 3.9 - Viable counts of listeria population during the storage at 4°C of hamburgers spiked with *Listeria monocytogenes* V7.
(l) Unpacked hamburgers; (n) Hamburgers packed with industrially developed bacteriocin activated PE-OPA film; (•) Hamburgers packed with untreated film.

3.4 DISCUSSION

For active antimicrobial packaging to be effective, an adequate procedure of activation is necessary in order to assure that the antimicrobial is linked to the film and to keep the antimicrobial activity during the film shelf life. Moreover, the activated film has to exert its preservative antimicrobial potential during packed food storage.

In this study different methods were used to bind the bacteriocin 32Y from *Lactobacillus curvatus* 32Y to PE-OPA films. All the activation procedures adopted, except extrusion, were successful, although the results of the antimicrobial activity were substantially different. The soaking procedure proved to be effective. However, the distribution of the bacteriocin on the surface of the soaked PE-OPA films was shown to be not homogeneous and the results of the antilisterial activity suggested that the bacteriocin irregularly diffused from the film into the agar. The further test consisted of spotting the bacteriocin on the surface of the PE-OPA film, which demonstrated that even a quick contact of the bacteriocin with the surface of the film conferred activation. As it appears in Figure 3.3, the bacteriocin seemed to migrate from the spots and it was not possible to determine whether the bacteriocin migrated into the film from the margins. This is an important characteristic of the substance to be employed because it is essential that preservatives have low diffusivity in their host film and remain at the surface of the food. In fact, diffusion into the food matrix may result in reduction of the preservative concentration at the surface increasing the possibility for the microorganisms to escape their antimicrobial effect (Han and Floros 1997; Scannel *et al.,* 2000). The spray-coating also yielded positive results. Also in this case the bacteriocin did not prove to bind homogeneously and firmly to the surface of the film. The spray-coated films proved to be effective in containing the surface-development of *Listeria monocytogenes* V7 on agar plates (Fig.3.5). As a matter of fact, this should be the function of an antimicrobial film used for fresh food packaging where the possible contamination of the food is on the surface and thus the

prevention of the surface microflora is important for long and safe food storage. Many studies have dealt with the activation of plastic films with bacteriocins. Several authors reported on the efficacy of antimicrobial films activated by nisin alone or in combination with other preservatives or chelators and by using different methods of activation (Siragusa *et al.,* 1999; Scannell *et al.,* 2000; Coma *et al.,* 2001; Cutter *et al.,* 2001). Some other authors (An *et al.,* 2000; Kim *et al.,* 2000; Lee *et al.,* 2004) described a coating of low density polyethylene films with bacteriocins different from nisin. However, the procedures used for the activation of the films were not shown to be adjustable to an industrial production, although the antimicrobial PE film proved to be effective in inhibiting the growth of *Listeria* and *Micrococcus* (An *et al.,* 2000; Kim *et al.,* 2000; Lee *et al.,* 2004). Similarly, Ming *et al.* (1997) developed pediocin-coated casings that showed useful in controlling the growth of L. monocytogenes in meat and poultry products. Also in this case, the spray-mediated activation procedure did not exactly fit an industrial model of production. The spray-coating was also used by Natrajan and Sheldon (2000) to develop antimicrobial films activated with nisin.

Therefore, to date little work has been done to prove the suitability of the bacteriocin and of the activation system for a real production. In this study, a film coating procedure with bacteriocin 32Y was developed using an industrial plant. The PE-OPA films used for the active packaging manufacture were checked for their necessary technological standard characteristics such as solderability, resistance to tensile stress, transparency etc. before and after the activation treatment and they were always shown to keep their quality after being coated with bacteriocin 32Y. Only about 1 l of PPBAC 32Y solution at 6400 AU/ml were used to activate about 300 m of PE-OPA film and this proved the process to be not expensive as only 500 ml of *Lactobacillus curvatus* 32Y in broth are needed to produce 1 litre of PPBAC 32Y ready for the coating. The bacteriocin coated films were active against *Listeria monocytogenes* V7 in agar plates assays and yielded clean, homogeneous and confined

inhibition areas, suggesting that the bacteriocin was uniformly bound to the surface of the film and did not diffuse irregularly into the agar. Therefore, the procedure used for the large-scale PE-OPA film activation, including spreading of the bacteriocin on the surface of the film and immediate air drying of the bacteriocin solution by a hot air flux, did not negatively affect the antimicrobial potential of the bacteriocin 32Y. Experiments of migration of the bacteriocin in water, used as simulator of meat products according to the regulation 2002/72/CE, demonstrated that the coated films lost the antilisterial activity in correspondence of the water-treated zones, although no residual antimicrobial activity was registered in the water drops after their contact with the activated film up to 1 h. The concentration of the bacteriocin released in water is probably below the detection limit of the agar diffusion assay used to detect the antagonistic activity. However, migration and activity of the bacteriocins incorporated in food packaging and their effect on microbial development should be assessed *in vivo* in challenge tests directly performed in foods. Before applications to food products can be considered, it is important to first ascertain, insofar as is possible, the shelf life of the bioactive films. Previous studies have shown that bacteriocins retain their activity when applied to various surfaces (Daeschel and Mc Guire 1992; Bower *et al.,* 1995a, 1995b; Ming *et al.,* 1997). Experiments used to qualitatively monitor the activity and stability of the bacteriocin coated PE-OPA films developed in this study demonstrated that the antilisterial activity was still stable after 4 months of film storage at room temperature. Moreover, the mechanical and standard required properties of the PE films were also stable during the storage proving to be unaffected by the bacteriocin treatment. Therefore, the developed active PE-OPA films, appearing suitable for a real production, were assayed for their antimicrobial activity against *Listeria monocytogenes* V7 in challenge tests involving storage of fresh meat products at refrigeration temperatures. The growth of the indicator during the storage of pork steaks was inhibited by both treated and untreated films and the

effect of the activated films in providing an increasing reduction of *Listeria* population compared to the control was fully different only after the first 24 h. In contrast, a significant decrease in *Listeria* viable counts was registered during the first 24 h of storage of hamburgers packed with antimicrobial PE-OPA film. The overall effect of control of the growth of *Listeria* resulted better in hamburgers than pork steaks storage. This may be due either to the higher superficially-concentrated contamination of *Listeria* on the pork steaks, which was more difficult to control, or to the nature of the meat products itself and their possible effect on bacteriocin release and action. In both cases, moreover, an increase in *Listeria* viable counts was registered after 48 hours of storage, which may be due to the particular mechanism of action of bacteriocins that can inhibit as many cells as molecules available in the medium (Moll *et al.*, 1999). Increasing the concentration of the bacteriocin in the coating solution may be also experimented with the aim of improving the preservative performance of the bacteriocin coated PE-OPA films in storage of meat as well as other food products. Addition for further hurdle molecules such as EDTA, lisozima, citric acid, lactic acid, lauric acid into the coating solution may improve the antimicrobial performance of bacteriocin activated films as reported in other studies. (Natrajan and Sheldon 2000).

Antimicrobial packaging can play an important role in reducing the risk of pathogen development, as well as extending the shelf-life of foods although it should not substitute for good quality raw materials and good manufactures practices. Studies of new food-grade bacteriocins as preservatives and development of suitable systems of bacteriocin treatment of plastic films for food packaging are important issues in applied microbiology and biotechnology, both for implementing and improving effective hurdle technologies for a better preservation of food products.

3.5 REFERENCES

- **An, D.S., Kim, Y.M., Lee, S.B., Paik, H.D. and Lee, D.S.** (2000) Antimicrobial low density polyethylene film coated with bacteriocins in binder medium. *Food Science and Biotechnology* **9**, 14-20.
- **Bower, C.K., Mc Guire, J. and Daeschel, M.A.** (1995a) Influences of the antimicrobial activity of surface absorbed nisin. *Journal of Industrial Microbiology* **15**, 227-233.
- **Bower, C.K., Mc Guire, J. and Daeschel, M.A.** (1995b) Suppression of *Listeria monocytogenes* colonization following adsorption of nisin onto silica surfaces. *Applied and Environmental Microbiology* **61**, 992-997.
- **Coma, V., Sebti, I., Pardon, P., Deschamps, A. and Pichavant, F.H**. (2001) Antimicrobial edible packaging based on cellulosic ethers, fatty acids, and nisin incorporation to inhibit *Listeria innocua* and *Staphylococcus aureus. Journal Food Protection* **64**, 470-475.
- **Cutter, C.N., Willett, J.L., and Siragusa, G.R.** (2001) Improved antimicrobial activity of nisin-incorporated polymer films by formulation change and addition of food grade chelator. *Letters in Applied Microbiology* **33**, 325-328.
- **Daeschel, M.A. and Mc Guire, J.** (1992) Antimicrobial activity of nisin adsorbed to hydrophilic and hydrophobic silicon surfaces. *Journal Food Protection* **55**, 731-735.
- European Community, Direttiva 2002/72/CE della Commissione, del 6 agosto 2002, *relativa ai materiali e agli oggetti di materia plastica destinati a venire a contatto con i prodotti alimentari* (G.U. 220 del 15/08/2002 pag. 18-58).
- **Han, J.H. and Floros, J.D.** (1997) Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. *Journal of Plastic Film Sheet* **13**, 287-298.
- **Johnson, J.L., Doyle, M.P., Cassens, R.G.** (1990) *Listeria monocytogenes* and other *Listeria* spp. in meat and meat products. A review. *Journal Food Protection* **53**. 81-91.
- **Kim, H.J., Lee, N.K., Paik, H.D. and Lee, D.S**. (2000) Migration of bacteriocin from bacteriocin -coated film and its antimicrobial activity. *Food Science and Biotechnology* **9**, 325-329.
- **Lee, C.H., An, D.S., Lee, S.C., Park, H.J. and Lee, D.S.** (2004) A coating for use as an antimicrobial and antioxidative packaging material incorporating nisin and •-tocopherol. *Journal of Food Engineering* **62**, 323-329.

- **Mauriello, G., Aponte, M., Andolfi, R., Moschetti, G. and Villani, F.** (1999) Spray-Drying of bacteriocin-producing lactic acid bacteria. *Journal Food Protection* **64**, 773-777.
- **Ming, X., Weber, G.H., Ayres, J.W. and Sandine, W.E.** (1997) Bacteriocins applied to food packaging materials to inhibit *Listeria monocytogenes* on meats. *Journal of Food Science* **62,** 413-415.
- **Moll, G.N., Konings, W.N. and Driessen, A.J.M.** (1999) Bacteriocins: mechanism of membrane insertion and pore formation. *Antonie van Leeuwenoek* **76**, 185-198.
- **Natrajan, N. and Sheldon, B.W.** (2000) Efficacy of nisin-coated polymer films to inactivate *Salmonella thyphimurium* on fresh broiler skin. *Journal Food Protection* **63**, 1189-1196.
- **Repubblica Italiana, DM 21 Marzo** (1973) Disciplina igienica degli imballaggi, recipienti, utensili, destinati a venire in contatto con le sostanze alimentari o con sostanze d'uso personale.
- **Scannell, A.G.M., Hill, C., Ross, R.P., Marx, S., Hartmeier, W. and Arendt, E.K**. (2000) Development of bioactive food packaging materials using immobilized bacteriocins Lacticin 3147 and Nisaplin. *International Journal of Food Microbiology* **60**, 241-249.
- **Siragusa, G.R., Cutter, C.N., Willett, J.L.** (1999) Incorporation of bacteriocin in plastic retains activity and inhibits surface growth of bacteria on meat. *Food Microbiology* **16**, 229-235.
- **Villani, F., Pepe, O., Mauriello, G., Salzano, G., Moschetti, G. and Coppola, S**. (1994) Antimicrobial activity of *Staphylococcus xylosus* from Italian sausage against *Listeria monocytogenes*. *Letters in Applied Microbiology* **18**, 159-161.

4. EFFECT OF A BACTERIOCIN-ACTIVATED POLYETHYLENE FILM ON *LISTERIA MONOCYTOGENES* AS EVALUATED BY VIABLE STAINING AND EPIFLUORESCENCE MICROSCOPY

4.1 INTRODUCTION

The aim of this third activity of project thesis was to examine the effect of the bacteriocin 32Y on resting and growing populations of *L. monocytogenes* in function of time and temperature of exposure to the bacteriocin. Moreover, the effect of bacteriocin activated polyethylene films on *L. monocytogenes* population was also evaluated both when the pathogen was directly in contact with the active film and when the film was used to pack a liquid medium contaminated by *L. monocytogenes* and frankfurters superficially contaminated with pathogenic strain.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains, growth conditions and bacteriocin production

Listeria monocytogenes V7 was used as indicator strain for all the challenge tests. The strain was daily cultivated in Tryptic Soy Broth (TSB, Oxoid) at 30°C. *Lb. curvatus* 32Y was used as bacteriocin 32Y producer strain and was cultivated in MRS broth (Oxoid) at 30°C. The bacteriocin solution to be used for the activation of polyethylene films was obtained as previously described in chapter 2 (see paragraph 2.2.5). After the extraction and partial purification with Amberlite, the concentration of the bacteriocin solution, expressed in arbitrary units (AU) per ml, was determined by an agar diffusion assay. The bacteriocin solution was stored at 4°C prior to use.

4.2.2 Viable staining

The reduction of *L. monocytogenes* V7 populations was evaluated by using Live/Dead® *Bac*Light™ Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR, USA). The stock solution of the two fluorochromes was prepared with 0.7 µl of SYTO 9 and 1 µl of propidium iodide in 330 µl of sterile deionized water. After the viable staining, the fluorescent cells were observed using a Nikon Eclipse E400 epifluorescence microscope equipped with an UV lamp and a 100 X magnification objective. An untreated control of *L. monocytogenes* V7 was analysed in each determination as control. Enumeration of cells was performed randomly counting 30 microscopic fields.

4.2.3 Effect of BAC 32Y activated polyethylene film on L. monocytogenes V7

A solution of bacteriocin in 70% of isopropanol was prepared with an activity of 6400 AU/ml against *L. monocytogenes*; the bacteriocin solution was used to industrially manufacture antimicrobial polyethylene films in a large scale coating plant as previously described in chapter 3 (see paragraph 3.2.3). The antagonistic activity of the polyethylene films was checked by observing a clear zone of growth inhibition in correspondence of the activated PE film in contact with TSA (Oxoid) inoculated with the indicator strain.

4.2.4 L. monocytogenes V7 in contact with activated polyethylene film

In order to monitor the behaviour of *Listeria* in contact with the antimicrobial film, the bacteriocin-activated film was stuck on a microscopy slide and 20 µl of BPS or TSB containing about 10^7 CFU/ml of *L. monocytogenes* V7 were spotted on the film. The viable staining solution (6 µl) was directly applied to the suspension on the film; live and dead cells were counted immediately and after 2, 5, 7, and 24 h of incubation at room temperature (RT) or at 4°C in a humid chamber. The epifluorescence microscopy counts were performed

directly on the film stuck onto the slide. The results were expressed as average number of cells per microscopic field calculated counting 30 microscopic fields.

Bacteriocin release from the polyethylene film was also evaluated. A sample of activated polyethylene film and a phosphate buffer saline (PBS: 50 mmol/L, pH 7.00) were pre-adapted at 4°C and RT. After 1 h, 20 µl of PBS were spotted onto the surface of the activated film, sucked every 5 min for 1 h and assayed for antimicrobial activity against *L. monocytogenes* V7 in agar diffusion assays as described in chapter 2 (see paragraph *2.2.3*).

4.2.5 Efficacy of a bacteriocin 32Y activated package for the inhibition of L. monocytogenes V7 *in liquid medium*

An active package was obtained by using antimicrobial films as internal coating of a plastic package. A sheet of antimicrobial film of 70 x 70 cm^2 was cut in a cross-like shape and the borders were thermally sealed to obtain a pack of 13 x 13 x 9 cm^3 (Fig. 4.1).

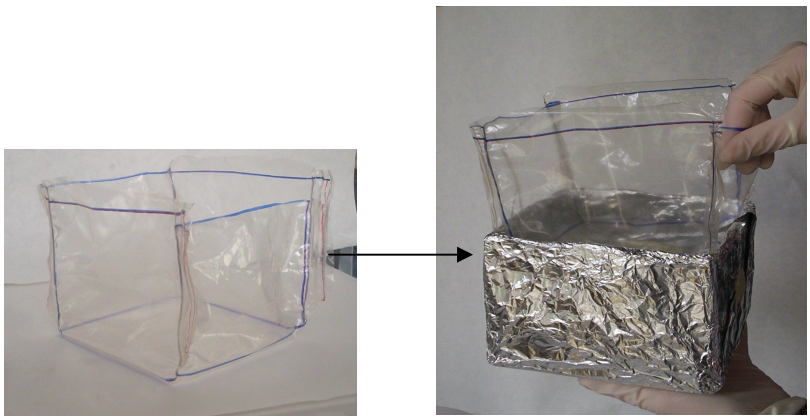


Fig. 4.1 – Antimicrobial package after thermally sealed

The package was filled with 1 L of *L. monocytogenes* V7 suspensions (10^7 CFU/ml) in PBS (Power buffer solution) or TSB and stored at 4°C and room temperature. Viable staining of the suspensions was performed immediately and after 2, 5, 7 and 24 h.

One ml aliquots of *L. monocytogenes* V7 suspensions after exposure to the antimicrobial film were filtered through a black, non-fluorescent polycarbonate membrane (25 mm diameter, 0.2 µm pore size, Sigma). Then 10 µl of the fluorochromes stock solution were applied to the filters and the membranes were incubated in the dark for 15 min at room temperature. The number of live and dead cells was calculated as follows:

$$\mathbf{N = x \bullet A/a \bullet V}$$

Where:

N = cells/ml;

x = the average number of cells per observation field (based on 30 fields);

A = filtration area (mm^2);

a = observation field area (mm^2);

V = volume of filtered sample.

4.2.6 Efficacy of a bacteriocin 32Y activated package for the inhibition of L. monocytogenes V7 *contaminating the surface of frankfurters*

Pork frankfurters were superficially contaminated by immersion in a cell suspension at 10^7 CFU/ml of *L. monocytogenes* V7. The frankfurters were then packed in bacteriocin 32Y activated film and stored at 4°C for 48 h. During the storage, the frankfurters were washed with 20 ml of PBS, the solution was filtered through a black, non-fluorescent polycarbonate membrane (25 mm diameter, 0.2 µm pore size, Sigma), and then subjected to viable staining. The results were expressed as number of live or dead cells per package. Frankfurters packed in untreated films were analysed as control. Each time, the viable staining was also performed on the film surface as above described in order to detect cells possibly attached to the film.

4.2.7 Statistical analysis.

All the experiments were repeated three times and the final results were calculated as the average of the replicates; standard deviations were calculated. For each sample, t-test analysis was carried out to ascertain that there were no significative differences between the averages calculated on the basis of the results of 30 microscopic fields count. Significance was declared at $P<0.05$.

4.3 RESULTS

4.3.1 L. monocytogenes V7 in contact with a bacteriocin activated polyethylene film

Bacteriocin 32Y activated polyethylene films were industrially obtained as previously described in chapter 3 (see paragraph *3.2.3*). Figure 4.2 showed antimicrobial activities against *L. monocytogenes* V7.

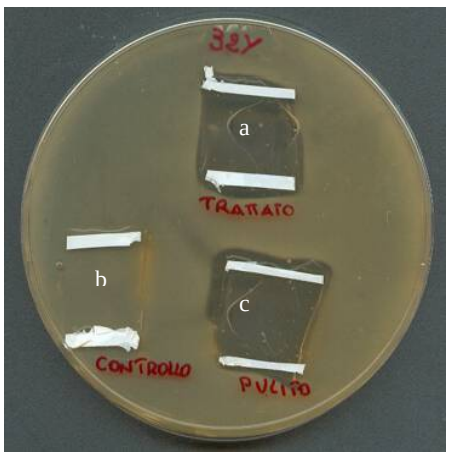


Fig. 4.2 – Antimicrobial activity of the polyethylene film coated in the industrial plant with PPBAC 32Y solution at 6400 AU/ml against the indicator strain *Listeria monocytogenes* V7. **(a),** treated film, (b) untreated film; (c) treated film after rubbing.

A PBS suspension (20µl) of *L. monocytogenes* V7 (about 10^7 CFU/ml) was spotted onto the surface of the film, incubated at RT and 4°C and monitored over the time by directly applying the viable staining procedure to the plastic film stuck on a microscopy slide. Immediate reduction of the number of viable cells was observed after the contact of the PBS suspension

with the activated film compared to the control (Fig. 4.3 panels A, B). However, while at RT the average number of live cells per microscopic field dropped to zero within two hours (Fig. 4.3A), at 4°C the initial number of live cells remained constant for 7 hours (Fig. 4.3B). When a TSB suspension with *L. monocytogenes* V7 (about 10^7 CFU/ml) was spotted on the activated film, an immediate reduction of the number of viable cells was registered compared to the control; regardless of the incubation temperature, the number of live cells per field decreased to zero within two hours (Fig. 4.3C, D). By contrast, both at RT and at 4°C, the population of *L. monocytogenes* V7 in contact with the untreated film rapidly increased (Fig. 4.3C, D).

Remarkably, after a few minutes of contact the decrease of live cell number was not linked to an increase in dead cells, indicating that the cells were directly damaged to lysis as result of the bacteriocin action. The time-dependent fate of *Listeria* population in contact with the bacteriocin activated film is shown in Figure 4.4. Immediately after the contact between film and suspension (time zero), both live and dead cells of *Listeria* could be observed, while after two hours of incubation no cells were present as result of lysis and background fluorescence caused by cellular debris was observed (Fig. 4.4).

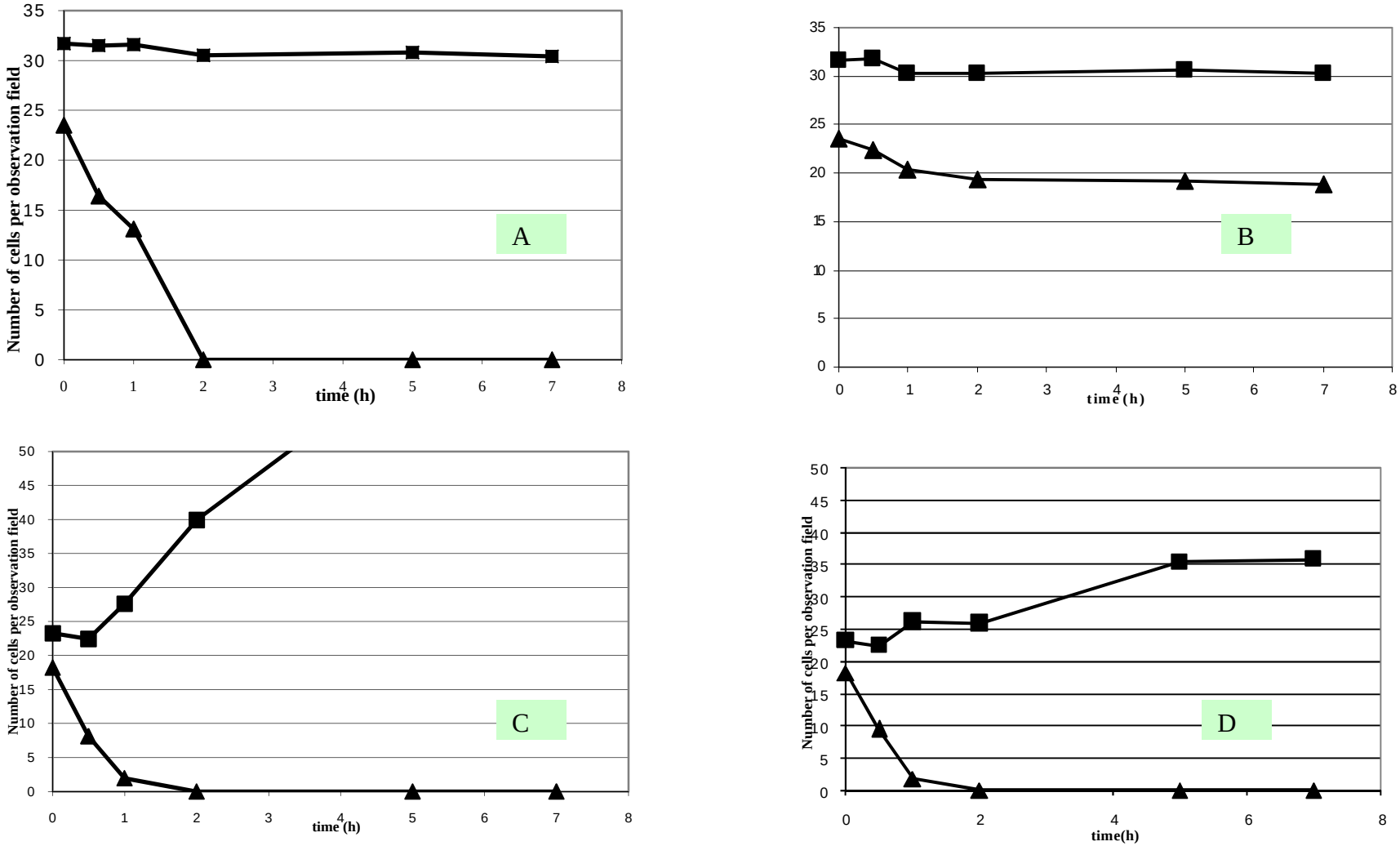
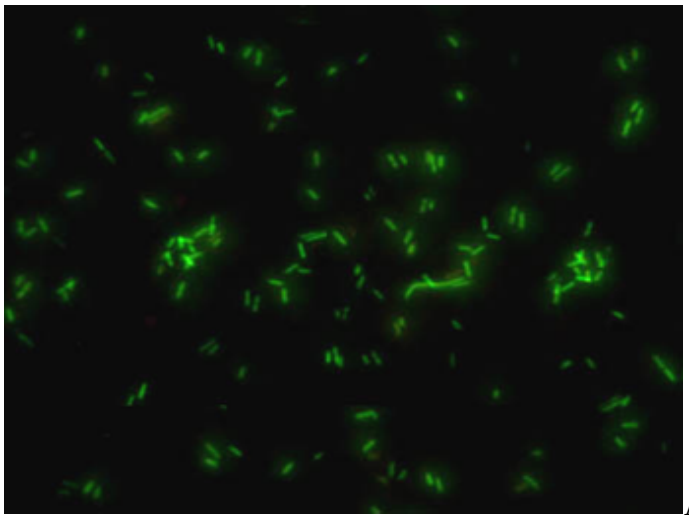
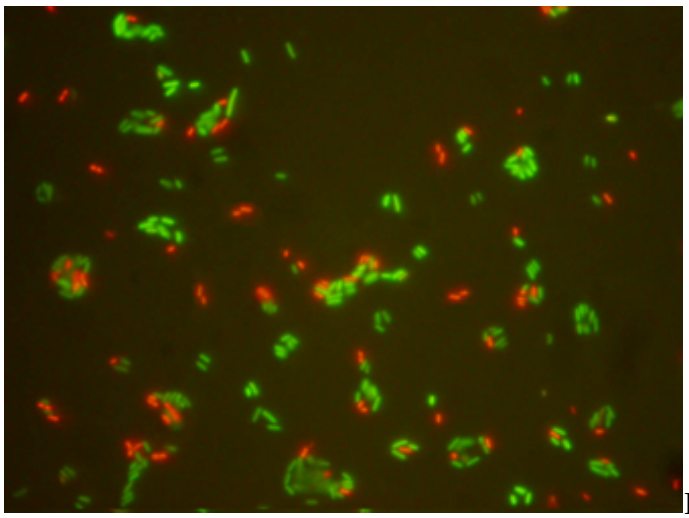


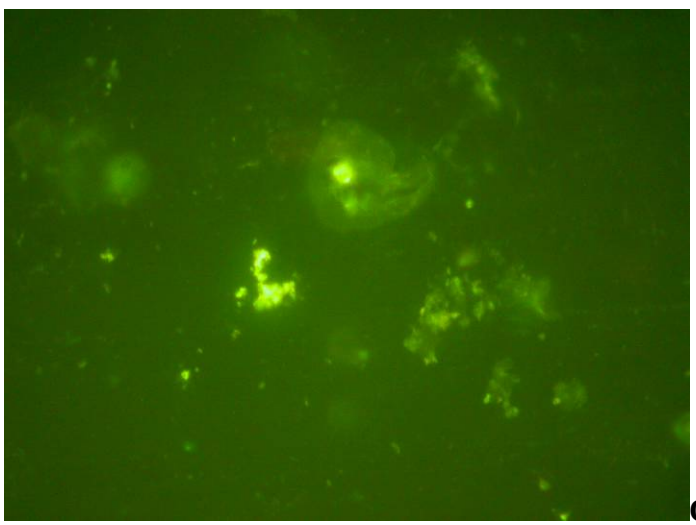
Fig. 4.3 – Counts of live cells of *L. monocytogenes* V7 after viable staining following the contact with the bacteriocin 32Y activated polyethylene film. (•) untreated film used as control (•) bacteriocin activated polyethylene film. (A) PBS suspension at RT; (B) PBS at 4°C; (C) TSB at RT; (D) TSB at 4°C.



A



B



C

Fig. 4.4 – Viable staining of *L. monocytogenes* V7 suspensions in direct contact with polyethylene films.All the observation were performed directly on the surface of the polyethylene film stuck on a microscopy slide. (A) live cells on untreated polyethylene film used as control; (B) live and dead cells of *L. monocytogenes* V7 on the bacteriocin activated film observed immediately after the contact (time zero) between cellular suspension and film; (C) cellular debris observed in epifluorescence microscopy after two hours of incubation of *L. monocytogenes* V7 suspensions spotted onto the surface of a bacteriocin activated film.

4.3.2 Behaviour of L. monocytogenes V7 in liquid media packed in bacteriocin activated polyethylene film

Liquid medium contaminating with listeria was packed in bacteriocin 32Y activated polyethylene films spread with industrial plant. An active package was manufactured as described in materials and methods, filled with 1L of *L. monocytogenes* V7 suspension in PBS or TSB (about 10^7 CFU/ml) and incubated at RT or 4°C.

Regardless of the incubation temperature, the live listeria population in PBS was reduced of about 1 log in 24 h (Fig. 4.5, Panels A, B). However, while an immediate appearance of almost 10^6 dead cells was observed at RT, the occurrence of dead cells at 4°C was registered in 2 h (Fig. 4.5B). The untreated pack used as control did not have effect on the population as dead cells were never observed and the number of live cells remained constant. When the activated film was used to control a growing population of *L. monocytogenes* V7 in TSB, a slight reduction of live cells was observed at both temperatures of incubation. The number of live cells was kept lower than the control at RT for the first 7 h (Fig. 4.5C). The treated and untreated films gave about the same result at 4°C where the growth was controlled for the first 24 h followed by an increase of live population after 24 h (Fig. 4.5D).

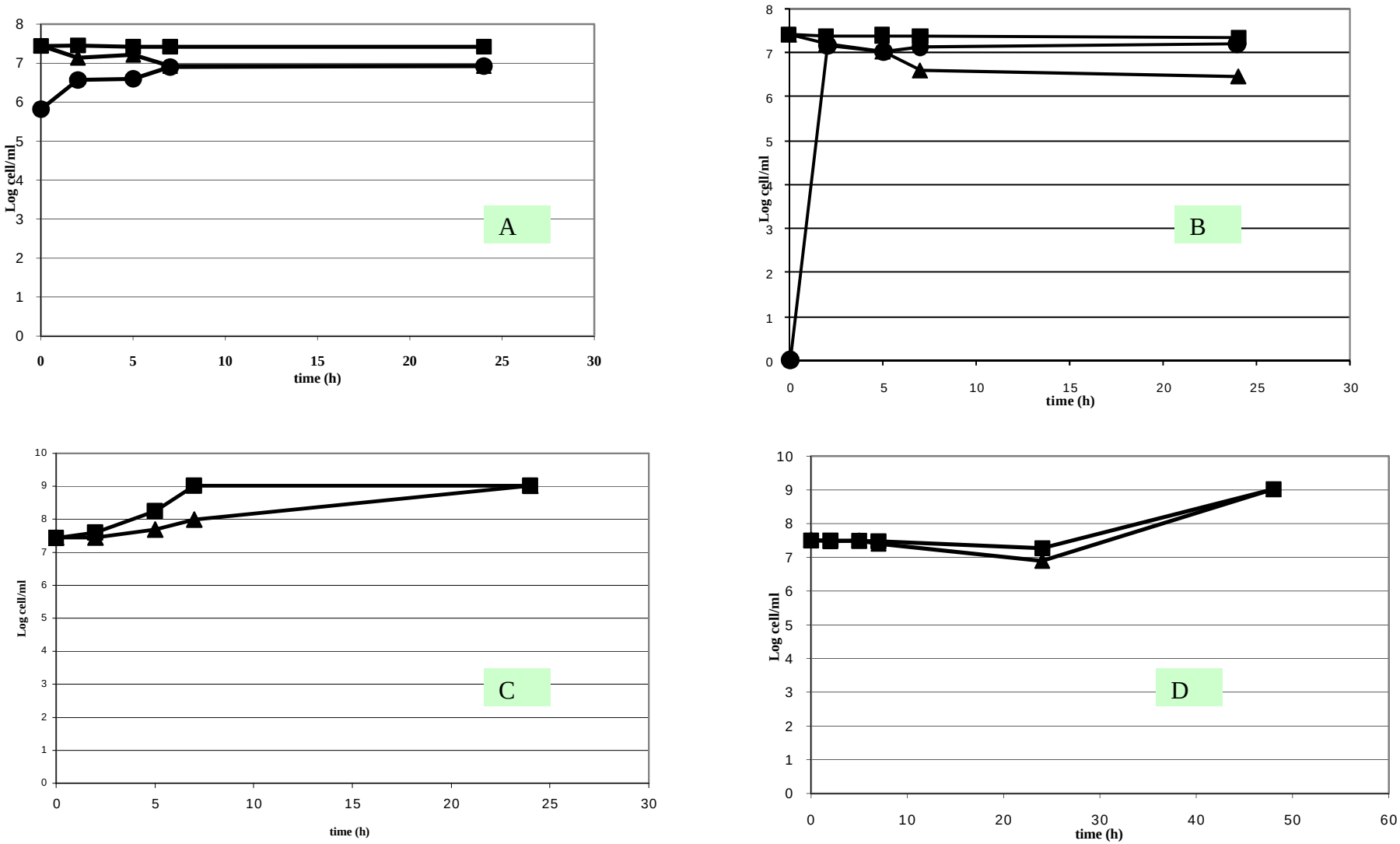


Fig. 4.5 – Trends of (•) live and (•) dead cells of *L. monocytogenes* V7 in liquid media packed in bacteriocin activated polyethylene film and (•) live cells of *L. monocytogenes* V7 packed in untreated film used as control. The counts were performed in 1 ml of filtered suspensions after viable staining. (A) PBS at RT; (B) PBS at 4°C; (C) TSB at RT; (D) TSB at 4°C.

4.3.3 L. monocytogenes V7 population in frankfurters

The effect of the activated polyethylene film was evaluated on *L. monocytogenes* superficially contaminating frankfurters during storage at 4°C. The results are reported in Figure 4.6. The frankfurters stored in untreated packaging did not show reduction of live cells per package during storage and did not show the presence of dead cells. However, when the frankfurters were stored in active package a 0.5 log reduction was obtained after 7 h of storage followed by a complete extinction of live cells of *Listeria* after 24 h. In addition, dead cells appeared soon after the frankfurters were packed (Fig. 4.6) although their number decreased to zero in 48 h as consequence of cell lysis.

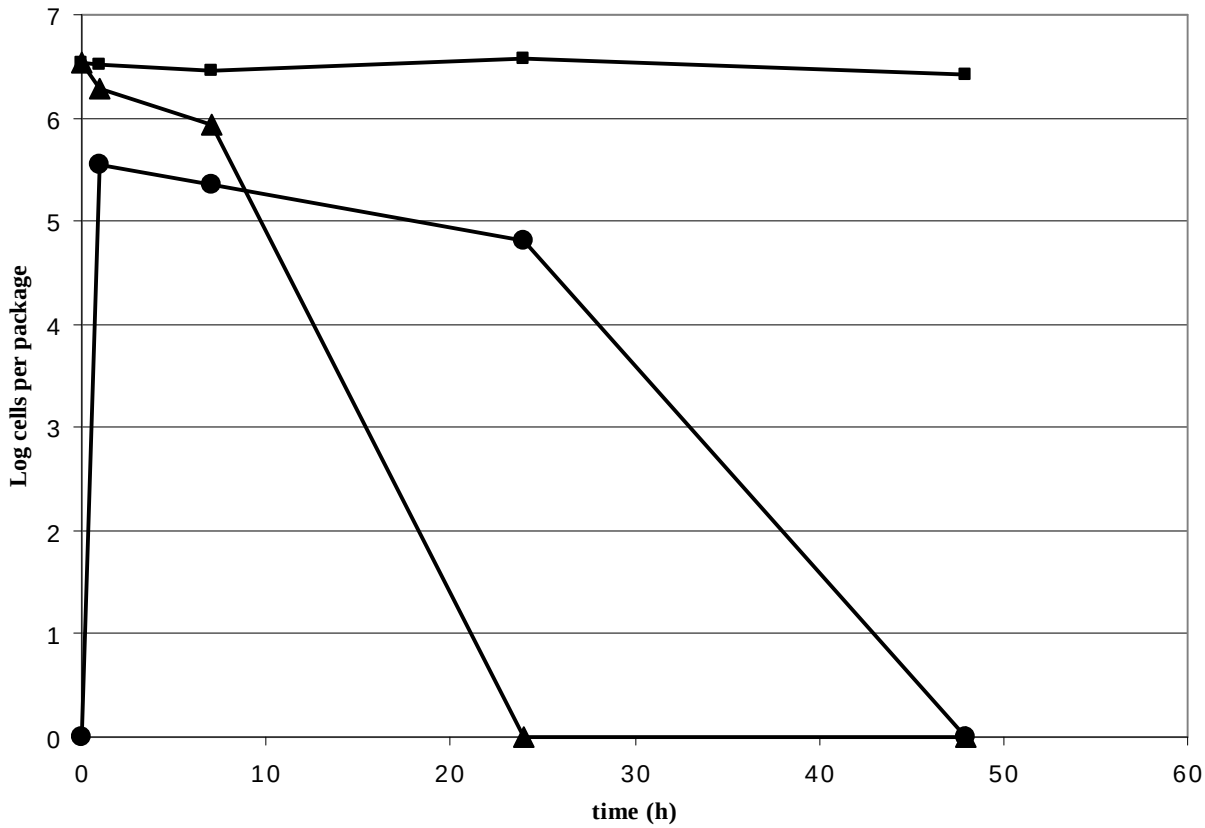


Fig. 4.6 – Changes of *L. monocytogenes* V7 population superficially contaminating frankfurters during storage at 4°C in bacteriocin activated polyethylene film. (•) live and (•) dead cells of *L. monocytogenes* V7 in bacteriocin activated packages; (•) live cells of *L. monocytogenes* V7 in untreated packages used as control.

No cells were found in 30 field of observation of the internal surface of treated and untreated polyethylene films at each time of analysis, indicating that the cells remained attached to the

sausage after the package was removed. The cells were efficiently removed from the surface of the frankfurters, as repeated washings of the frankfurters did not lead to more cells to be analysed.

4.4 DISCUSSION

In this work, the efficacy of a bacteriocin 32Y activated polyethylene film was evaluated on resting and growing populations of *L. monocytogenes*. The viable staining procedure by using the Live/Dead® *Bac*Light™ Bacterial Viability Kit was chosen to rapidly assess the bacterial viability in the bacteriocin treated samples. This method is used to microscopically count live and dead bacteria in environmental samples or in laboratory conditions, and several applications can be found in the recent literature (Boulos et al., 1999; Decker, 2001; Bunthof et al., 2001; Nohynek et al., 2003; Lee et al., 2003). In the Live/Dead® *Bac*Light™ bacterial viability kit the SYTO 9 labels all the bacteria in a population staining them green, while propidium iodide penetrates only bacteria whose cell membrane has been damaged, staining them red. Therefore, undamaged cells will be stained green and scored as live whereas cells whose membrane is damaged will appear red and scored as dead. In each experiment performed in this work, the contact of the bacteriocin 32Y with cells of *L. monocytogenes* turned all or part of the cells into red. This not only confirms the antimicrobial activity of BAC32Y against *L. monocytogenes* carried out before in other experiments, but also supports the hypothesis that the action of this bacteriocin against listeria cells involves the cytoplasmic membrane as target of the bactericidal effect. Membrane damage and cell permeabilisation caused by bacteriocins (Cleveland et al., 2001) would be responsible for propidium iodide entry inside the cell and for cell colour turning red. Beyond supporting the mechanism of action of bacteriocins this effect also makes it possible to use the viable staining technique to study bacterial viability following exposure to bacteriocins.

When the activated film was used for the packaging of PBS containing high loads of *L. monocytogenes* V7, dead cells promptly appeared indicating that the bacteriocin was exerting its bactericidal effect into the buffer. This effect was shown to be dependent on the temperature and we supposed this to be caused to a slower release of the bacteriocin from the polyethylene film at 4°C. Therefore, the release was immediate at RT, causing an immediate occurrence of dead cells; while the effect of the bacteriocin release into the buffer was registered only 2 hours later at 4°C (Fig. 4.5A, B). The effect of temperature on bacteriocin release from the polyethylene film was confirmed by the release experiments. The bacteriocin release experiment showed that at RT the spots of PBS solution were active after 5 min of contact showing a 1.10 cm inhibition halo in agar diffusion assay. At 4°C, the same antimicrobial activity was registered only after 1 h of contact of the PBS solution with the activated film. This clearly showed that the low temperature delays the release of active bacteriocin from the film. An improved efficacy of the antimicrobial treatment would be obtained at RT even though this does not fit the optimal storage conditions of most food products. However, a more effective action of bacteriocins at low temperatures has been recently reported by other authors (García et al., 2004). When growing populations of *L. monocytogenes* V7 in TSB were stored into the active package, a control of the growth was obtained both at RT and at 4°C. However, the faster release of the bacteriocin at RT kept the number of live cells lower than the control up to 7 h; at 4°C the growth was delayed of 24 h but in this case there was only a slight influence of the active package because the low temperature contained the growth also in the untreated control. The concentration of active bacteriocin molecules represents a limiting factor due to the single-hit mechanism of action (Tagg *et al*., 1976). Therefore, in applications of bacteriocin activated antimicrobial films, the quantity of bacteriocin that is possibly released or that is actually able to exert the bactericidal action has to be considered in order to predict the reduction of the population and the probable amount of survivors that may keep growing in the food matrix. We also evaluated the effect

of the antimicrobial film on the cells of *L. monocytogenes* V7 by observing the behaviour of the listeria population directly on the surface of the film. The antimicrobial film did not interfere with the observations in epifluorescence microscopy as it did not give background fluorescence. The cells of *L. monocytogenes* V7 in PBS were killed at RT in two hours after which no more live cells could be observed in 30 fields of observation. The system did not prove as much successful for the same PBS suspension at 4°C where after an early reduction in 2 h, the number of live cells remained constant for 7 h. Regardless of the incubation temperature, in both cases there was an immediate reduction of live cells after the contact compared to the control indicating that an immediate effect was exerted on the population. Also in this case the temperature influenced the release of the bacteriocin in PBS making the system more effective at RT. By contrast, the live *Listeria* population in TSB rapidly decreased regardless of the incubation temperature, probably due to a higher sensitivity of the metabolically active cells. Interestingly, the growing cells of *L. monocytogenes* V7 in TSB were rapidly killed in 2 h after the contact with the activated film, while population of *L. monocytogenes* V7 in contact with the untreated film grew rapidly in the first hours of incubation both at RT and at 4°C. The close contact of the cell suspension with the bacteriocin-activated film determined an exposure of the cells to a high concentration of bacteriocin causing a rapid inactivation of the population. In contrast with the previous experiment, in this case there was a decrease in live cells that was not accompanied by appearance of dead cells. Immediately after the contact between the activated film and the cell suspension a number of dead cells were observed, while no dead cells could be observed after a few minutes of incubation and only cellular debris occurred after 2 h (Fig. 4.4). This result indicates that cells die when they come in contact with the film and cell lysis follows immediately afterwards. With the viable staining procedure a red cell with a damaged membrane appears red and is scored as dead; however, it may be repaired in appropriate conditions. Therefore, the contact with the listeria with the antibacterial film resulted

particularly effective as the cells died and lysed at the same time and in this case no cell recover can take place. While the active packaging system was not satisfactorily effective in controlling the growth of listeria in liquid media, the close contact of the cell suspension with the active film reduced the population with efficacy. This result indicates that the system would probably work better for solid foods superficially contaminated by the pathogen. The results of the challenge test against *L. monocytogenes* V7 contaminating frankfurters packed in the activated film supported this hypothesis by revealing that the antimicrobial package was effective in reducing the live population of listeria during the storage at 4°C. The direct contact between the cells and the bacteriocin 32Y coated on the film caused an immediate reduction of live and appearance of dead cells just after 15 min from the packaging. A reduction to complete elimination of both live and dead cells followed in 24 h satisfactorily assuring the control of both presence and growth of *Listeria* on the surface of the frankfurters. Luchansky and Call (2004), in a similar experiment could not control *L. monocytogenes* in frankfurters by nisin activated casings unless additional antimicrobials, such as potassium lactate and sodium diacetate, were employed.

4.5 CONCLUSION

The overall results demonstrate that the developed active polyethylene film for food packaging has an antimicrobial effect on both resting and growing populations of *L. monocytogenes*. The number of live resting cells could be reduced while the growth in liquid medium could be delayed as a consequence of the bacteriocin treatment. Moreover, the direct contact of the population with the active film surface resulted effective for an irreversible inactivation of the *L. monocytogenes* V7 population. According to these results, the use of the antimicrobial film is encouraged especially for solid food products were the superficial contaminants come immediately in contact with the antimicrobial film. The effect would be a fast inactivation of the population, which coupled with appropriate condition of storage, might

improve the quality and safety and prolong the shelf life of the food products packed in antimicrobial films.

4.6 REFERENCES

- **Boulos, L., Prevost, M., Barbeau, B., Coallier, J. and Desjardins. R.** (1999) LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal Microbiology Method* **37**, 77-86.
- **Bunthof, C. J., van Schalkwijk, S., Meijer, W., Abee, T. Hugenholtz, J.**. (2001) Fluorescent method for monitoring cheese starter permeabilization and lysis. *Applied Environmental Microbiology* **67**, 4264-71.
- **Cleveland, J., Montville, T. J., Nes, I. F. and Chikindas, M. L.** (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal Food Microbiology* **71**, 1-20.
- **Lee, J., Kolling, G. L., Matthews, K. R., and Chikindas, M. L.** (2003) Cold and carbon dioxide used as multi-hurdle preservation do not induce appearance of viable but non-culturable *Listeria monocytogenes*. *Journal Applied Microbiology* **94**, 48-53.
- **Luchansky, J. B., and Call J. E.** (2004) Evaluation of nisin-coated casings for the control of Listeria monocytogenes inoculated onto the surface of commercially prepared frankfurters. *Journal Food Protection* **67**, 1017-1021.
- **Nohynek, L., Saski, E., Haikara, A., and Raaska, L.** (2003) Detection of bacterial contamination in starch and resin-based papermaking chemicals using fluorescence techniques. *Journal Ind. Microbiology Biotechnology* **30**, 239-244.
- **Tagg, J. R., Dajani, A. S. and Wannamaker L. W. (**1976). Bacteriocins of gram-positive bacteria. *Bacteriology Reviews* **40**, 722-756.
- **Villani, F., Pepe, O., Mauriello, G., Salzano, G., Moschetti, G. and Coppola, S**. (1994) Antimicrobial activity of *Staphylococcus xylosus* from Italian sausage against *Listeria monocytogenes*. *Letters in Applied Microbiology* **18**, 159-161.

5. NISIN AND EDTA APPLIED TO FOOD PACKAGING MATERIALS TO INHIBIT MICROBIAL POPULATION ON MEATS PRODUCTS

5.1 INTRODUCTION

A plastic active packaging for the storage of milk was never employed before, especially for raw milk, although a nisin activated chitosan package was used for pasteurized milk storage by Lee *et al*. (2004). The bacteriocin nisin, discovered in England in 1928, is produced by certain strains of *Lactococcus lactis* subsp. lactis. Structurally, it is a 34-aminoacid polypeptide, a cationic molecule due to combination of three lysine residues and one or more histidine residues (Cleveland et al., 2001). Nisin is believed to bind to the outer membrane receptors by conjugation with other cell components (i.e., phospholipids), or by aggregation with other proteins (i.e., glycoproteins). Such binding creates ion channels in the cytoplasmic membrane, causing the cell permeable (Delves-Broughton, 1990). Nisin is generally protected by food ingredients to which it is added, and does not lose its activity by processes such as pasteurization or sterilization (Henning et al., 1986).

The objective of this study was to determine the effectiveness of a packaging film coated with nisin to inhibit *Micrococcus luteus* ATCC 10240 in TSB and the microbial population during the storage of milk and to examine the release of the nisin from the activated film. The aim of this activities was to study the spoilage related microbial populations in beef steaks and hamburgers to investigate the effect of a nisin activated antimicrobial packaging on the beef spoilage development at refrigerate condition.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains and growth conditions

Micrococcus luteus ATCC 10240, which is usually used as an indicator strain in the nisin bioactivity assay, was daily propagated in Tryptone Soya Broth (TSB, Oxoid) supplemented with 0.5% yeast extract at 30°C.

5.2.2 Bacteriocin preparation and activity

1 g of nisin (2.5% pure, Sigma Chemical Co.) was suspended in 5 mL of 0.02 N HCl and it was centrifuged at 19000 x *g* for 5 min. The pellet was resuspended by stirring in 5 ml of 0.02 N HCl and recentrifuged at 19000 x *g* for 5 min. The supernatant, showing an activity of 51200 AU/ml against *M. luteus* ATCC 10240, represented a stock solution of nisin and it was stored at 4°C prior to use. The concentration of the nisin solution was determined by an agar diffusion and critical dilution assay as previously described in chapter 2.

5.2.3 Preparation of antimicrobial plastic film and its activity

The nisin was coated onto low-density polyethylene (LDPE) film. As a preliminary step the stock nisin solution was diluted at a concentration of 6400 AU/ml and it was coated manually on one side of the film using a coating rod (Fig. 5.1) (Lee *et al*., 2003).

Fig. 5.1 - Coating rod

The film was dried by exposure to warm air and assayed for antimicrobial activity against the indicator *M. luteus* ATCC 10240. Samples (2 x 2 cm^2) of activated film were placed in a Petri dish and then covered with TSA soft agar inoculated with 2.5% of an overnight culture of *M. luteus* ATCC 10240. After the incubation at 30°C for 24 h, the antimicrobial activity was observed as a zone of inhibition of the indicator organism around the packaging material. Besides, the film was rubbed and then assayed for residual antimicrobial activity, in order to evaluate the possible removal of the bacteriocin from the film during handling. Untreated film was assayed as control.

5.2.4 Antimicrobial efficacy of the nisin-coated polymer film on Micrococcus luteus ATCC 10240 in TSB

An active package (13x13x9 cm^3) was obtained from the nisin-coated film by thermo-welding and it was placed into a rigid support in order to simulate the internal side of a brick as showed in chapter 4. The active package was filled with 1 liter of *M. luteus* suspension (10^7 CFU/ml) in TSB and stored at 4°C and 25°C. The cultures were analyzed by viable staining and plate counts as described below at 0, 1, 2, 5, 7, 24 and 48 h. An untreated package was included in each determination as control.

5.2.5 Antimicrobial efficacy of the nisin-coated plastic film on the microbial stability of milk during storage

In the challenge tests three different types of milk were used: raw cow milk, pasteurized milk and U.H.T. milk, the latter of which was artificially inoculated with a *M. luteus* ATCC 10240 suspension obtaining a contamination of about 10^7 CFU/ml.

One litre of the milk was poured into the package and it was then stored at 4°C for a week. During the storage period, samples of milk were taken periodically to measure pH (0, 1, 2, 3,

4 and 7 days) and to estimate microbial growth (0, 1, 3 and 4 days). Total viable counts were performed on Plate Count Agar (PCA, Oxoid) after incubation at 30°C for two days.

5.2.6 Bacterial enumeration

A rapid epifluorescence staining method using the LIVE/DEAD® Bacterial Viability Kit (*Bac*Light™, Molecular Probes Inc., Eugene, OR, USA) was applied to estimate the reduction of *M. luteus* ATCC 10240 populations in TSB. The stock solution of the two stains was prepared mixing 330 µl of sterile deionized water, 0.7 µl of SYTO 9 and 1 µl of propidium iodide. The number of live green and dead red cells was calculated on one ml aliquots or dilutions of *M. luteus* ATCC 10240 suspensions as described in Materials and Methods of Chapter 4.

5.2.7 Release of nisin from the antimicrobial coated film

The study of the nisin release was performed using a film coated with a solution showing an activity of 25600 AU/ml.

In a first experiment 20 µl of sterile deionized water were spotted onto the surface of the treated film, the film was incubated in a humid chamber and the water was removed every 5 min for 1 h. Water spots were then assayed for antimicrobial activity against *M. luteus* ATCC 10240. In a second experiment a circular treated film (Ø 8.5 cm) was placed into a Petri dish as showed in figure 5.2, and covered with 15 ml of two different simulating solutions: sterile water and phosphate buffer saline (PBS) at pH 3.5. The dishes were shaken continuously at 75 rpm by an orbital shaker at room temperature (25°C). After 1, 2, 3, 4, 5, 6, 7, 24, 30, 48 and 72 h, samples of 10 µl of both solutions were taken and tested for antimicrobial activity. The remaining simulating solution was lyophilized, resuspended in 250 µl of 0.02 N HCl and analyzed by HPLC in order to quantify the nisin released in the contact solution. A C18 reversed-phase column (250 x 4.6 mm) and a gradient elution with water-acetonitrile gradients (1 ml/min) containing 0.1% TFA were used (Buonocore *et al*., 2003). The gradient

was 20-60 % of acetonitrile over 25 min, with nisin eluting at about 17-18 min. The calibration curve was constructed for peak area against concentration of standard solutions of nisin (94,6% pure, donated by Aplin and Barrett, Dorset, UK) from 10 to 100 ppm, with three replicate samples for each nisin concentration. Furthermore, the film resulting from each release experiment was assayed for antimicrobial activity in agar diffusion assays. The last experiment was performed to evaluate the effect of exposure temperature on nisin release into liquid media. The active package was filled with different media (water, PBS and TSB, PBS and TSB inoculated with *M. luteus* ATCC 10240) and stored at 4°C and 25°C. After a 48 h contact, the film was dried and assayed for the antimicrobial activity against the indicator strain.

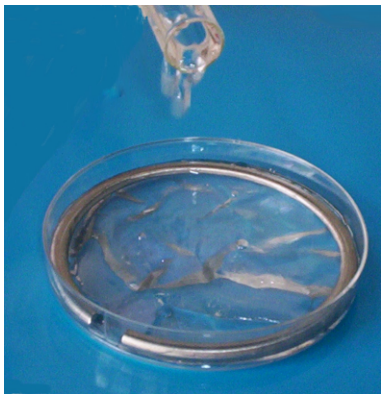


Fig. 5.2 - Release of nisin from the antimicrobial coated film

5.2.8 Nisin-EDTA activated plastic film for the storage of meat products

A nisin antimicrobial solution (NIS) was prepared as follows. Briefly, 1 g of nisin (2.5% pure, Sigma-Aldrich, Milan - Italy) was suspended in 10 ml of 0.4 N lactic acid with 0.1 g/ml of EDTA. The mixture was centrifuged at 19000 x g for 5 min. The pellet was resuspended by stirring in 10 ml of 0.4 N lactic acid and centrifuged at 19000 x g for 5 min. The supernatant represented the NIS solution which at a concentration of 6400 AU/ml against *M. luteus* ATCC 10240 was used to coat polyethylene film. Beef steaks were packed in nisin activated film under vacuum and stored at 4°C. Before packaging and after 2, 7, 15 days of storage,

selective viable counts of natural spoilage microorganisms (Microbial total count, *Brochothrix thermosphacta*, *Enterobacteriaceae*, *Pseudomonas* spp. and lactic acid bacteria) were performed on 10 g of beef steak stored at 4°C. The counts of spoilage microorganisms were performed in triplicate and the results were expressed as CFU/g. Similarly, 45 g hamburger were packed on both faces with the nisin–activated film and stored at 4°C. Furthermore, minced meat mixed with 2.5% NIS solution was utilized to make hamburger packed with untreated film. Samples packed with untreated film was analysed as control. The behaviour of spoilage bacteria was analysed after 0, 1, 5 and 7 days of storage and the results were expressed as CFU/g.

The experiments were performed in triplicate and the results were expressed as CFU/g. For lactic acid bacteria (LAB), MRS agar (Oxoid) was employed with incubation at 30°C for 48h; for *Enterobacteriaceae* Violet Red Bile Glucose Agar (Oxoid) was employed with incubation at 30°C for 48h; for *Pseudomonas* spp. Pseudomonas Agar with CFC selective supplement (Oxoid) was employed with incubation at 30°C for 48h; *Brochothrix thermosphacta* STAA (Sreptomycin Thallous Acetate Agar) (Oxoid) with STA selective supplement (Oxoid) was employed with incubation at 20°C for 48h

5.3 RESULTS AND DISCUSSION

5.3.1 Antimicrobial efficacy of the nisin-coated polymer film against Micrococcus luteus ATCC 10240 in TSB

The nisin-coated films showed antimicrobial activity against *M. luteus* ATCC 10240 (Fig. 5.3) and the inhibition area spread beyond the film perimeter. Moreover, the activated film maintained its activity even after rubbing (Figure 5.3, d). In both cases the untreated film did not show any activity against the indicator strain.

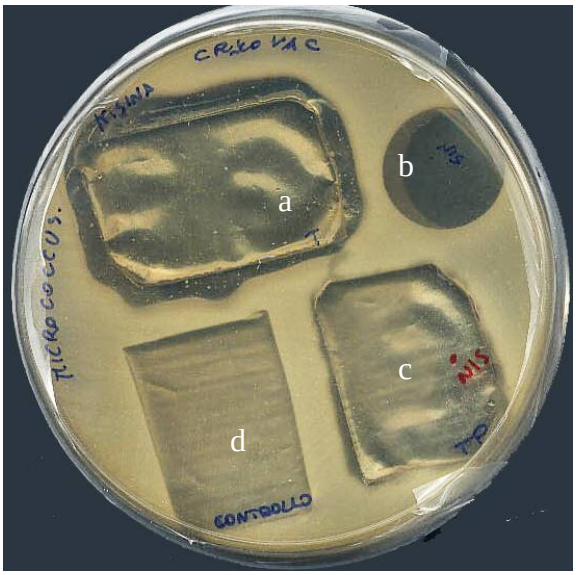


Fig. 5.3 - Antimicrobial activity of nisin-coated film against *Micrococcus luteus* ATCC 10240: (a) treated film; (b) spot of a nisin solution (c) treated film after rubbing; (d) untreated film.

In the antimicrobial package at 4°C a slight reduction of live cells was observed by viable staining after 48 h compared with the control in which the number of live cells remained constant (Fig. 5.4). When the *M. luteus* ATCC 10240 population in TSB was incubated at 25°C there was a remarkable reduction of live cells in the bacteriocin-coated package (Fig. 5.4).

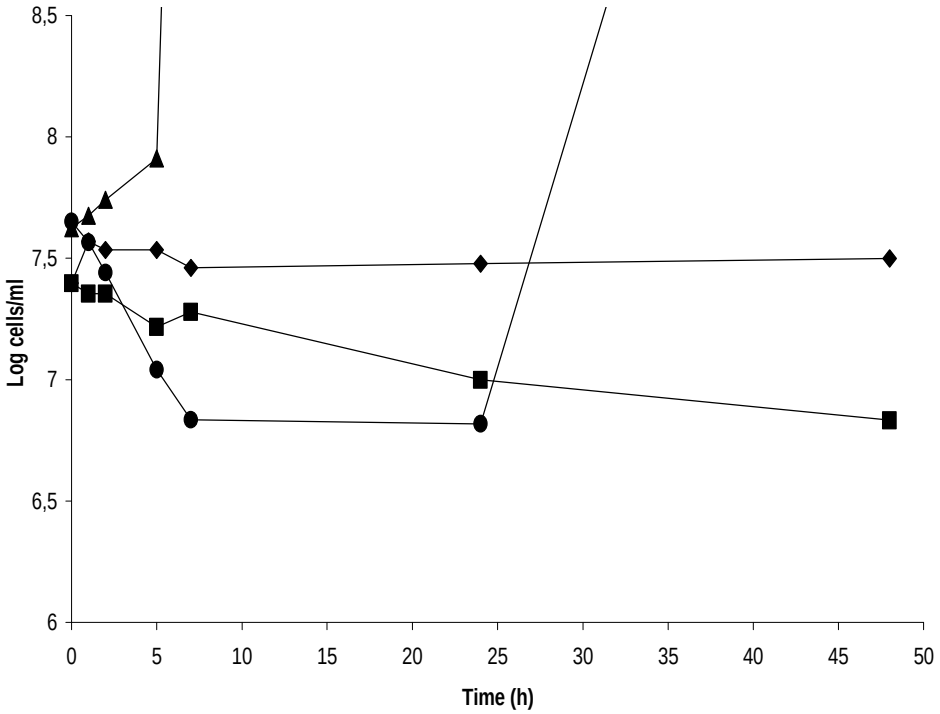


Fig. 5.4 - Trends of live cells of *Micrococcus luteus* ATCC 10240 in TSB packed in activated and non-activated film during storage at 4°C and 25°C. (•) Untreated package at 25°C; (•) Treated package at 25°C; (•) Untreated package at 4°C; (•) Treated package at 4°C. Values in the graph are means of two experiments and the result of each single experiment was calculated as average of counts of 30 microscopic fields.

In fact after 5 h of contact with active film the live population was reduced of 0.9 log compared with the untreated package, and after 7 h of storage the number of live cells continued to decrease in the treated package, while it increased rapidly in the control. A remarkable increase of *M. luteus* ATCC 10240 viable cells was registered after 25 h of storage, which may be due to the particular mechanism of action of bacteriocins that can inhibit as many cells as molecules available in the medium (Moll *et al*., 1999). In fact the total viability in the active package was restored after 48 h of storage. The results of viable staining by *Bac*Light Kit were compared with those from plate counts. The counts on TSA plates at 4°C showed the same trend as the counts after viable staining; however, during the first hours of incubation up to 7 h the difference between treated sample and control at 25°C, detected by plate counts, was narrow compared to the difference appreciated by viable staining. A

possible explanation is that red cells scored as “dead” in the *Bac*Light assay under certain condition may be able to recover and reproduce. When the number of red cells decreased as a consequence of cell lysis, after 24 h of storage, plate counts showed a difference of 2.4 log between control and active package. The viable staining technique supported the hypothesis that the mechanism of action of nisin involves the cytoplasmic membrane as target (Cleveland *et al*., 2001), causing the cell permeabilisation that is responsible of propidium iodide entry inside the cell.

5.3.2 Antimicrobial efficacy of the nisin-coated polymer film against Micrococcus luteus ATCC 10240 in milk

Another experiment was carried out in order to monitor the microbial growth in milk. The results are reported in Fig. 5.5 A, B and C. The antimicrobial package retarded the microbial growth and lowered the maximum growth levels in raw milk, pasteurized milk and U.H.T. milk, confirming the results of a similar study (Lee *et al*., 2004). Only in the raw cow milk a decrease of the pH was observed, and the final pH value was lower in the control than in treated sample. In fact a difference of 1.1 units of pH between the treated and control samples was observed after 7 days suggesting that the nisin affected the activity of the acidifying microbiota (Fig.5.5, panel C).

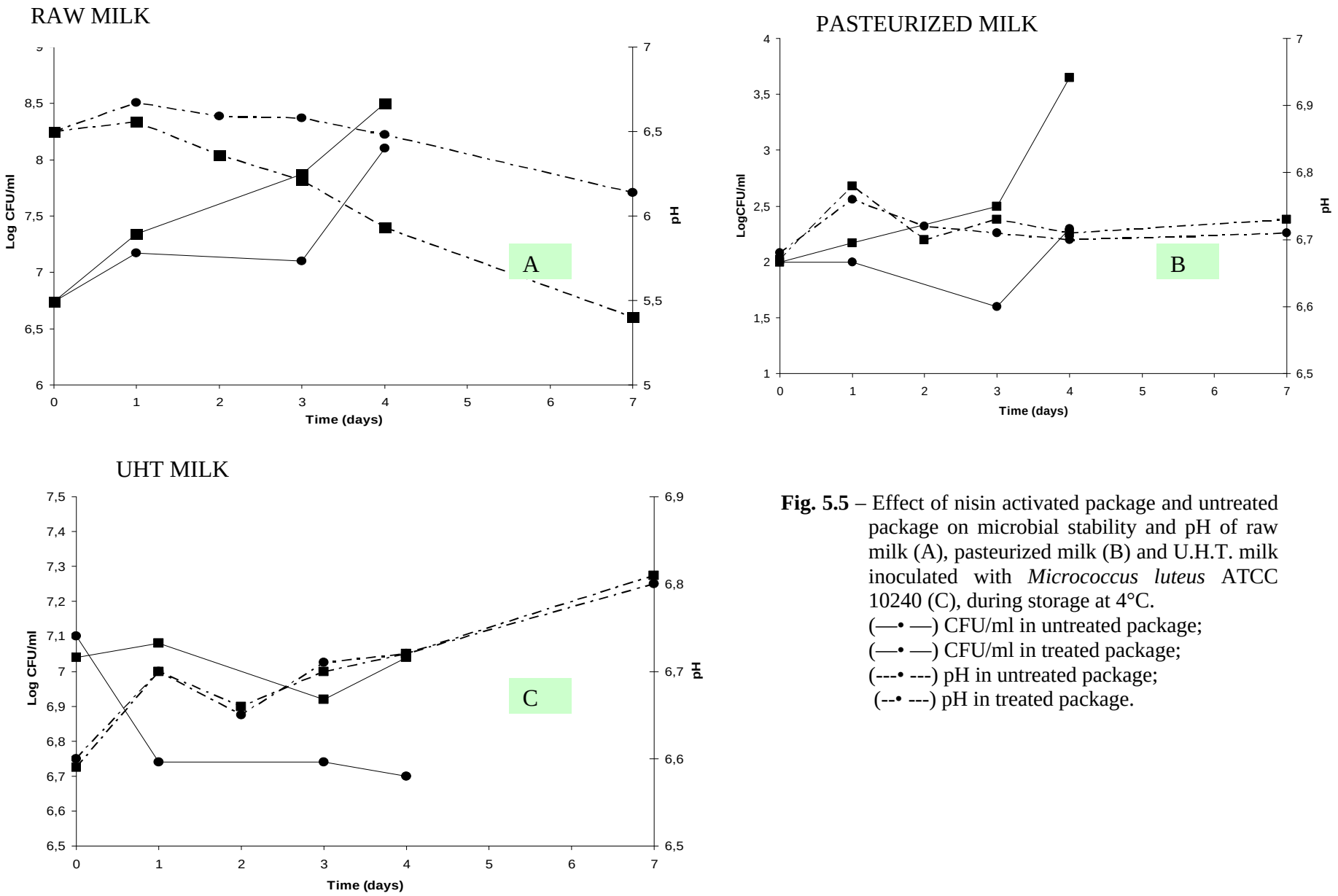


Fig. 5.5 – Effect of nisin activated package and untreated package on microbial stability and pH of raw milk (A), pasteurized milk (B) and U.H.T. milk inoculated with *Micrococcus luteus* ATCC 10240 (C), during storage at 4°C.
(—• —) CFU/ml in untreated package;
(—• —) CFU/ml in treated package;
(---• ---) pH in untreated package;
(--• ---) pH in treated package.

5.3.3 Release of nisin from the antimicrobial-coated film

The nisin-coated films were also subjected to studies of release of the bacteriocin in water or PBS at pH 3.5. All the experiments suggested a mechanism of release and back-absorption of nisin from/to the film. Back-absorption is to be intended as absorption of nisin to the film after the release. In fact, during the contact time between active film and simulating solutions high values of antimicrobial activity alternated to low values of antimicrobial activity (Table 5.1).

Table 5.1 – Antimicrobial activity of simulating solutions and activated film in the nisin release experiment.

	Water		**PBS pH 3.5**	
Time (h)	Diameter of inhibition halos (mm)	Residual activity of the activated film	Diameter of inhibition halos (mm)	Residual activity of the activated film
1	2	+	8	-
2	2	+	9	-
3	3	+	9	-
4	3	+	9	-
5	4	-	9	-
6	5	-	9	-
7	5	-	9	-
24	0	+	6	-
30	0	+	6	-
48	0	+	8	+
72	4	-	9	+

Particularly, when antimicrobial activity increased in the simulating solutions, the antimicrobial activity of the activated films ceased and *vice versa*. This behaviour was also observed in the study of Grower *et al*., (2004) who evaluated the release of nisin into peptone water from LDPE film coated with cellulose. This result was also confirmed by the quantitative determination of nisin in the surrounding solution by means of HPLC. At this

stage of the research is not possible to speculate on the reason of this back absorption behaviour because the mechanism of nisin binding to the plastic film is still unknown.

Lower pH favoured the migration of the active compound from the film in fact at pH 3.5 higher values of activity of the simulating solution and higher concentrations of nisin, as determined by HPLC, were registered. The explanation may be that the nisin is more soluble at acid than neutral pH as reported by Liu and Hansen (1990). On the other hand, the nisin is more active at lower pH and consequently, higher activity may be registered with the same quantity of nisin at lower pHs (Huot et al., 1996). Moreover, the results showed that the low temperature delayed the release of the nisin from film, since after contact with a liquid medium bacteriocin-coated film maintained its antimicrobial activity at 4°C but not at 25°C. Similar results were obtained by Dawson *et al.* (2003) and in the study described in chapter 4.

The nisin-activated film was shown to be effective in inhibiting a population of *M. luteus* ATCC 10240 in TSB. Moreover, the antimicrobial package could contribute to control the development of bacterial flora in milk. Finally, the release of nisin from the plastic film was shown to be unpredictable but temperature and pH-dependent.

5.4 REFERENCES

- **Buonocore, G.G., Del Nobile, M.A., Panizza, A., Corbo, M.R. and Nicolais, L.** (2003) A general approach to describe the antimicrobial agent release from highly swellable films intended for food packaging applications. *Journal Control Release* **90**, 97-107.
- **Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.L.** (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal Food Microbiology* **71**, 1-20.
- **Dawson, P.L., Hirt, D.E., Rieck, J.R., Acton, J.C. and Sotthibandhu, A.** (2003) Nisin release from films is affected by both protein type and film-forming method. *Food Research International* **36**, 959-968.
- **Delves-Broughton, J.,** (1990) Nisin and its uses as a food preservative. Food *Technology* **44** , 100–117.
- **Henning, S., Metz, R., Hammes, W.P.,** 1986. Studies on the mode of action of nisin. *International Journal Food Microbiology* **3**, 121–134.
- **Huot, E., Barrena Gonzalez C. and Petitdemange, H.** (1996) Comparative effectiveness of nisin and bacteriocin J46 at different pH values. *Letters Applied Microbiology* **22**, 76-79.
- **Lee, C.H., An, D.S., Lee, S.C., Park, H.J. and Lee, D.S.** (2003) A coating for use as antimicrobial and antioxidative packaging material incorporating nisin and α-tocoferol. *Journal Food Engineering* **62**, 323-329.
- **Lee, C.H., Park H.J. and Lee, D.S.** (2004) Influence of antimicrobial packaging on kinetics of spoilage microbial growth in milk and orange juice. *Journal Food Engineering* **65**, 527–531.
- **Liu, W., and Hansen, N.** (1990) Some chemical and Physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis*. *Applied Environmental Microbiology* **56**, 2551-2558.
- **Moll, G.N., Konings, W.N. and Driessen, A.J.M.** (1999) Bacteriocins: mechanism of membrane insertion an pore formation. *Antonie van Leeuwenoek* **76**, 185-198.

6. ANTIMICROBIAL ACTIVITY OF A NISIN-ACTIVATED PACKAGING AGAINST SPOILAGE MICROORGANISMS DURING STORAGE OF MEAT PRODUCTS

6.1 INTRODUCTION

Numerous types of food packaging, in combination with different storage techniques can be used in order to extend the shelf life of meat. One of the key technological measures needed during storage is the preservation of the meat from microbial spoilage and contamination/proliferation of pathogenic microorganisms. Active packaging is one of the innovative food packaging concepts that has been introduced as a response to demands of consumers for high quality, safety and extended shelf-life of food products (Vermrein *et al.*, 1999; Quintavalla and Vicini, 2002; Cagri *et al.*, 2004). Among the active packaging applications, the incorporation of antimicrobials is receiving considerable attention as a means of inactivating bacterial cells, slowing the growth rate of microorganisms and maintaining food quality and safety (Han, 2000; Gill, 2003; Guerra *et al.*, 2005). The use of bacteriocins and other biologically derived antimicrobials in packaging material (Ming *et al.*, 1997; Siragusa *et al.*, 1999) is recently attracting increasing interest. Bacteriocin activated plastic films for food packaging have been profitably developed for the storage of milk (Mauriello *et al.*, 2005), hamburgers (Mauriello *et al.*, 2004), frankfurters (Ercolini *et al.*, 2006) and cooked ham (Marcos *et al.*, in press). In all the cases the activation of plastic films with bacteriocin solutions was helpful to retard the growth of pathogenic and/or spoilage bacteria. Among the known bacteriocins, nisin is currently the only bacteriocin whose employment in food as antimicrobial is allowed. The shelf-life of meat mostly depends on the number and types of microorganisms initially present and their subsequent growth (Borch *et al.*, 1996). Many groups of organisms contain members potentially contributing to meat spoilage under

appropriate conditions. This makes the microbial ecology of spoiling raw meat very complex and thus the spoilage very difficult to prevent. The main bacteria implicated in the spoilage of refrigerated beef include *Brochothrix thermosphacta*, *Lactobacillus* spp., *Leuconostoc* spp., *Carnobacterium* spp., *Pseudomonas* spp. and *Enterobacteriaceae* (Dainty, *et al.*, 1992; Borch *et al.*, 1996; Huis in't Veld, *et al.*, 1996; Jay *et al.*, 2003). The aim of research described in this chapter was to determine the effectiveness of a packaging film coated with nisin-EDTA to inhibit the natural microflora of hamburgers and beef steaks during storage

6.2 MATERIALS AND METHODS

6.2.1 Antimicrobial solution preparation and activity

A nisin antimicrobial solution (NIS) was prepared as follows. Briefly, 1 g of nisin (2.5% pure, Sigma Chemical Co.) was suspended in 10 ml of 0.4 N lactic acid with 0.1 g/ml of EDTA. The mixture was centrifuged at 19000 x g for 5 min. The pellet was resuspended by stirring in 10 ml of 0.4 N lactic acid and centrifuged at 19000 x g for 5 min. The supernatant, represented a stock solution of nisin and it was stored at 4°C prior to use. The concentration of the nisin solution was determined by an agar diffusion and critical dilution assay as previously described. The supernatant represented the NIS solution, showing an activity of 102400 AU/ml against *M. luteus* ATCC 10240.

6.2.2 Preparation of antimicrobial plastic film and its activity against bacterial strains

The NIS solution diluted at concentration of 6400 AU/ml against *M. luteus* ATCC 10240 was used to spread manually one side of the polyethylene film using a coating rod. Antimicrobial activity of NIS treated film was determined against *M. luteus* ATCC 10240, *Brochothrix thermosphacta* 7R1, *Pseudomonas fragi* 6P2, *Enterococcus faecalis* 227 and *Lactobacillus spp* 3A. The indicator strains was from the culture collection of the Department of Food Science, the University of Naples Federico II, Italy.

6.2.3 Antimicrobial efficacy of the nisin-coated plastic film on the microbial stability of beef steak

The films spread with NIS solution were used to study the behaviour of natural spoilage microorganisms on beef steaks stored under vacuum at 4°C. Before packaging and after 2, 7, 15 days of storage, selective viable counts of natural spoilage microorganisms such as microbial total count, *Brochothrix thermosphacta, Enterobacteriaceae, Pseudomonas* spp. and lactic acid bacteria (LAB) were performed on 10 g of beef steak. Beef steaks packed under vacuum in untreated film were examined as control samples. The counts of spoilage microorganisms were performed in triplicate and the results were expressed as CFU/g.

6.2.4 Antimicrobial efficacy of the nisin-coated plastic film on the microbial stability of hamburgers

Hamburgers of 45 g each were packed on both faces with the NIS–activated film and stored at 4°C. Furthermore, minced meat mixed with 2.5% NIS solution was utilized to make hamburger packed with untreated film. Samples packed with untreated film were examined as control. The behaviour of spoilage bacteria was analysed after 0, 1, 5 and 7 days of storage and the results were expressed as CFU/g.

6.2.5 Microbial analysis

Samples (25 g) arising from each samples were aseptically weighed, homogenized in quarter strength Ringer's solution (Oxoid) for 2 min in a stomacher (LAB Blender 400, PBI, Italy) at room temperature. Decimal dilutions were prepared and aliquots of 0.1 ml of the appropriate dilutions were spread in triplicate on the following media: Plate Count Agar (PCA, Oxoid) incubated at 30°C for 72 h; Violet Red Bile Glucose Agar (VRBGA, Oxoid) for the *Enterobacteriaceae*, incubated at 30°C for 24-48 h; MRS agar (Oxoid) for LAB,

incubated at 30°C for 48 h; Pseudomonas Agar with cetrimide-fucidin-cephaloridine (CFC) selective supplement (Oxoid) for *Pseudomonas*, incubated at 30°C for 48 h; STAA medium (Oxoid) for *Brochothrix thermosphacta*, incubated at 25°C for 48 h. Results were calculated as the means of three determinations. MRS and VRBGA plates were incubated in anaerobic conditions by using an Anaerogen kit (Oxoid).

6.3 RESULTS AND DISCUSSION

6.3.1 Plastic films activation

The antimicrobial activity of the plastic films tested in agar plates (Mauriello *et al.*, 2004) against the indicator strain of *B. thermosphacta* 7R1, *Enterococcus faecalis* 227 and *Lactobacillus* spp. 3A proved that the NIS solution, and also the antimicrobial activity, were homogeneously distributed on the surface of the plastic. The active packaging did not show inhibition of *Pseudomonas fragi* 6P2. Figure 6.1 showed the antimicrobial activity against *B. thermosphacta* 7R1.

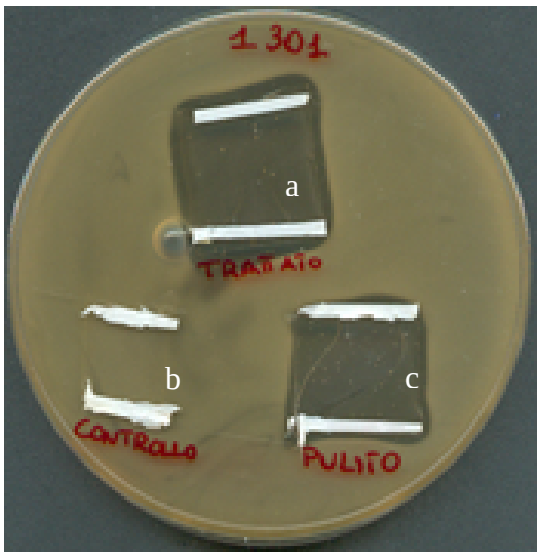


Fig. 6.1 - Antimicrobial activity of the polyethylene film coated with NIS solution against *B. thermosphacta* 7R1 (a), treated film, (b) untreated film; (c) treated film after rubbing

6.3.2 Effect of activated film on natural bacterial population in meat storage

The results of viable counts on appropriate media of the meat spoilage target groups from beef steaks are reported in table 6.1. The nisin-coated polymer film was effective to control *Brochothrix thermosphacta* population and showed its antagonistic power from the beginning, in fact the counts of *B. thermosphacta* in beef steaks storage in antimicrobial packaging was more than about 5, 4, and 3 log cycles lower than the counts in meat storage in untreated film after 2, 7 and 15 days, respectively. The sample stored in active packaging did not show reduction of *Pseudomonas* sp. On the other hand, after 15 days of refrigerated storage LAB population showed $1.9x10^3$ and $1.1x10^6$ CFU/g in meat packed in treated and untreated film, respectively. At the same time the level of *Enterobaceriaceae* decreased by 1 log cycle in antimicrobial film packed meat. The results of hamburger experiments are reported in Figure 6.2 and values are means of three replicates. Before packaging the hamburger prepared with the meat mixed with NIS solution showed an immediate reduction of *Brochotrhix thermosphacta* (Fig. 6.2 panel A); in fact the counts of the test organisms presented 2 log cycles lower than control samples. After 5 and 7 days of refrigerated storage the counts of *Brochotrhix* population was of 1x10 e 3.1 $x10^2$ CFU/g while in the control samples was of 3.5 $x10^6$ e 3.1 $x10^8$ CFU/g, respectively (Fig. 6.2 panel A). At the same times the hamburgers packed with Nisin-coated film exhibited a *Brochotrhix* population of $1.8x10^5$ CFU/g. After 5 and 7 days in every hamburger that was prepared with NIS solution and that was packed in treated film, *Pseudomonas* level decreased of 1 log cycles (Fig. 6.2, panel B); LAB and microbial total count decreased of 2 log cycles (Fig. 6.2 panel C and D). No reduction of *Enterobacteriaceae* population was registered in any hamburger sample (Fig, 6.3). The inhibition on *Pseudomonas* population could be explained by the use of EDTA in the NIS solution developed in this study. In fact, it has been previously reported that coupling nisin and EDTA, an improved antimicrobial effect could be obtained (Gill and Holley, 2002; Cutter *et al.*, 2001) and, in some cases, also an enhancement of nisin efficacy against Gram negative

bacteria (Delves-Broughton, 1993). On the other hand, it was interesting to note that antagonistic activity of NIS solution in minced meat was more efficacy than the NIS solution spread on plastic film. However, this evidence is in contrast with results of other authors (Han *et al.*, 2000), whose reported the reduction of efficacy of antimicrobial substances added directly in foods because of their interaction with food components.

Table 6.1 - Microbiological analysis of beef steaks in antimicrobial packaging stored at 4°C

Behaviour of natural bacterial population in beef steaks CFU/g [a]										
	Microbial total count		***Enterobacteriaceae***		***Brochothrix thermosphacta***		***Pseudomonas spp.***		**Lactic acid bacteria**	
Time (days)	**C**	**A**	**C**	**A**	**C**	**A**	**C**	**A**	**C**	**A**
0	$2.2x10^4$	$1.9x10^4$	$1.5x10^2$	$1.6x10^2$	$4.7x10^3$	$3.5x10^3$	$2.9x10^4$	$5.5x10^4$	$4.0x10^2$	$1.4x10^2$
2	$4.5x10^4$	$8.5x10^4$	$3.1x10^2$	$1.0x10^2$	$3.5x10^4$	<10	$2.0x10^4$	$2.3x10^4$	$4.4x10^3$	$2.0x10^3$
7	$1.5x10^4$	$2.5x10^4$	$4.0\ x10^2$	$1.0\ x10^2$	$2.0x10^5$	<10	$3.7x10^5$	$8.5x10^5$	$8.0x10^4$	$5.0x10^4$
15	$2.8x10^6$	$1.1x10^6$	$1.5x10^4$	$1.5x10^3$	$1.1x10^6$	$1.9x10^3$	$2.0x10^6$	$3.3x10^6$	$1.0x10^6$	$3.9x10^3$

[a]: values are means of three replicates;
C: beef steaks packed in untreated film nisin
A: beef steaks packed in treated film

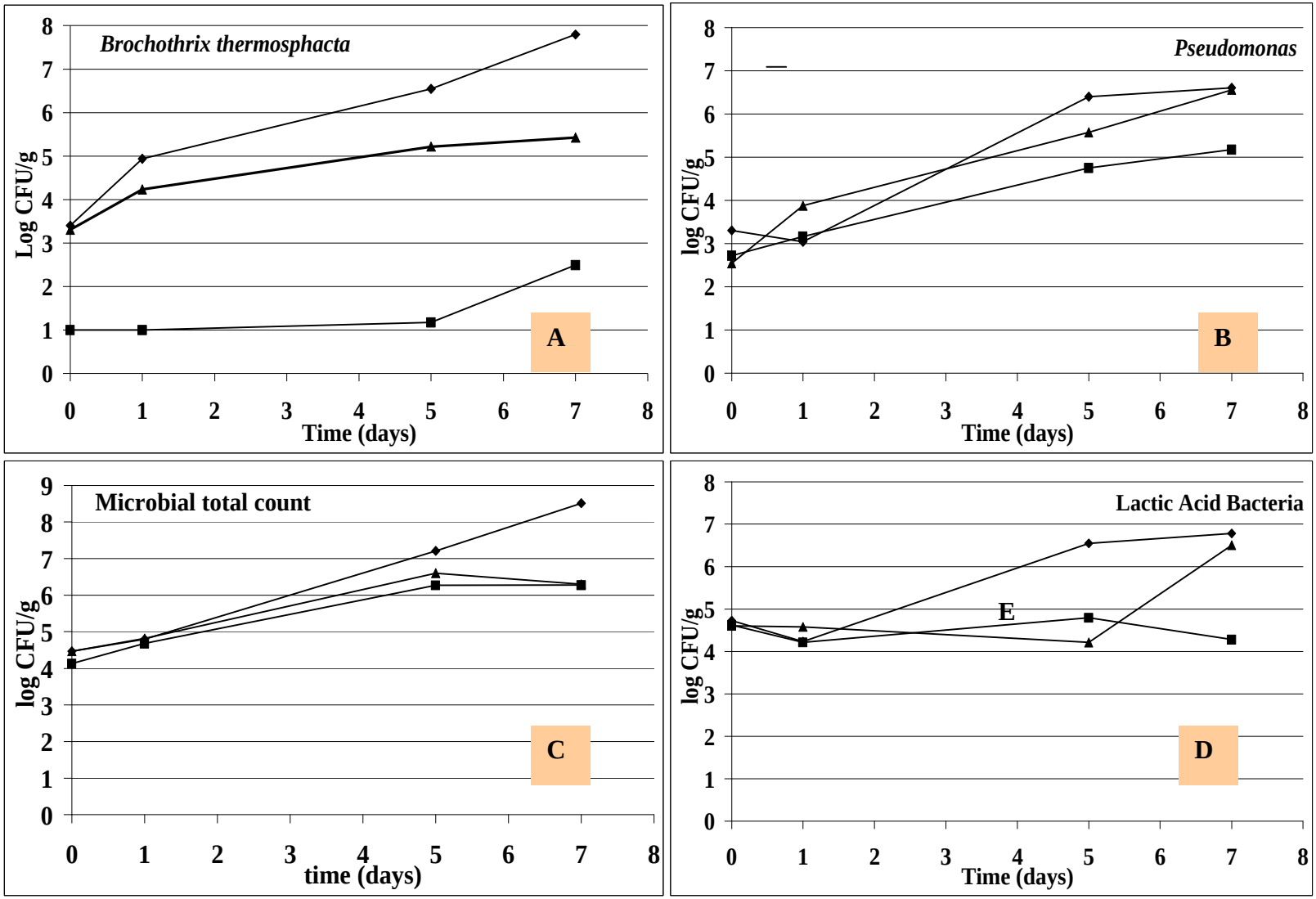


Fig. 6.2 - Effect of NIS solution, nisin activated film and untreated film on spoilage bacteria during storage at 4°C in hamburgers. (—•—) log CFU/g in control sample; (—• —) log CFU/g with treated film; (—• —) log CFU/g in hamburger mixed with NIS solution.

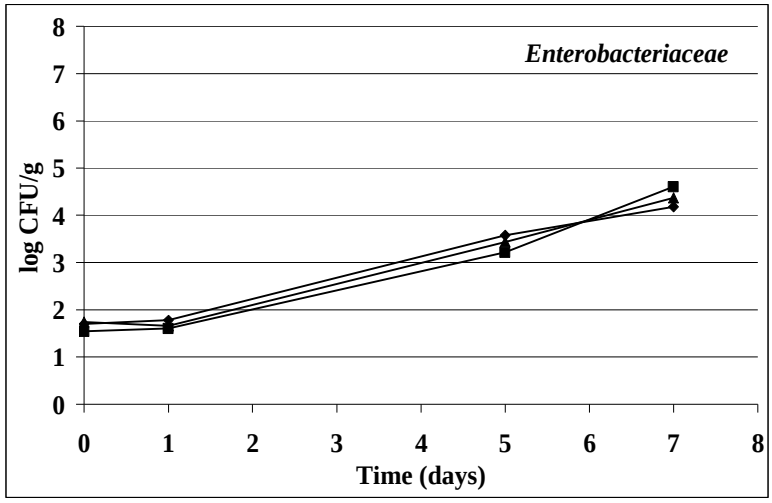


Fig. 6.3 - Effect of NIS solution, nisin activated film and untreated film on *Enterobacteriaceae* during storage at 4°C in hamburgers. (—• —) log CFU/g[1] in control sample; (—• —) log CFU/g with treated film; (—• —) log CFU/g in hamburger mixed with NIS solution.

6.4 REFERENCES

- **Borch, E., Kant-Muermans, M-L., and Blixt, Y.** (1996) Bacterial spoilage of meat and cured meat product. *International Journal of Food Microbiology* **33**, 103-120.
- **Cagri, A., Ustunol, Z. and Ryser, E.T.** (2004) Antimicrobial edible films and coatings. *Journal Food Protection* **67**, 833-848.
- **Cutter, C.N., Willet, J.L., and Siragusa, G.R.** (2001) Improved antimicrobial activity of nisin-incorporated polymer films by formulation change and addition of food grade chelator. *Letters Applied Microbiology* **33**, 325-328.
- **Dainty, R.H. and Mackey, B.M.** (1992) The relationship between the phenotypic properties of bacteria from chill-stored meat and spoilage processes. *Journal Applied Bacteriology* **73**, 103-114.
- **Delves-Broughton, J.,** 1990. Nisin and its uses as a food preservative. *Food Technology* **44**, 100–117.
- **Ercolini, D.**, **La Storia, A., Villani, F., and Mauriello, G.** (2006) Effect of a bacteriocin-activated polyethylene film on *Listeria monocytogenes* as evaluated by viable staining and epifluorescence microscopy. *Journal Applied Microbiology* **100**, 765-772.

- **Ercolini, D., Russo, F., Torrieri, E., Masi, P. and Villani, F.** (2006) Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. *Applied Environmental Microbiology* **72**, 4663-4671.
- **Gill, C.O.** (2003) Active packaging in practice: meat. *In* Ahvenainem H, Novel food packaging technology. Woodhead publishing limited and CRC Press LLC. 378-396.
- **Guerra N.P., Macias, C.L., Agrasar A.T. and Castro, L.P.** (2005) Development of a bioactive packaging cellophane using Nisaplin as biopreservative agent. *Letters Applied Microbiology* **40,** 106-110.
- **Han, J. H.** (2000) Antimicrobial food packaging. *Food Technology* **54**, 56-65.
- **Huis in't Veld, J.H.J.** 1996. Microbial and biochemical spoilage of foods: an overview. Int. *Journal Food Microbiology* **33**, 1-18.
- **Jay, J.M., Vilai, J.P., and Hughes, M.E.** (2003) Profile and activity of the bacterial biota of ground beef held from freshness to spoilage at 5-5°C. *International Journal Food Microbiology* **81**, 105-111.
- **Lee, C.H., An, D.S., Lee, S.C., Park, H.J. and Lee, D.S.** (2003) A coating for use as antimicrobial and antioxidative packaging material incorporating nisin and α-tocoferol. *Journal Food Engineering* **62**, 323-329.
- **Mauriello G., Ercolini, D., La Storia, A., Casaburi, A., and Villani, F.** (2004) Development of polyethylene films for food packaging activated with an antilisterial bacteriocin from *Lactobacillus curvatus* 32Y. *Journal Applied Microbiology* **97**, 314-322.
- **Mauriello, G., De Luca, E., La Storia, A., Villani, F. and Ercolini D.** (2005) Antimicrobial activity of a nisin-activated plastic film for food packaging. *Letters Applied Microbiology* **41**, 464-469.
- **Ming, X., Weber, G.H., Ayres, J.W. and Sandine, W.E.** (1997) Bacteriocins applied to food packaging materials to inhibit *Listeria monocytogenes* on meats. *Journal Food Science* **62**, 413-415.
- **Quintavalla, S. and Vicini, L.** (2002) Antimicrobial food packaging in meat industry. *Meat Science* **62**, 373-380.
- **Siragusa, G. R., Cutter, C. N., Willett, J. L.** (1999). Incorporation of bacteriocin in plastic retains activity and inhibits surface growth of bacteria on meat. **Food Microbiology 16**, 229-235.

- **Vermeiren, L., Devlighere, F., van Beest, M., de Kruijf, N. and Debevere, J.** (1999) Developments in the active packaging of foods. *Trends Food Science Technology* **10**, 77-86.

7. CHARACTERIZATION OF BACTERIOCIN COATED ANTIMICROBIAL POLYETHYLENE FILMS BY ATOMIC FORCE MICROSCOPY

7.1 INTRODUCTION

Antimicrobial packaging is a promising and rapidly emerging technology in which antimicrobial agents are incorporated into or coated onto food packaging materials to prolong the shelf-life of the packed food. Different kinds of antimicrobial packaging are described in the current literature and the bacteriocin coating of polyethylene surface is reported as an effective method to confer antimicrobial properties to food packages (Lee *et al.* 2003, 2004; Grower *et al.* 2004; Vartiainen *et al.* 2004; Mauriello *et al.* 2004, 2005; Ercolini *et al.* 2006). Film composition, bacteriocins as active compounds, effects of processing conditions, film microstructure, nature of foods in contact with film and controlled release are the major properties affecting the activity of antimicrobial packaging and have been the topic of several works (Han, 2000; Kim *et al.* 2002; Cha *et al.* 2003; Grower *et al.* 2004). However, unsatisfactory information is available on the interactions between the bacteriocin and the surface of the plastic film. Along with adhesion and release mechanisms of the antimicrobials, such interactions are fundamental in determining the antimicrobial efficacy of the activated film. Atomic Force Microscopy (AFM) is a powerful technique that can provide direct spatial mapping of surface morphology with nanometer resolution allowing investigations on surface topography and roughness.

7.1.1 Atomic Force Microscopy

Exhaustive explanations of AFM and its application are available in the literature (Smith *et al.* 1996; Fang *et al.* 2005). Typically, AFM is carried out to investigate the correct surface allocation of ions during superconductors preparation (Staszczuk *et al.* 2006). However, a lot of researches have been performed by using AFM to monitor the change in the surface features of the same material after different treatments (Jones *et al.* 2005). The atomic force microscope used in this study is the EasyScan 2 (Nanosurf AG, Switzerland). The representative model is showed in Figure 7.1.

Fig. 7.1 - EasyScan 2 (Nanosurf AG, Switzerland)

The Atomic Force Microscopy (AFM) allows studying the surface topography and roughness. The core of the microscope is constituted by a cantilever, which has a typical length of about 100-400 µm, and the probe, also called tip (Fig. 7.2). The tip long and often less than 100 Å in diameter is located at free end of the cantilever.

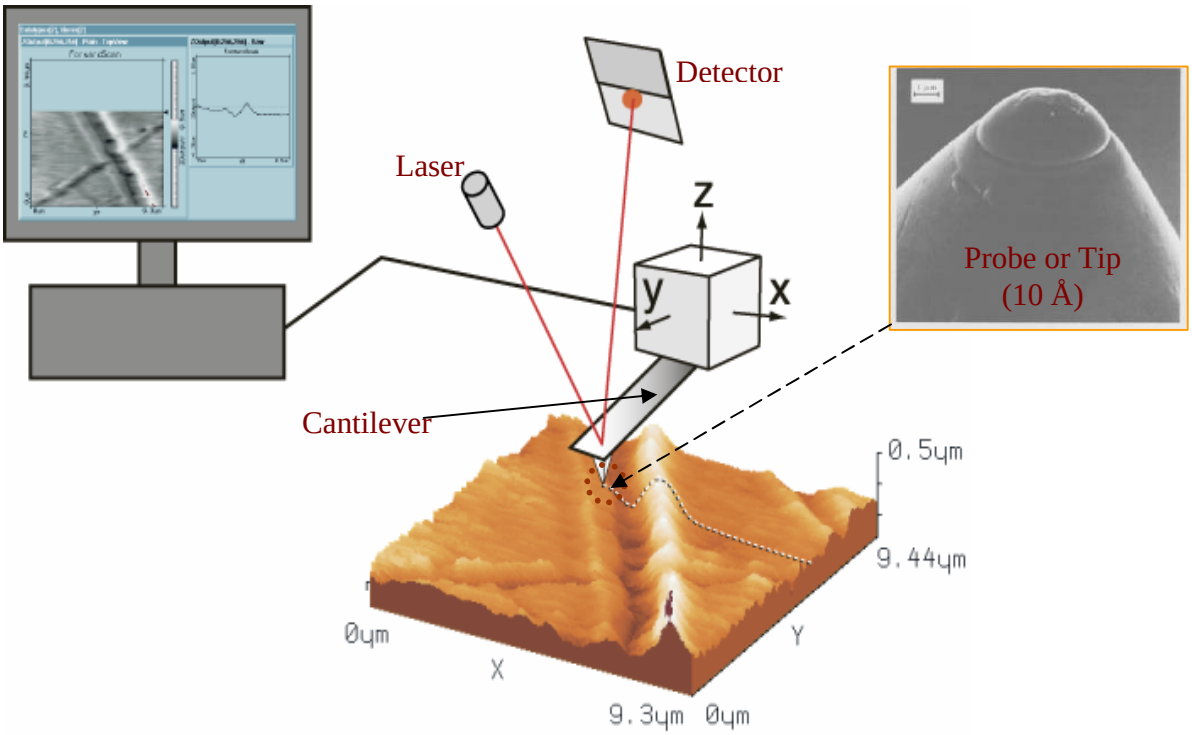


Fig. 7.2 – Schematic representation of an AFM scanning

The cantilever and the probe move on the surface of the sample in the *x* direction until all the surface is scanned in the *y* direction. Contemporaneously, they deflects in *z* direction according to the surface shape. The probe motion is determined by the forces between the probe and the sample: such forces identified with Van der Waals interaction. The bends and deflections of the cantilever are detected by a laser ray which is reflected to a special detector amplified by an optical lever system combined with a photodetector. Finally, the signal is transferred to the PC and a specific software processes the data. Each scanning line produces just one profile characterized by a bearing area and by peaks and valleys. Finally, the signal is transferred to the specific software. All the profiles registered in *x* direction are then assembled and the software creates bi-dimensional and three-dimensional topographies (Fig. 7.3).

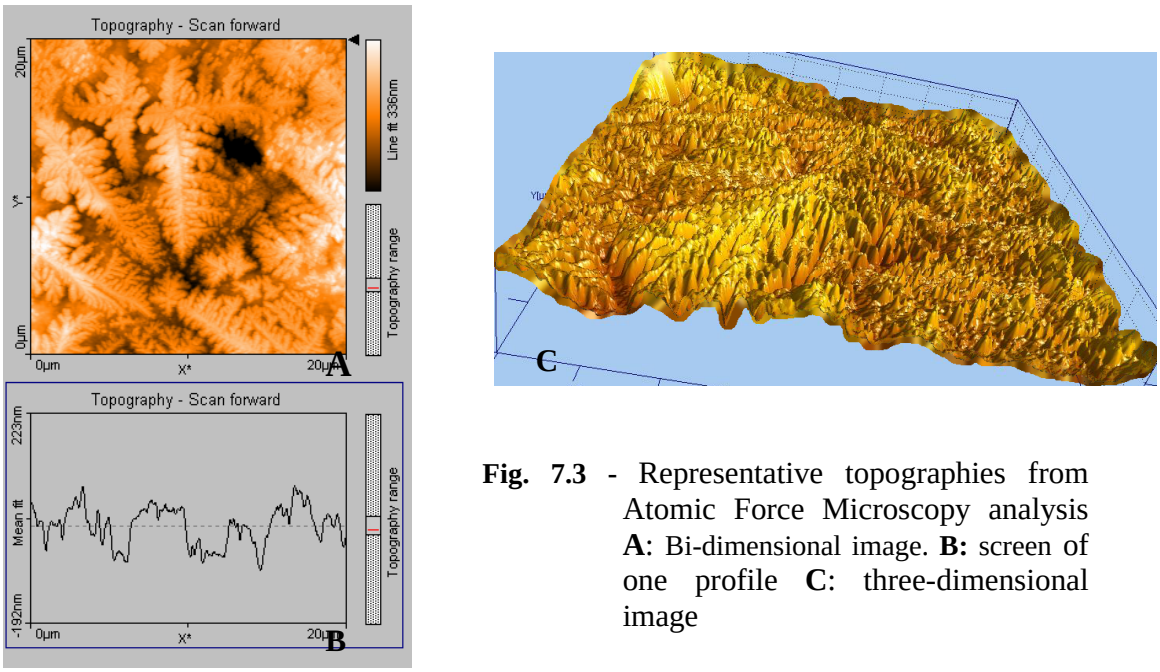


Fig. 7.3 - Representative topographies from Atomic Force Microscopy analysis **A**: Bi-dimensional image. **B:** screen of one profile **C**: three-dimensional image

Additionally, AFMs require no specific sample preparation procedure: measurements can be operated in environmental condition and provides information about the sample surface in a non destructive way. With the advent of Atomic Force Microscopy, new possibilities are opened to evaluate the features of polymers, natural polymers and biopolymers. New developments see application of AFM to investigate mechanical properties of edible films and biomaterials (Herrmann *et al.* 2004; Puskas *et al.* 2003). The instrument is suitable to monitor changes on surface topography of a sample after different treatments (Smith *et al.* 1996; Jones *et al.* 2005). Furthermore, AFM is often applied to characterize differences in the properties of individual components of heterogeneous materials, is also useful for compositional mapping in polymer blends and copolymers, and for heterogeneity mapping in polymer coatings (Magonov *et al.* 1997; Cleveland, *et al.* 1998). Because of its high resolution in describing surface topography, AFM may help explaining the adhesion mechanisms of different antimicrobials used to activate plastic films by coating. However, no literature is available on the possible use of AFM to study the surface topography of plastic films activated with bacteriocins. Therefore the aim of this

study was to investigate the interaction between three bacteriocins and five different polyethylene films for food packaging by using AFM.

7.2 MATERIALS AND METHODS

7.2.1 Bacterial strains

Listeria monocytogenes V7 was used as indicator strain of bacteriocin solutions and to reveal the antimicrobial packaging activity.

7.2.2 Preparation and activity of bacteriocin solutions

A partial purified preparation of both bacteriocins, named Bac162W and BacAM09, was obtained as previously described in chapter 2 with an antimicrobial activity against *Listeria monocytogenes* V7 of 102400 and 56200 AU/ml, respectively. A nisin antimicrobial solution (NIS) used to activate plastic films was prepared as reported in chapter 6. The antimicrobial concentration of NIS solution was determined by an agar diffusion and critical dilution assay as previously described in chapter 2. Solutions of Bac162W, BacAM09 and NIS were prepared in 70% isopropanol at a final concentration of 6400 AU/ml against *Listeria monocytogenes* V7 and used for plastic film activation experiments.

7.2.3 Characteristics of plastic films

In this study five different commercial Linear Low Density Polyethylene films, differing in vinyl acetate ethylene (EVA) and erucamide contents (Table 7.1), were used for the coating

treatment with bacteriocin solutions. The films underwent a Corona treatment (Jones *et al.* 2005) before the bacteriocin coating.

Table 7.1 - Composition of the five Linear Low Density Polyethylene films used in this study.

Plastic film	EVA[a] (%)	Erucamide (ppm)
A	5	500
B	0	500
C	0	600
D	3	550
E	0	500

[a]EVA: Ethylene Vinyl Acetate

7.2.4 Preparation of antimicrobial plastic films and their activity

All bacteriocin solutions were spread manually onto one side of the film using a coating rod (Mauriello *et al.* 2005). Then, the plastic films were dried by exposure to warm air for the solvent removal and assayed for antimicrobial activity against *L. monocytogenes* V7. Samples of activated film were placed in the bottom of an empty Petri dish, then overlaid with Tryptone Soya Broth (Oxoid) added of 0.75% agar and seeded with an overnight culture of the indicator strain. After the incubation at 30°C for 24 h, the antimicrobial activity was observed as an inhibition zone of the indicator organism around the plastic material. Untreated film samples were assayed as control.

7.2.5 Surface characterization by AFM: calculation of surface roughness

The atomic force microscope EasyScan 2 (Nanosurf AG, Switzerland) was used in this study (Figure 7.1). Measurements were carried out in dynamic force no-contact mode; scan size was set

to 20 x 20 μm^2, with a 1024 x 1024 pixels resolution and a 20 μm/sec scan speed. Scan force was kept low (<0.10 nN), ensuring a complete absence of deformations of the measured films. Samples of treated and control films were analysed on ten different positions, randomly selected all over the sample. The output results of the AFM analysis were further analysed by SPIP (Scanning Probe Image Processor, Image Metrology A/S, Lyngby - Denmark). A simple and repeatable quantitative approach, widely applied for characterization of surface functionalities is the study of surface roughness. In particular, the attention was focused on analysis of these roughness parameters that best describe the smoothness of a surface and the dimension of the exchange area at the interface. These are: the mean roughness (Sa) and the surface area ratio (Sdr) (Blunt *et al.* 2000). Mean roughness Sa (Equation 1) is expressed in nm and is defined as the arithmetic average of the deviation from the median plane; surface area ratio Sdr (Equation 3), expressed as a percentage, gives the ratio between actual surface area and projected area on a flat horizontal plane.

$$Sa = \frac{\int_{y=0}^{y_r} \int_{x=0}^{x_r} \left| z(x,y) - \mu \right| dxdy}{x_r \cdot y_r} \tag{1}$$

with

$$\mu = \frac{\int_{y=0}^{y_r} \int_{x=0}^{x_r} z(x,y)\, dxdy}{x_r \cdot y_r} \tag{2}$$

$$Sdr = \frac{\int_{y=0}^{y_r} \int_{x=0}^{x_r} \left(z(x,y) \cdot \sqrt{1 + \left(\frac{dz}{dx}\right)^2 + \left(\frac{dz}{dy}\right)^2} - 1 \right) dxdy}{x_r \cdot y_r} \tag{3}$$

where:

- a *x,y,z* coordinate system is taken as reference for the measurement, with the origin ($x = 0$, $y = 0$) coincident with the beginning of the scan (bottom left corner of the measured topographies);
- z is the vertical elevation of the surface measured by the AFM in the position (x,y);
- x_r and y_r are the measured scan sizes, respectively in x and y direction;
- μ is the mean value, as reported in the Equation 2.

7.3 RESULTS

7.3.1 Antimicrobial activity of the bacteriocin solutions

The results of the antimicrobial activity of the bacteriocins used in this study are reported in Table 7.2. The bacteriocins were shown to be active against strains of *Listeria* spp. and *Lactobacillus* spp. (Table 7.2). In addition, Bac162W was also active against *Brochothrix thermosphacta* 7R2 and *Enterococcus faecalis* 227. By contrast, nisin antimicrobial solution (NIS) with lactic acid and EDTA showed antimicrobial activity against all the indicator strains tested. The best activity was obtained against *Brochothrix thermosphacta* 7R2.

Table 7.2 Antimicrobial spectrum of bacteriocins produced by *L. curvatus* 162W and *L. plantarum* AM09 and the NIS solution

Indicator strains	Source	Growth conditions	Antimicrobial activity of bacteriocins (AU/ml)		
			162W	AM09	NIS
Staphylococcus aureus DSM 20231	DSM	[a]TSB 24h at 37°C	0	0	6400
Listeria monocytogenes V7	Carminati	TSB 24h at 30°C	102400	56200	102400
Listeria monocytogenes CAL	Carminati	TSB 24h at 30°C	12800	12800	102400
Listeria monocytogenes ATCC 7644	ATCC	TSB 24h at 30°C	12800	12800	102400
Listeria welshmani 3Z	Meat	TSB 24h at 30°C	12800	12800	102400
Escherichia coli O157:H7 25	Meat	TSB 24h at 37°C	0	0	6400
Salmonella enterica serovar Thompson	Poultry	TSB 24h at 37°C	0	0	6400
Brochothrix thermosphacta 7R2	Meat	TSB 24h at 20°C	12800	0	204800
Pseudomonas sp. 6P2	Meat	TSB 24h at 30°C	0	0	6400
Lactobacillus sp. 3A	Meat	[b]MRS 24h at 30°C	12800	6400	6400
Enterococcus faecalis 227	NWC	TSB 24h at 30°C	6400	0	6400

[a]TSB: Tryptone Soya Broth (Oxoid, Milan - Italy) supplemented with 0.5% yeast extract.
[b]MRS: de Man Rogosa Sharp (Oxoid)

7.3.2 Antimicrobial activity of activated plastic films

The activation of the polyethylene films was performed with a coating rod using the three different bacteriocin solutions with concentration of 6400 AU/ml against *L. monocytogenes* V7. The films showed to be always active against *L. monocytogenes* V7 in agar inhibition assays (Fig. 7.4). However, some active films showed a homogeneous inhibition of the indicator strain in the agar assay, while other films displayed a spot-like antimicrobial activity. In Figure 7.4A, an

example is given of a homogeneous activity displayed by film A activated by the bacteriocin AM09 solution. In the cases of spot-like activity the inhibition was strictly confined in some points of contact between film and agar plate (Fig. 7.4B). Such activity was shown by films B, C, D and E activated with bacteriocins 162W and AM09. The NIS-treated films showed the most effective antimicrobial activity highlighted by a very clear inhibition area spread beyond the film perimeter (Fig. 7.4C). The untreated films did not show any antimicrobial activity (Fig. 7.4D).

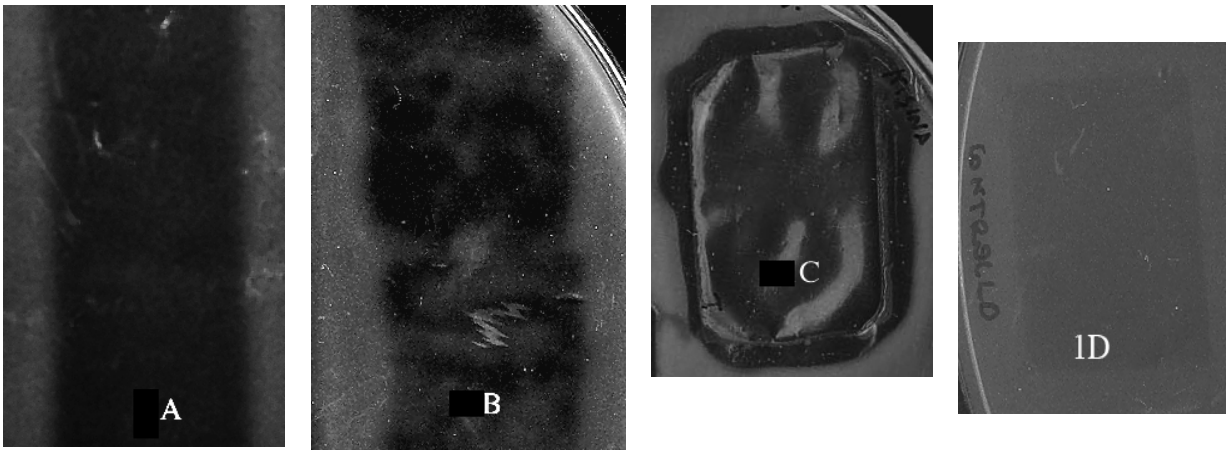


Fig. 7.4 - Antimicrobial activity of bacteriocin treated polyethylene films against *Listeria monocytogenes* V7. A, homogeneous inhibition of the indicator strain (film A activated by BacAM09); B, spot-like inhibition of the indicator strain (film D activated by BacAM09); C, inhibition beyond the film perimeter (Film A activated by NIS solution); 1D, untreated film.

7.3.3 Active film surface topography by AFM

Figure 7.5 shows representative images of the films surface with the scan size of 20x20 μm^2 for each sample. The distribution of bacteriocin solutions on the surface of the plastic films was investigated by AFM images. The control images evidenced the same topography surface in all the directions. By contrast, deposits of the bacteriocin solutions could be observed on the surface of the treated plastic films. However, the three antimicrobial solutions were not evenly distributed in the same way (Fig. 7.5).

As shown in Figure 7.5, the control samples displayed different topographies suggesting that their peculiar surface characteristics may influence the coating attitude of each film. All the bacteriocin solutions could be homogeneously spread on film A, which appeared as completely covered by surface layer after AFM analysis (Fig. 7.5). The NIS solution was coated as agglomerates of crystals upon the surface of the films probably due to the EDTA salt content. However, only on the film A the NIS solution was homogeneously distributed, while in films B to E the NIS distribution was uneven (Fig. 7.5). Overall, the Bac162W showed the most homogeneous distribution except for film B. As already pointed out, AFM can provide not only qualitative, but also quantitative characterization of interfaces. Therefore we tried to correlate functional behaviour of the analyzed films to topographic properties using the roughness parameters. Analyses and comparisons of the roughness parameters are presented in Figure 7.6; Sa (Fig. 7.6A) and Sdr (Fig. 7.6B) values are shown for every film-bacteriocin couple. All data processing was performed by means of SPIP™ software. Parameters were directly evaluated after a standard pre-processing operation, consisting of a first order profile levelling. Qualitative analyses performed on topographies were confirmed by quantitative analyses. Surface treated with NIS, showing a sort of micro-texturing, always gave the highest roughness values, in terms of both Sa and Sdr (Fig. 7.6). This was true for the different tested contents of EVA and erucamide (Table 7.2). For the NIS treated surface, Sdr values up to ten times higher compared to the other bacteriocin solutions were detected (Fig. 7.6B).

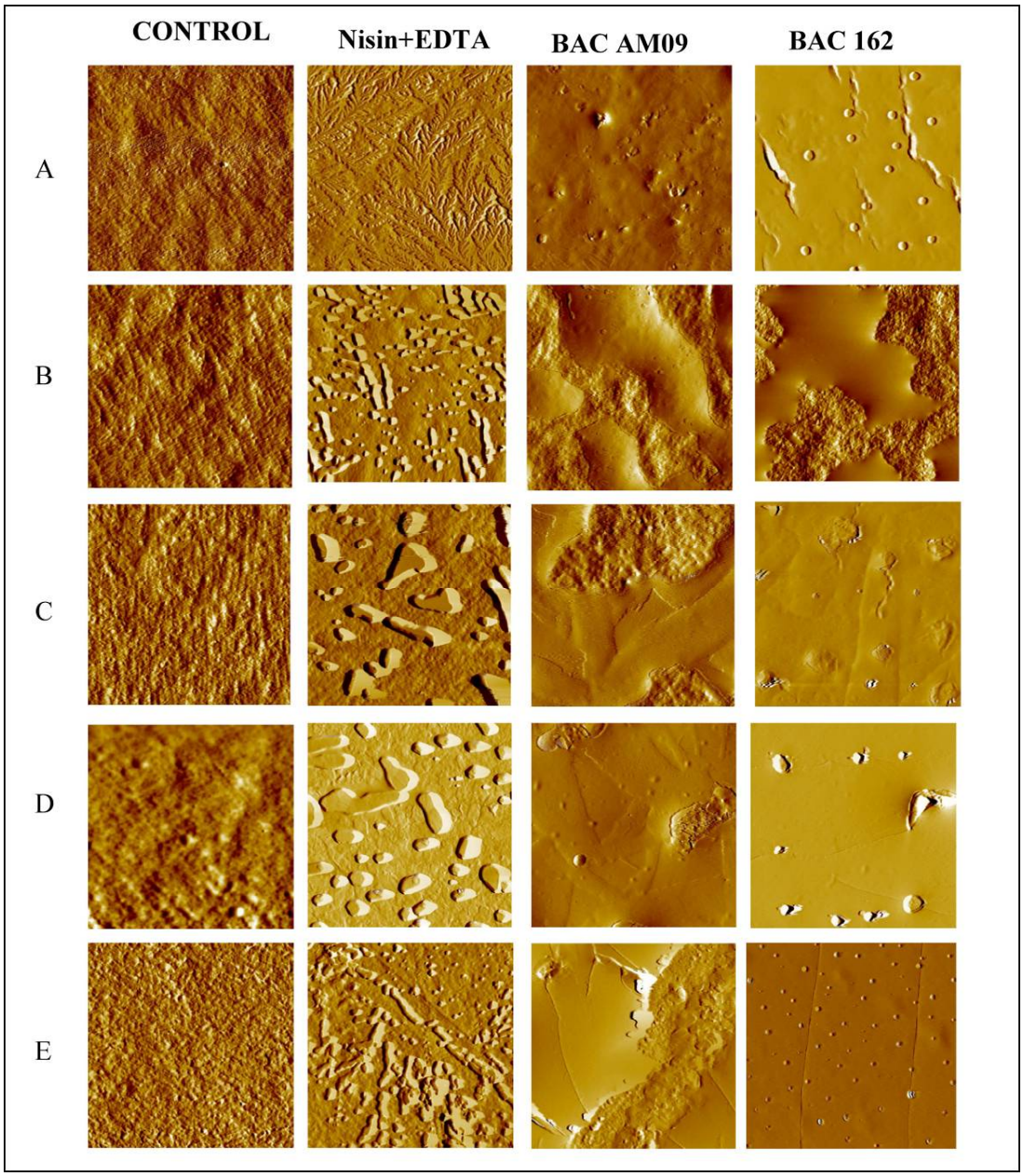


Fig. 7.5 - Representative images from Atomic Force Microscopy analysis of polyethylene films (20 x 20 μm^2) treated with different bacteriocin solutions.

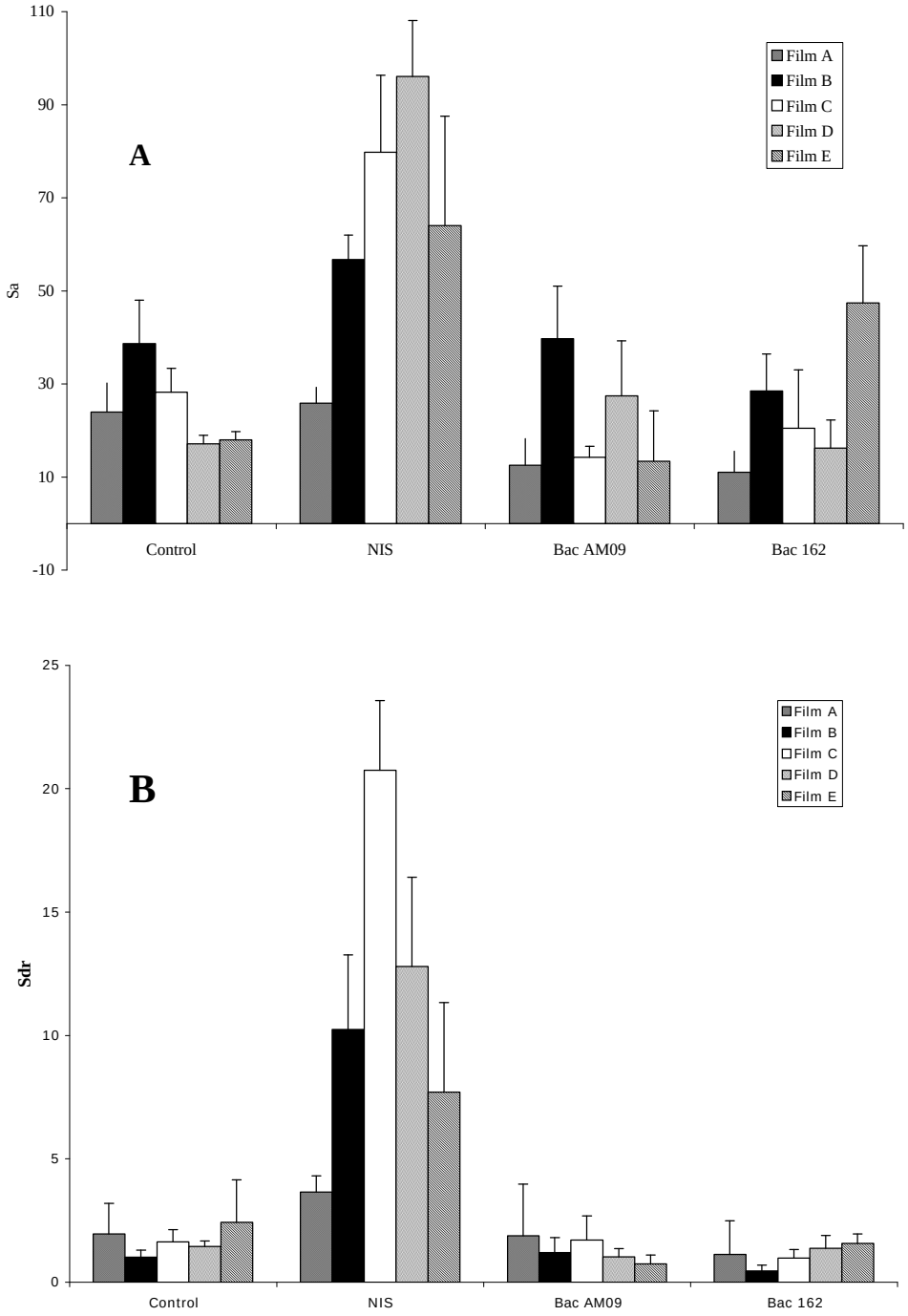


Fig. 7.6 - Mean roughness (Sa, panel A) and surface area ratio (Sdr, panel B) of different polyethylene films coated by three bacteriocin solutions.

7.4 DISCUSSION

In this study we prepared antimicrobial polyethylene films by using different bacteriocin preparations and evaluated the coating of the antimicrobial solutions on the surface of the plastic films by AFM. Furthermore, we carried out a roughness analysis on AFM images. The bacteriocin solutions used were active against most of the indicator strains used. However, the NIS solution showed the broader spectrum of activity. The usual antimicrobial activity of the nisin is exclusively exerted against Gram positive bacteria. However, nisin is often used in combination with other substances in order to make it active also against Gram negative bacteria (Gill *et al.* 2000; Ukuku *et al.* 2004; Samelis *et al.* 2005). In our case the antimicrobial activity of NIS solution may be related to the ability of EDTA to bind magnesium and other bivalent cations from the outer membrane of Gram negative bacteria destabilizing the lipopolysaccharide layer and producing cells with increased outer membrane permeability allowing the nisin to penetrate easily and kill the cells (Stevens *et al.* 1991).

All the developed antimicrobial films, activated by coating, proved to be active against *L. monocytogenes* V7. However, as clear from Figure 7.4, each couple film-bacteriocin showed a different behaviour in inhibiting the indicator strain in the antimicrobial tests performed in agar plates. We had three different behaviours: a homogeneous, spot-like and expanded distribution of the antimicrobial activity of the plastic films (Fig. 7.4 panels A, B and C, respectively).

Overall, all the activated Film A (with the highest EVA content) showed the best antimicrobial performances as well as all the film types treated with the NIS solution. Interestingly, although all the bacteriocin solutions were employed at the same concentration (6400AU/ml) the NIS-treated films showed the most effective antimicrobial activity highlighted by a very clear inhibition area spread beyond the film perimeter (Fig. 7.4C). This behaviour may be due to the different interaction between film and bacteriocin solutions and to the mechanisms of release of the bacteriocin from the plastic surface. A similar result was

already observed in one of our previous researches when different methods for film activation with bacteriocins were used leading to different results in terms of size and diffusion of the antimicrobial in the agar (Mauriello *et al.* 2004). Differences were also observed comparing the antimicrobial activity of the five different films. In particular, the film A displayed a good performance with all the three bacteriocin solutions used.

AFM images were used to evaluate the morphology and the roughness of the surface of antimicrobial polyethylene films activated with bacteriocin solutions, providing qualitative as well as quantitative information.

As shown in Figure 7.5, the control samples displayed different topographies suggesting that their peculiar surface characteristics may influence the coating attitude of each film. In fact, the surface topography of the activated plastic films resulted very different depending on film and bacteriocin solution used. Such evidence was appreciated by both surface images (Fig. 7.5) and by the evaluation of the roughness parameters (Fig. 7.6).

The film A gave the most homogeneous distribution of the bacteriocin solutions, related to a homogeneous appearance of the antimicrobial activity in agar plates. The NIS solution also gave a satisfactory antimicrobial performance in all the plastic films associated to a considerable increase of the surface roughness. In fact, for the NIS treated surfaces, Sdr values were up to ten times higher compared to the other bacteriocin solutions.

This means that the exchange area at the interface for the NIS treated surface is up to ten times the one available in the case of the same films activated with the other bacteriocin solutions. This is in agreement with the crystallized structure of the spread NIS solution on film A evidenced in Figure 7.5; the presence of the agglomerates increased the spread surface with a consequent increase of Sdr values compared to the smooth covering layers obtained with the other bacteriocins. This may be relevant; in fact a larger interface area is normally synonymous of higher chemical reactivity. Furthermore, the presence of agglomerates with dimension in the order of a few microns could be noticed. The role and the behaviour of such

agglomerates are to be further investigated; their presence may provide a sort of slow release deposit protecting the surface for a long time. A slight growth in the dimensions of the agglomerates was also observed for increased contents of erucamide, corresponding to higher values of Sa and Sdr. For the bacteriocin solutions BacAM09 and Bac162W, differences were much more limited. Sensibly lower values of Sa and Sdr are symptomatic of a flatter surface, with a reduced area at the interface. Such topography can explain the lower antimicrobial activity of the BacAM09 and Bac162W activated films. As above pointed out, Bac162 generally seems to give a more homogeneous surface topography (Fig. 7.5). However, this was not confirmed or put in evidence by the roughness analysis. This results seem to be reasonable and in agreement with the similar behaviour of the two bacteriocin solutions.

As a matter of fact, specific couples film-bacteriocin showed different interactions leading to different results in film topography after coating and differences in the antimicrobial activities. Although all the films displayed the antimicrobial activity against the indicator strain, the higher EVA content of the film A seems to cause a homogeneous distribution of the antimicrobial solutions with a consequent higher antimicrobial efficacy. Moreover, the NIS solution, though interacting differently with the films used, gave the highest roughness values associated with clear and homogeneous inhibition halos of the activated films in the antimicrobial assays.

The AFM analysis proved to be useful to investigate the coating attitude of polyethylene films with different bacteriocin solutions and to correlate the surface topography to the antimicrobial activities. However, further studies are necessary to understand the chemical interactions between films and bacteriocin solutions in order to predict and improve the release of the antimicrobials from the packages.

Significant differences were found between the bacteriocin activated and control films and the activated surfaces showed lower values of average roughness and surface area ratio. A homogeneous distribution of the bacteriocin preparation could not always be obtained

following the coating procedure. This result was dependent on the bacteriocin used and its distribution on the different plastic films. Information on the interactions between plastic film and antimicrobial preparations could improve the production and implementation of bacteriocin activated food packages.

7.5 REFERENCES

- **Blunt, L. and Stout, K.J.** (2000) Three-Dimensional Surface Topography.
- **Cha, D.S., Cooksey, K., Chinnan, M.S. and Park, H.J**. (2003) Release of nisin from various heat-pressed and cast films. *Lebensmittel Wissenschaft und Technology Food Science and Technology* 36, 209-213.
- **Cleveland, J.P.; Anczykowski, B.; Schmid, A.E.; Elings, V.B.,** (1998) Energy Dissipation in Tapping-Mode Atomic Force Microscopy. *Applied Physical Letters* **72,** 2613-2615.
- **Ercolini, D., La Storia, A., Villani, F. and Mauriello, G.** (2006) Effect of a bacteriocin-activated polythene film on *Listeria monocytogenes* as evaluated by viable staining and epifluorescence microscopy. *Applied Microbiology* **100**, 765-768.
- **Fang, T.E., Chang, W.J. and Weng, C.I.** (2005) Surface analysis of nanomachined films using atomic force microscopy. *Materials Chemistry and Physics* **92**, 379-383.
- **Gill, A.O. and Holley, R.A.** (2000) Surface application of lysozyme, nisin and EDTA to inhibit spoilage and pathogenic bacteria on ham and bologna. *Journal of Food Protection* **63**,1338-1346.
- **Grower, J.L., Cooksey, K. and Getty, K.J.K.** (2004) Development and characterization of an antimicrobial packaging film coating containing nisin for inhibition of *Listeria monocytogenes*. *Journal of Food Protection* **67**, 475-479.
- **Grower, J.L., Cooksey, K. and Getty, K.J.K.** (2004) Release of nisin from methylcellulose-hydroxypropyl methylcellulose film formed on low-density polyethylene film. *Journal of Food Science* **69**, 107-111.
- **Han, J.H.** (2000) Antimicrobial food packaging. *Food Technology* 54, 56-65.
- **Herrmann, P.S.P., Yoshida P.,M.,C., Antunes, A.J.A., and Marcondes J.A.** (2004) Surface evaluation of whey protein films by atomic force microscopy and water vapour permeability analysis. *Packaging Technology and Science* **17**, 267-273.
- **Jones, V., Strobel, M. and Prokosch, J.M.** (2005) Development of Poly(propylene) surface topography during Corona treatment. *Plasma Processing Polymers* **2**, 247-553.
- **Kim, Y.M., An, D.S., Park, H.J., Park, J.M. and Lee, D.S.** (2002) Properties of nisin-incorporated polymer coatings as antimicrobial packaging materials. *Packaging Technology and Science* **15**, 247-254.

- **Lee, C.H., An, D.S., Lee, S.C., Park, H.J. and Lee, D.S.** (2004) A coating for use as an antimicrobial and antioxidative packaging materials incorporating nisin and α-tocopherol. *Journal of Food Engineering* **62**, 323-329.
- **Lee, C.H., An, D.S., Park, H.J. and Lee, D.S.** (2003) Wide-spectrum antimicrobial packaging materials incorporating nisin and chitosan in the coating. *Packaging Technology and Science* **16**, 99-106.
- **Magonov, S.N., Reneker, D.H.** (1997) Characterization of Polymer Surfaces with Atomic Force Microscopy. *Annual Reviews Material Science* **27**, 175-222.
- **Mauriello, G., De Luca, E., La Storia, A., Villani F. and Ercolini, D.** (2005) Antimicrobial activity of a nisin-activated plastic film for food packaging. *Letters in Applied Microbiology* **41**, 464–469.
- **Mauriello, G., Ercolini, D., La Storia, A., Casaburi, A. and Villani, F.** (2004) Development of polyethylene films for food packaging activated with an antilisterial bacteriocin from *Lactobacillus curvatus* 32Y. *Journal of Applied Microbiology* **97**, 314–322.
- **Puskas J.E. Chen, Y., Prince A., KwonY., Kovar M., Harbottle, R.R., De Jong, K. Norton, P.R., Cadieux, P., Burton, J., Reid, G., Beiko, D., Watterson, J.D., and Denstedt, J.** (2003). Atomic Force Microscopic and Encrustation Studies of Novel Prospective Polyisobutylene-Based Thermoplastic Elastomeric Biomaterials. *Polymers for Advanced Technology* **14**, 763–770.
- **Samelis, J., Bedie, G.K., Sofos, J.N., Belk, K.E., Scanga, J.A. and Smith, G.C.** (2005) Combinations of nisin with organic acids or salts to control *Listeria monocytogenes* on sliced pork bologna stored at 4 degrees C in vacuum packages. *Lebensmittel Wissenschaft und Technologie Food Science and Technology* **38**, 21-28.
- **Smith, P.F., Chun, I., Liu, G., Dimitrievich, D., Rasburn, J. and Vancso, G.J.** (1996) Studies of optical haze and surface morphology of blown polyethylene films using atomic force microscopy. *Polymer Engineering and Science* **36**, 2129-2135.
- **Staszczuk, P., Sternik, D., Chadzynski, G.W., Robens, E. and Blachnio, M.** (2006) Studies of heterogeneity properties of selected high-temperature superconductor surfaces. *Journal of Thermal Analysis Calorimetry* **86**, 133-136.
- **Stevens, K.A., Sheldon, B.W., Klapes, N.A. and Klaenhammer, T.R.** (1991) Nisin treatment for inactivation of *Salmonella* species and other gram-negative bacteria. *Applied and Environmental Microbiology* **57**, 3613-3615.

- **Ukuku, D.O. and Fett, W.F.** (2004) Effect of nisin in combination with EDTA, sodium lactate, and potassium sorbate for reducing *Salmonella* on whole and fresh-cut cantaloup. *Journal of Food Protection* **67**, 2143-2150.
- **Vartiainen, J. and Shytti, F.** (2004) Properties of antimicrobial plastics containing traditional food preservatives. *Packaging Technology and Science* **16**, 223-229.
- **Villani, F., Pepe, O., Mauriello, G., Salzano, G., Moschetti, G. and Coppola, S.** (1994) Antimicrobial activity of *Staphylococcus xylosus* from Italian sausage against *Listeria monocytogenes. Letters in Applied Microbiology* **18**, 159–161.
- **Villani, F., Salzano, G., Sorrentino, E., Pepe, O., Marino, P. and Coppola, S.** (1993) Enterocin 226NWC, a bacteriocin produced by *Enterococcus faecalis* 226, active against *Listeria monocytogenes*. *Journal Applied Bacteriology* **74**, 380-387.