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Contrôle de la croissance microbienne par une combinaison de nisine, de lysozyme et d'acide lactique : Application à l'emballage actif

Microbiological growth control by nisin, lysozyme and lactic acid combination: Application to active packaging

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Chapter I: Literature Review

Chapter II: Materials and methods

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INTRODUCTION

Food and packaging are closely related and depend themselves. Many chemical, physical reactions exist between a food, its packages and the environment, which alter the composition, quality and physical properties of the food and/or the package. These studies about interactions have increased during recent years, as consequence of higher demands on food quality protection by packaging and rapid development of new packaging materials or technologies (Hotchkiss 1995b).

Novel and advanced polymeric material are being developed for enhanced food packaging. The development of these materials is based on conventional polymers, as well as newer technologies including biopolymers, nanotechnology and nanocomposites, active, antimicrobial intelligent, and packaging (Bugusu and Bryant 2006, Mahalik *et al.* 2010)

Food preservation is closely related with microbiological quality (Bureau 1985). Food spoilage may occur at any stages between the acquisition of raw materials and the consumption of a food product. These stages can include processing, packaging, distribution, retail display, transport, storage and use by the consumer. They are under varying degrees of control, with the aim of delivering the satisfactory shelf-life and finally-consumed product of high quality. Spoilage is characterized by any change in a food product that renders it unacceptable to the consumer from a sensory or health point of view. This may be physical damage, chemical changes (oxidation, colour changes) or appearance of off-flavours and off-odours resulting from microbial growth and metabolism in the product (Boddy and Wimpenny 1992, Gram *et al.* 2002).

Microbial growth on food surfaces is a major cause of food spoilage and bacterial contamination of dairy, meat or ready-to-eat products and moulds decay in fruits and vegetables. Attempts have been made to improve safety and to delay spoilage by using antimicrobial sprays or dips (Torres *et al.* 1995). However direct surface applications onto food have limited benefits, because the active substance can be neutralized on contact with food or diffuse rapidly from the surface into food mass (Padgett *et al.* 1998). An alternative is the use of antimicrobial packaging, and it is a promising form of active packaging. Antimicrobial packaging could be more efficient than direct surface application by controlling migration of antimicrobial agents from packaging material to the product surface (Suppakul *et al.* 2003).

Thanks to active or antimicrobial packaging, food product can be distributed over a wide geographical area over a long period of time without unacceptable loss quality and within economical constraints (Sonneveld 2000).

The present thesis is focused on the improvement of paper wrapping materials by adding active antimicrobial agent in the packaging structure to ensure the high quality of the packaging material and a sanitizing effect on food product. Nisin is the only active component allowed for food contact through all Europe. It is efficient to reduce Gram positive bacteria. This molecule is one of the active components studied in this task. Nevertheless, other active components, such as lysozyme and lactic acid can be combined with nisin to extend the microbial quality of food. Several combinations and interaction between active components ratio are to study and then define the most appropriate active system.

The first section presents the bibliographical information about antimicrobial agent and active packaging systems used in food packaging. The main objectives of bibliographical review were to select active components ratio exist and the ration between inhibitors have to be studied in order to ensure food hygiene and determine packaging concepts able to ensure good antimicrobial activity for food.

The materials and methods of this study are presented in second chapter. The results section is presented in third part and is composed of four parts.

First part, antibacterial activity of nisin and lysozyme, used alone or in combination was studied against some *Bacillus* ssp. The objective of the second part was to examine the antimicrobial activity of nisin, lysozyme and lactic acid alone or in combination against *Listeria monocytogenes* CIP 82.110 and *Staphylococcus aureus* CIP 4.83. Third the optimized mixture of nisin, lysozyme and lactic acid was incorporated onto paper packaging. The purpose of the last part was to study diffusion of nisin incorporated in a cellulose matrix.

LITERATURE REVIEW

I. LITERATURE REVIEW

I. ANTIMICROBIAL AGENTS

Antimicrobial agents are components that hinder growth of microorganisms. Some of these compounds are called food preservatives. According to the definition used by the Commission of the European Communities, preservatives are substances which extend the shelf life of foodstuffs by protecting them against deterioration caused by microorganisms (Directive 95/2/EC). Similar rules were applied in the USA, where FDA defines preservatives as any chemical that when added to food tends to prevent or retard deterioration. It does not include common salt, sugars, vinegar, spices or oils extracted from spices, substances added to food by direct exposure such as wood smoke, or chemicals applied for their insecticidal or herbicidal properties (FDA, Code of Federal Regulations: 21 CFR 172, 2000). Antimicrobials are used in food to control natural spoilage and to prevent or control growth of microorganisms, including pathogenic microorganisms (Burt 2004).

Natural antimicrobials can be defined as substances produced by living organisms in their fight with other organisms for space and their competition for nutrients. The main sources of these compounds are plants (secondary metabolites in essential oils and phytoalexins), microorganisms (bacteriocins and organic acids) and animals (lysozyme from eggs, lactoferrins from milk). Across the various sources the same types of active compounds can be encountered, e.g. enzymes, peptides and organic acids (Meyer *et al.* 2002). Reducing the need for antibiotics, controlling microbial contamination in food, improving shelf-life extension technologies to eliminate undesirable pathogens, delaying microbial spoilage, decreasing the development of antibiotic resistance by pathogenic microorganisms or strengthening immune cells in humans are some of the benefits (Tajkarimi *et al.* 2010). Most of approved food antimicrobials have limited application due to pH or food component interactions. Most food antimicrobials are amphiphilic and they can solubilize or be bound by lipids or hydrophobic proteins in foods making them less available to inhibit microorganisms in the food product (Davidson and Zivanovic 2000).

1. Organic acid

The commercially most important preservatives are still the organic acids **(Table 1)**. They are all naturally occurring, although the bulk amount of these substances used in foods are synthetically produced. Some acids, especially benzoic and sorbic, are very effective

inhibitors of microbial growth (Dziezak 1986). Other acids including: acetic fumaric, propionic and lactic are added to foods to prevent or delay the growth of pathogenic or spoilage bacteria (Dziezak 1986, Greer and Dilts 1995, Podolak *et al*. 1996).

Name	Target microorganisms	pKa	Concentration	Application
acetic acid	*Bacillus, Clostridium, Listeria monocytogenes, Staphylococcus aureus* and *Salmonella.* some strains *Aspergillus, Penicilium Rhizopus,Saccharomyces*	4.75	0.1 to 0.4% 0.05 to 0.1%	baked goods, cheese, condiments and relishe, dairy product analogues, fats and oils, gravies and sauces, meats,
benzoic acid	*Bacillus cereus, Listeria monocytogenes. Staphylococcus aureus*, and Vibrio parahaemolyticus	4.19	1000- 2000 µg/ml	beverages, syrups, cider, margarine, olives, relishes, soy sauce, jams, jellies, preserves, pie and pastry fillings, fruit salads, salad dressings and in the storage of vegetables
	Byssochlamys nivea, Pichia membranaefaciens, Talaromyces flavus		20-700 µg/ml	
propionic acid	*E. coli. Staphylococcus aureus, Sarcina lutea Salmonella, Proteus vulgaris, Lactobacillus plantarum.* and *Listeria monocytogenes*	4.87	0.1 to 5.0%	baked goods and cheeses
sorbic acid	*Acinetobacter, Aeromonas, Bacillus, Campylobacter, Clostridium, Escherichia, Lactobacillus, Listeria, Pseudomonas, Salmonella, Staphylococcus, Vibrio, Yersinia Byssochlamys, Candida, Saccharomyces, Zygosaccharomyces Aspergillus, Fusarium,* and *Penicillium,*	4.75	0.5 to 1% 0.3 to 0.5%	beverages, jams, jellies, preserves, margarine, chocolate syrup, salads, dried fruits, dry sausages, salted and smoked fish, cheeses, and various lactic acid fermentations

Table 1. Inhibitory action of some organic acids on some pathogenic bacteria (Long and Barker 1999).

The antibacterial effectiveness of organic acids is thought to stem from the fact that protonated acids are membrane soluble, and can enter the cytoplasm by simple diffusion (Lambert and Stratford 1999, Ricke 2003). If the rate of intracellular proton release exceeds cytoplasmic buffering capacity or the capability of proton efflux system internal pH begins to fall and cellular functions are eventually inhibited (Booth 1985). Indeed, the antibacterial action of organic acids has been ascribed to cytoplasmic acidification from proton release, and subsequent inhibition of acid sensitive enzymes such as those involved in glycolysis (Davidson 2001).

Organic acids are usually added as sodium, potassium or calcium salts because they are more soluble in water.

Organic acids are easily applied by wash, spray, or dip to decontaminate surfaces of fresh produce and meats, while the salts of organic acids are simply included in product formulations to prevent outgrowth of pathogens in a variety of ready-to-eat (RTE) foods (Carpenter and Broadbent 2009). Acidification of foods with short-chain organic acids, either by fermentation or by deliberate addition, is an important and widespread mechanism for controlling food-borne pathogens in a variety of foods (Barker and Park 2001).

1.1 Benzoic and sorbic acid

Benzoic acid was first described as a preservative in 1875. The benzoic acid occurs naturally in high amounts in cranberries and some other fruits and spices. Sodium benzoate has a pKa value of 4.19, which gives optimal activity below pH 4.0. Benzoates have an advantage of low cost compared to other preservatives. Sorbic, benzoic and propionic acid show antimicrobial activity only when they are present as undissociated acids. The efficiency of these acids depends on the dissociation constant, pKa. As the pKa of most acids is between 3 and 5, these preservatives are only active at lower pH-values (Skirdal and Eklund 1993). Sorbic acid is an unsaturated fatty acid with a high pKa value 4.76, therefore it is applied in a great variety of food products. This acid is active against yeasts, moulds and many bacteria. Microorganisms and moulds resistant to sorbate are able to degrade sorbate, producing strong off-flavours by the decarboxylation of sorbic acid into *trans*-1, 3- pentadiene (Liewen and Marth 1985, Kinderlerer and Hatton 1990).

1.2 Lactic acid

The lactic acid and lactate are used in food industry for the following properties: the acidification potential of lactic acid, pH regulation, property of sodium and potassium lactate, and antimicrobial activity (Bogaert and Naidu 2000). Lactic acid is the main metabolic end-product of lactic acid bacteria during fermentation of a variety of food products and beverages, and plays a key role for flavour and texture, shelf-life and safety of the products. The produced isomer depends on the bacterial species. For example, *Lactococcus* and *Carnobacterium* produce L-lactic acid, while *Leuconostoc* produces D-lactic acid (**Figure 1**) (Liu 2003). Lactic acid is a weak organic acid (pK_a 3.85 at 25ºC) and one of smallest molecules (90.80) with an asymmetrical carbon in location α of the carboxylic function (Bogaert and Naidu 2000).

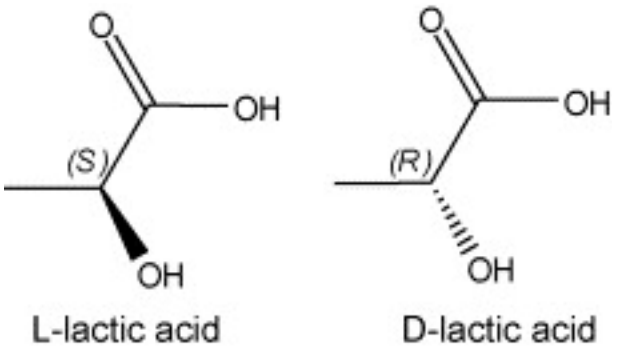


Figure 1. Chemical structure of L(+) and D(-) isomers of lactic acid (Norton *et al.* 2007).

The lactic acid lower the pH of the food and thus add stress to the microorganism, or in the undissociated form migrate through the cell membrane into the cytoplasm of the microorganism where they dissociate and lower the internal pH of the cell (Meyer *et al.* 2002). Lactic acid elicits antibacterial effects on a variety of microorganism. Lactic acid is an excellent inhibitor of spore forming bacteria at pH 5.0, but is ineffective against yeast and molds (Woolford 1975).

Lactates of calcium, potassium and sodium reduce potential risk of *Clostridium perfringens* (Juneja 2006, Reddy Velugoti *et al.* 2007) or *Clostridium botulinum* (Meng and Genigeorgis 1993) spore germination and outgrowth in meat product. Growth of *Listeria monocytogenes* and *Salmonella* species in meat decreased by using sodium lactate (Deumier and Collignan 2003) or with diacetate (Mbandi and Shelef 2002). *Listeria monocytogenes* is sensitive to the antibacterial activity of lactate (Nerbrink *et al.* 1999, Stekelenburg 2003) alone or in combination with others antimicrobial agents (Ye *et al.* 2008, Maks *et al.* 2010). Lactate salts at pH 5.5 prevent the anaerobic growth of *Serratia liquefaciens, Yersinia enterocolitica, Enterobacteur cloacae* and *Areomonas hydrophila* (Grau 1981).

Lactic acid is a hygroscopic, syrupy liquid with a moderately strong acid taste. Lactic acid is used in the manufacture of jams, jellies confectionery products and beverages. It is used to adjust acidity in brines for pickles and olives. Lactic acid spray has been effective in limiting microbial growth on meat carcasses and in poultry industry (Bogaert and Naidu 2000).

It is important to move the research to study the interactions of food matrix, bacteria, and organic acids. This could allow the food industry to expand the flexibility and effectiveness of organic acids as antibacterial agents to better ensure food safety (Carpenter and Broadbent 2009).

Currently, chemical preservatives are employed to limit the number of microorganisms capable of growing within foods, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to examine the possibility of using bacteriocins produced by lactic acid bacteria LAB as biopreservatives (Abee *et al.* 1995, Dykes 1995, Topisirovitz *et al.* 2008).

2. Bacteriocins

Bacteriocins are ribosomal synthesized antimicrobial peptides or proteins (Jack *et al.* 1995). Nowadays, the term bacteriocin is mostly used to describe the small, heat-stable cationic

peptides synthesised by Gram positive bacteria, namely lactic acid bacteria (LAB), which display a wider spectrum of inhibition (Cotter *et al.* 2005). The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation. They are generally recognised as safe substances, no active and non-toxic on eukaryotic cells become inactivated by digestive proteases and having little influence on the gut microbiota. Bacteriocins are usually pH and heat-tolerant, they have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria. They show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane. They not have cross resistance with antibiotics and their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (Galvez *et al.* 2007, Garcia *et al.* 2010b).

Bacteriocins comprise heterogeneous group regarding their primary structure, composition and physico-chemical properties and their classification is still a matter of debate (Heng and Tag 2006). A scheme has been recently proposed by Heng and Tag (2006), which involves from previous classification schemes and takes into account the nature of colicins (**Table 2**). Class I or lantibiotics include post-translationally modified peptides characterized by the distinctive thioether-based intermolecular rings of lanthionine and ß-methyl-lanthionine. Class II encompasses heat stable non modified peptides and is by far the largest class among Gram positive bacteriocins. In general, they are short cationic peptides with high isoelectric points and particular relevance for food biopreservation is the potent anti-*Listeria* activity display by the pediocin-like bacteriocins (Class IIa). Class III comprises large heat labile proteins with modest prospects as food biopreservatives. With the exception of colicin V and microcins, Gram negative bacteriocins fall in this class. Finally, circular peptides characterized by a peptide bond between the C- and N-terminus are clustered in class IV. Examples of bacteriocins **(Table 2)**, whose activity resides on the concerted action of two independent peptides are found in both classes I and II. Most lactic acid bacteria bacteriocins which have been applied in food biopreservation belong to Class I, IIa and IV (Klaenhammer 1993, Nes and Holo 2000, McAuliffe *et al.* 2001).

Table 2. Bacteriocins classification according to Heng and Tag (2006).

Class	General features	Produced by lactic acid bacteria
I-Lantibiotics	Modified, heat stable, <15 kDa	
Ia-Linear	Pore forming, cationic	Nisin, Lacticin 481, Plantaricin C
Ib-Globular	Enzyme inhibitors, no cationic	None
Ic-Multi-component	Two peptides	Lct3147, Plantaricin W
II-Unmodified peptides	Heat stable, <15 kDa	
IIa–Pediocin-like	anti-listeria, YGNGV consensus	Pediocin PA1/AcH, Enterocin A, Sakacin A
IIb-Miscellaneous	Non-pediocin-like	Enterocin B, L50, Carnobacteriocin A
IIc- Multi-component	Two peptides	Lactococcin G, Plantaricin S, Lactacin F
III-Large proteins	Heat labile, >30 kDa	
IIIa-Bacteriolytic	Cell wall degradation	Enterolysin A, Lcn972[a]
IIIb-Non-lytic	Cytosolic targets	Colicins[b] E2-E9
IV-Circular peptides	Heat stable, tail-head peptide bond	AS-48, Gassericin A, Acidocin B

[a] Lcn972 binds to the cell wall precursor lipid II and blocks cell wall biosynthesis, 15 kDa.
[b] Colicins are synthesised by *E. coli*.

Bacteriocins inhibit pathogenic and spoilage bacteria during food processing. Nisin and lacticin 3147, or pediocin, enterocin AS-48 have proven to be very effective against a wide range of spoilage and food-borne pathogens in several foodstuffs including dairy, meat and vegetable products.

Bacteriocins may be applied basically in two different approaches: *in situ* production by protective cultures, as an ingredient (fermentation of a bacteriocinogenic strain) or an additive in a semi- or purified preparation. Protective cultures, which do not contribute to the sensory attributes of food, have been mainly applied to enhance the hygienic quality of raw meat and fish products (Devlieghere *et al.* 2004). The use of bacteriocins as ingredients or additives requires new strategies for large scale production in suitable low-cost food-grade media. Besides food biopreservation, bacteriocins have been shown to accelerate cheese ripening by promoting the release of intracellular enzymes to the cheese matrix and a subsequent increase in the concentration of volatile and other sensorial compounds attributes of the matured cheese (Martínez-Cuesta *et al.* 2006).

2.1 Nisin

Nisin was discovered in England in 1928, when problems had been arisen in cheese making. The commercial development of the nisin preparation called Nisaplin was carried out by Aplin and Barrett in 1957. The commercial preparation, Nisaplin contains 2.5% of nisin, 74.4% sodium chloride, 23.8% denatured milk solids and 1.7% moisture (Delves–Broughton 1996,

Delves–Broughton *et al.* 2000, Deegan *et al.* 2006, Taylor *et al.* 2007). In 1969, the Food and Agriculture Organization (FAO/WHO) Expert Committee on Food Additives reviewed the toxicological data for nisin and recommend its use as a food preservative, with an acceptable daily intake (ADI) of 0.0825 mg kg^{-1} of body weight per day. Consequently it was admitted into the European food additive list, where it was assigned the number E 234 (Directive 83/463/ EEC). The activity of nisin is expressed in terms of International Units (IU) and 1 g of pure nisin is usually equivalent to 40×10^6 IU, while 1g of Nisaplin, a commercial nisin reference (Aplin and Barrett, UK), is equivalent to 1×10^6 IU (Davies *et al.* 1999).

2.2 Structure of nisin

Nisin is an antimicrobial peptide composed of 34 amino acid residues, with a molecular mass of 3.5 kDa and is classified as class Ia bacteriocins or lantibiotic produced by certain strains of *Lactococcus lactis* subsp. *lactis* (Hurst *et al.* 1981). It's contains unusual thioether amino acids: lanthionine (ALA-S-ALA) at the positions 3-7 and β-methyl lanthionine (ABA-S-ALA) at the position 8-11, 13-19, 23-36 and 25-28. It has a sulfur content of 5-6% due to these residues (Berridge *et al.* 1952). Five sulfide bridges are present: ring A is formed by lanthionine and ring B-E by the four β-methyl lantionine residues. Nisin also contains three αβ unsaturated amino acids: dehydroalanine (DHA) at the position 5 and 33 and dehydrobutyrine (DHB) or β-methyl-dehydroalanine at the position 2 (**Figure 2**). The N-terminal domain encompasses residues 1-19 and comprises the first three lanthionine rings (A, B, and C).

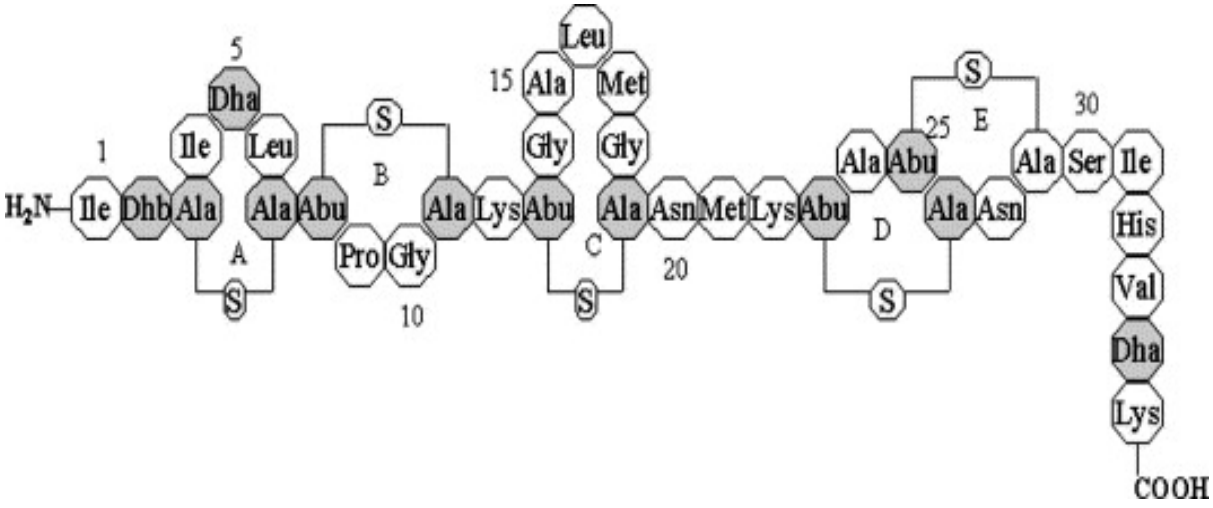


Figure 2. Primary structure of nisin showing 34 amino acid residues (Hsu *et al.* 2004).

The other domain is formed by residues 23-28 and consists of the intertwined rings D and E, followed by a flexible stretch of the 6 C-terminal residues. The dehydro residues have been

suggested to play a role in the antimicrobial activity of nisin by reacting with the sulfhydryl groups of enzymes responsible for cell wall synthesis (Gross and Morell 1971).

2.3 Physico-chemical properties of nisin

Nisin is a catonic molecule due to the combination of three lysine residues and hystidine residues together with a lack of a glutamate and aspartame.

The side chain in histidine has a pKa of 6.5 and those in lysine have pKa 10.0. It's make the net charge of nisin dependent on pH. Nisin is most soluble at acid pH (Tramer and Fowler 1964) and becomes less soluble as the pH approaches neutrality. At the pH 2.2 the solubility is around 56 mg $m.L^{-1}$, at pH 5.5 the value is 3 $mg.mL^{-1}$ (Delves – Broughton *et al.* 2000).

Nisin is in soluble in non polar solvents (Liu and Hansen 1990). Nisin has an isoelectric point in the alkaline range; thus, the net charge on its surface should be higher at acidic pH than at neutral pH. In addition, the decrease in nisin solubility with increasing pH indicates that hydrophobic associations are more favorable at neutral pH.

Net charge may also affect the adsorption mass by repulsion between adsorbed and adsorbing molecules at the interface. The adsorption of nisin to bacterial cells is pH-dependent (Bhunia *et al.* 1991), with a maximum in adsorption observed at pH 6.5, and less than half of that being adsorbed at pH 4.4 (Yang *et al.* 1992). Nisin is a highly surface active molecule whose adsorptive properties were recognized as early as 1949 when it was noted that nisin in culture remained with *Lactococcus lactis* cells rather than in the culture broth at pH 6 (Berridge 1949). Friedman and Epstein (1951) recognized that nisin could adsorb onto glass test tubes and found it only partially elutable upon introduction of sterile, nisin-free broth to the empty tubes. They also found that boiling in the presence of detergents and oxidizing agents was not sufficient to remove all the adsorbed nisin. Nisin adsorption at silanized silica and the retention of its antibacterial activity after adsorption has been studied (Bower *et al.* 1995a,b, Lakamraju *et al.* 1996). It was observed that nisin can adsorb to solid surfaces in a manner stable to rinsing, maintain activity, and kill bacterial cells that have subsequently adhered. Nisin has an effect similar to cationic detergents on the bacterial cell membrane, as leakage of ultra-violet absorbing material from treated bacterial cells was observed (Jack *et al.* 1995).

2.4 Antimicrobial activity and mode of action

Nisin is an effective bactericidal agent against Gram positive bacteria including strains of *Lactococcus, Streptococcus, Staphylococcus, Micrococcus, Pediococcus, Lactobacillus, Listeria*

and *Mycobacterium* (Sahl *et al.* 1995). Gram-positive spores like *Bacillus* and *Clostridium spp*. are particularly susceptible to nisin, with spores being more sensitive than vegetative cells (Delves-Broughton 1996).

Nisin action against vegetative cells can br bacteriosatic or bactericidal, depending on the nisin concentration, bacterial concentration, physiological state of bacteria and the prevailing conditions. Nisin shows a bactericidal effect, when conditions of test are optimum for the growth of bacteria: optimum temperature, pH, water activity, redox potential, and nutrient availability and bacteria are in an energized state. However the nisin action against spores is caused by binding of the nisin with a sulfhydryl groups on protein residues. Phospholipides are not implicated (Delves-Broughton 1996)**.**

The bactericidal efficacy of nisin has been compromised by the occurrence of nisin resistance in various Gram positive bacteria. It could be acquired by exposure of sensitive strains to increasing concentrations of nisin through alterations in the expression of genes involved in cell wall and cytoplasmic membrane biosynthesis, which is referred to as a physiological adaptation (Peschel *et al.* 1999, Gravesen *et al.* 2001, Mantovani and Russel 2001). Nisin-resistant variants include many microorganisms: *Listeria monocytogenes* (Martínez *et al.* 2005, Naghmouchi *et al.* 2007), *Listeria innocua* (Maisnier-Patin and Richard 1996), *Streptococcus thermophilus* (Garde *et al*. 2004), *Staphylococcus aureus* (Peschel *et al.* 1999) and *Streptococcus bovis* (Mantovani and Russell, 2001), *Bacillus licheniformis* (Kang *et al.* 2001), *Bacillus subtilis* (Hansen *et al.* 2009).

Changes in the membrane composition and fluidity and polysaccharide production are examples of resistance mechanisms towards nisin (Kramer *et al.* 2004, Martínez and Rodríguez 2005). The cell wall in Gram-positive bacteria consists of a relatively thick, multi-layered peptidoglycan sacculus that, depending on the bacterial species, may contain proteins, lipoteichoic acid (LTA), wall teichoic acid (WTA) and polysaccharides. Notably, not all Gram-positive bacteria harbour LTA or WTA. The cell wall has a net negative charge, mainly because of the LTA and WTA content. LTA in its most common form consists of a polyglycerol phosphate that is linked to the membrane via a glycolipid anchor (Kramer *et al.* 2008). The resistance resulted in an increase and a decrease of two different phosphoenolpyruvate-dependent phosphotransferase systems (PTSs), which are responsible for the uptake and concomitant phosphorylation of a number of sugars in both Gram-negative and Gram-positive bacteria (Gravensen *et al.* 2002). The mechanism of *L. monocytogenes* resistance to nisin has been correlated with changes in

membrane fatty acid composition, cell wall structure and requirements for divalent cations (Mantovani and Russell, 2001; Crandall and Montville, 1998).

Nisin-producing bacteria are protected against nisin by two self-protection mechanisms: a lipoprotein, NisI, which likely binds and inactivates nisin, and an export system, NisEFG, which presumably extrudes nisin from the cell (Kuipers *et al*. 1993a,b). The existence of this nisI promoter is likely an evolutionary adaptation of the nisin gene cluster to enable its successful establishment in other cells following horizontal transfer (Li and O' Sullivan 2006).

In non-nisin-producing *L. lactis*, nisin resistance is conferred by a specific nisin resistance gene (*nsr*), which is located on a 60-kb plasmid and encodes a 35-kDa nisin resistance protein (NSR). This NSR-mediated proteolytic cleavage represents an mechanism for nisin resistance in non-nisin-producing *L. lactis* (Sun *et al.* 2009).

Gram-positive bacteria have been shown to be resistant to nisin due their ability to synthesize an enzyme, nisinase, which could inactivate nisin (Mazotta and Montville 1997). An altered gene expression was detected in nisin resistant mutants in *Listeria monocytogenes* (Martínez *et al.* 2005). Cell changes induced in two nisin resistant variants of *Listeria innocua* after growth in the presence of high concentrations of nisin (Maisnier-Patin and Richard 1996).

The bacteriocin nisin is not generally active against Gram-negative bacteria (*Escherichia coli, Salmonella, Shigella, and Pseudomonas*) fungi and virus (Boziaris and Adams 1999). The inability of nisin to attack Gram negative bacteria is due to the protective outer membrane, which cover the cytoplasmatic membrane and peptidoglycan layer of Gram negative cells. This membrane contains glycerophospholipidis in inner leaflet, but the outer leaflet is built of lipopolysaccharide molecules. Lipopolysaccharides are composed of a lipid part and a complex heteropolysaccharide with a partly anionic character. It formed a tight layer endowed with a hydrophilic surface. As results, outer membrane is barrier that excludes hydrophobic substance and macromolecules. Nisin is a hydrophobic macromolecule, so is unable to traverse a normal outer membrane, and thus can not reach the Gram negative bacteria (Helander and Mattila-Sandholm 2000).

2.5 Mechanism of antibacterial action

Nisin displays four different activities: auto induction of its own synthesis, inhibition of the target bacteria growth by membrane pore formation (**Figure 3**), inhibition of bacterial growth by interfering with cell wall synthesis and inhibition the outgrowth of spores (Rink *et al.* 2007).

Main antimicrobial activity of nisin is to form pores in the cytoplasmic membrane, which leads to a loss of small intracellular molecules and ions and a collapse of the proton motive force. To exert its antimicrobial activity, nisin seems to require a specific receptor (Hasper *et al.*2006a,b) or a sufficient trans-negative electrical membrane potential (Abee *et al.*1995).

Production of nisin is encoded by a cluster of genes *nisABTCIP*, *nisRK*, and *nisFEG*, (Immonen and Saris 1998). This gene cluster encodes the nisin precursor protein (NisA), as well as proteins involved in posttranslation modifications, immunity for the producing cell, transcriptional regulation, transport, and processing of the prepeptide (Kuipers *et al.* 1993a, Engelke *et al.* 1994). The precursor is an inactive peptide and is chemically modified by the products of *nisB* and *nisC* (Siegers *et al.* 1996). The modified precursor peptide is transported by NisT and processed by a subtilisin-like protease, NisP, which cleaves the 23-amino-acid leader peptide to form an extracellular mature nisin peptide (Kuipers *et al.*1993b). The mature nisin peptide can then function as an autoinducer to regulate expression of the nisin genes through a two-component regulatory system, NisRK (Kuipers *et al.* 1995).

Rings A and B physically interact with lipid II, and this results in membrane permeabilization by hybrid pores of nisin and lipid II (Breukink *et al.* 1999a,b, 2003) and inhibition of cell wall synthesis via lipid II abduction (Hasper *et al.* 2006a,b).

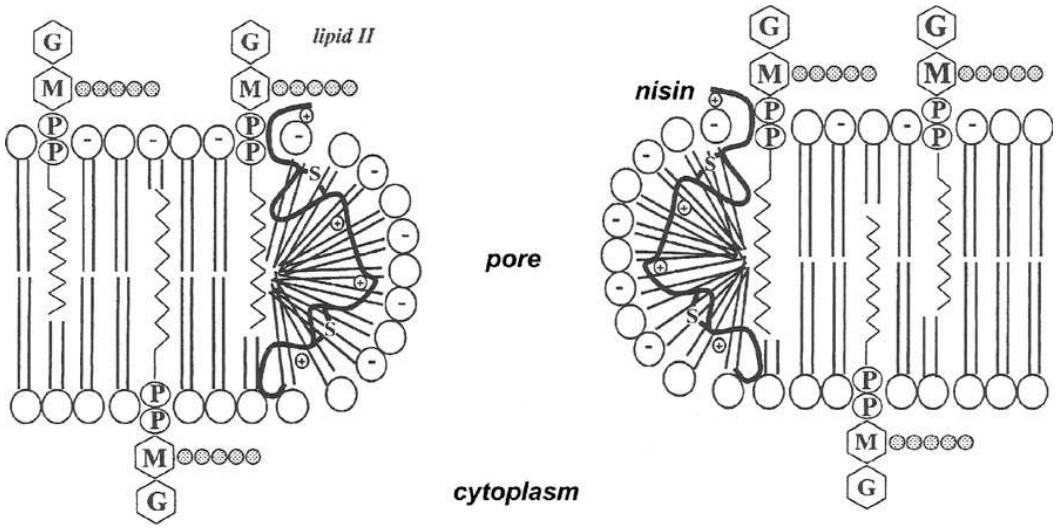


Figure 3. General mode of action of nisin: lipid II serves as a docking molecule which energetically facilitates the formation of pores by binding the molecule of nisin and allowing adopting the correct position for pore opening (Sorbino- López and Martín-Belloso 2008)

Nisin permeabilizes the membrane by forming trans membrane hybrid pores composed of lipid II and nisin (van Heusden *et al.* 2002, Hasper *et al.* 2004, Hasper *et. al.* 2006a,b) and inhibits cell wall synthesis (Breukink *et al.* 1999, Breukink *et al.* 2003) by displacing lipid II The binding of nisin to lipid II involves a pyrophosphate cage, formed by

lanthionine rings A and B of nisin (Hsu 2004). This formation is followed by the assembly of nisin into a pore complex, together with lipid II, that has a stochiometry of eight nisins and four lipid IIs (Hasper *et al.* 2004). The N terminus of nisin is also involved in binding with lipid II (Brotz *et al.* 1998, Hsu *et al.* 2004).

Nisin inhibits the outgrowth of spores of several *Bacillus* species (Mansour *et al.* 1999, Mansour *et al.* 2001, Vessoni Penna *et al.* 2002, Montville *et al.* 2006). Nisin's dehydroalanine in position 5 is involved in nisin inhibitory activity (Morris *et al.* 1984). Nisin inhibits the outgrowth of spores with Dha5, which react with protein thiol groups in the spore wall. Dha5 is indeed a reactive residue and likely the least-stable residue of ring A that is of functional importance (Rink *et al. 2007*). In the presence of nisin, spores lose heat resistance and become hydrated, because nisin initiates the germination. Nisin also rapidly and irreversibly inhibits growth by preventing the establishment of oxidative metabolism and the membrane potential in germinating spores (Gut *et al.* 2008). Replacement of the dehydroalanine with an alanine at position 5 of nisin strongly reduced the capacity to prevent the outgrowth of spores (Chan *et al.* 1996).

2.6 Application of nisin in food

Nisin is suitable for use in a wide range of food: liquid or solid, packaged or canned, chilled or warm ambient storage. Based on target microorganism, usage of nisin can be divide on three category: to prevent spoilage by Gram positive endospore formers in heat processed food; to prevent spoilage by lactic acid bacteria; to kill or inhibit Gram positive bacteria such as: *L .monocytogenes, Bacillus* ssp., *Clostridium* ssp. The additional level of nisin depends on type of food, its heat process, pH, storage conditions and required shelf life. Addition levels of a commercial extract such as nisaplin vary from 10 to 750 mg/kg, which is equivalent to 0.25 -18.7 μg nisin/g (Delves- Broughton 1996).

Nisin has been shown to be effective in the microbial control of a number of dairy products and its use has been widely assessed in cheese manufacturing at low pH. The use of nisin-producing and nisin-resistant starter cultures appears to be a viable means of incorporating and maintaining this bacteriocin through the cheese-making process, to control food-borne pathogenic and spoilage bacteria (Rodríquez *et al.* 2005). *Lc. lactis* subsp. *lactis* TAB50 and its lactose negative proteinase-negative mutant strain TAB50-M4 have been tested and selected as useful starter cultures or adjuncts in semi-hard cheese from raw or pasteurized milk, providing

protection against contamination of milk or curd by *S. aureus* (Rodríguez *et al.* 2000). The combination nisin-producing strains with other strains – nisin resistant can be applied in tailor-made starter cultures for improving the safety of traditional Domiati cheese (Ayad 2009). The addition of *Lactobacillus. bulgaricus* UL12 together with a nisin-producing strain increased in cheese proteolysis and and improved in Cheddar cheese texture (Sallami *et al*. 2004). Starter cultures, containing of nisin Z-producing *Lc. Lactic* subsp. *lactis diacetylactis* UL 719, offer control over undesirable microflora in Gouda cheese (Bouksaim *et al.* 2000).

Nisin alone, or combined with other treatments as heat and non-thermal treatments, such as high pressure, pulsed electric fields and other antimicrobials, could represent a promising advance for the microbiological safety, maintenance of sensory properties in dairy products (Galvez *et al.* 2008, Sorbino-López and Martín- Belloso 2008) and extension in shelf life (Sarkar 2006). The addition of nisaplin to dairy-based beverages, such as a chocolate milk drink, reduces the thermal resistance of selected bacterial spores (Beard *et al.* 1999).

The use of nisin in cured and fermented meat is equivocal. Compared to dairy products, nisin used in meat products has not been very successful because of its low solubility, uneven distribution and lack of stability. Moreover the required dose to be effective is uneconomical and exceeding the acceptable daily intake for a consumption of 100g/day and an average weight of 60 Kg (Hugas 1998). Sprayed nisin has been effective for the decontamination of meat surfaces (Cutter and Siragusa 1997) raw pork (Murray and Richard 1997). Nisin in combination with 2% sodium chloride is an antilisterial agent in minced raw buffalo meat (Pawar *et al.* 2000). Nisin in combination with nitrite was effective against *Clostridium, Listeria* and *Staphylococcus* in frankfurters, pork slurries and raw meat (Chung *et al.* 1989). Combination nisin and plused light treatment can be used as an effective antilisterial step in the production of ready-to-eat sausages (Uesugi and Moraru 2009).

Nisin promote the microbial stability of vegetable food products, through using nisin strains as starter cultures, protective cultures or co-cultures (Settanni and Corsetti 2008). Nisin is used to preserve kimchi by inhibiting lactobacilli (Choi and Park 2000). Nisin has been tested to control *Bacillus* and *Clostridium* growth in potato-based products (Thomas *et al.* 2002). Nisin addition to fruit juices, fruit juice-based drinks, not heat-treated or pasteurized, completely prevented *Alicyclobacillus acidoterrestris* under all temperature and time of storage conditions (Pettipher *et al.*,1997, Komitopoulou *et al.* 1999). Washing fresh-cut lettuce with nisin and other bacteriocins decreases the viability of *Listeria monocytogenes* immediately after treatment, during storage at 4ºC (Allende *et al.* 2007).

Nisin can also be used in distilled alcohols production for beverages and industrial products. Added to fermentation mashes naturally contained lactic acid bacteria control contamination and allow the yeast less competition for substrates, thus resulting in increased alcohol yield (Delves Broughton 1996)

3. Lysozyme

Lysozyme is well known as an antimicrobial protein and considered as a natural food preservative. Lysozyme is the preservative E1105 in cheese, according to EU legislation and the Codex Alimentarius.

Lysozyme was first discovered in 1922 by Alexander Fleming. Later in 1945, Alderton identified lysozyme in hen's egg albumem. Lysozyme is described as N-acetylhexosaminodase and is classified as a muridase. The Commission on Enzyme has assigned the numbers 3.2.17 to lysozyme.

Lysozyme structure is clearly characterized as a compactly folded molecule (**Figure 4**), the rigidity of which is stabilized by the four-disulfide bonds (6Cys–127Cys, 30Cys–115Cys, 64Cys–80Cys, and 76Cys–94Cys) and four tryptophan residues (Trp 62, Trp 63, Trp 123,Trp 108) (Canfield and Liu 1965).

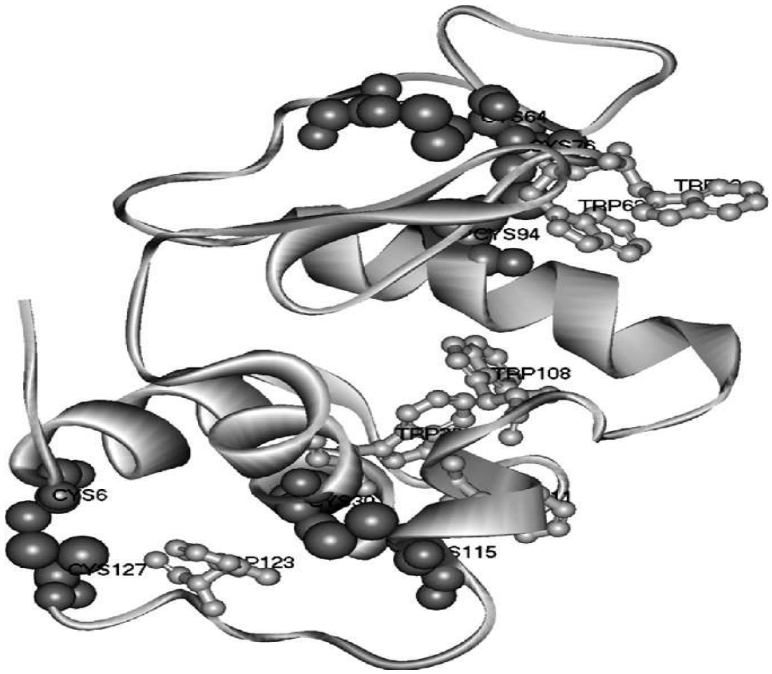


Figure 4. The structure of hen egg white lysozyme with the four pairs of disulfide bonds and the six tryphophan residues (Wu *et al.* 2008).

These disulfide bonds are well known to be stable to denaturing agents and heat treatment, but easily disrupted with reducing agents, and reduction of these S–S bridges is conducive to a

greater molecular flexibility and dramatic increase in exposed hydrophobic regions (Hayakawa and Nakamura 1986, Volkin and Klibanov 1987, Li-Chan and Nakai 1989, Joseph and Nagaraj 1995).

The molecular weight of lysozyme is approximately from 14300 to 14600 Da and the isoelectric point is 10.7. Due to its high an isoelectric point lysozyme has a positive net-charge over a large pH range (2-11) (Kvasicka 2003). The active site of hen eggs white lysozyme consist of six subsites A, B, C, D, E and F, where the active catalytic group Glucosoamine 35 and Asp 52 are between sub sites D and E (Losso *et al.* 2000).

3.1 Antimicrobial activity

The highest lysozyme activity rate is from pH 3.5 to 7. The lysozyme is most effective against some specific Gram-positive bacteria such as *Staphylococcus aureus, Micrococcus lysodeikkticus, Bacillus cereus, Bacillus stearothermophilus, Clostridium thermosaccarolyticum,* and *Clostridium tyrobutyricum*, and is largely ineffective against Gram negative bacteria (Proctor and Cunningham 1988, Masschalack and Michelis 2003, Mine *et al.* 2004).

Gram-positive bacteria are sensitive to lysozyme because their petidoglycan is directly exposed, but some are intrinsically resistant due to a modified petidoglycan structure (Bera *et al.* 2007). Gram negative bacteria are generally resistant to lysozymes due to the presence of an outer membrane exterior to the petiglycan, which shields the petidoglycan from lysis. The barrier function of the outer membrane can be partially overcome by membrane perturbing compounds or treatments. For example high hydrostatic pressure (100- 1000 MPa) has been demonstrated to sensitize resistant bacteria to several antimicrobial peptides including lysozyme (Masschalck *et al.* 2000, Nakimbugwe *et al.* 2006).

3.2 Mechanism of antimicrobial activity

One of the mechanisms proposed by which lysozyme inhibits bacteria is the degradation of the glycosidic β-linkage between the Nacetylglucosamine (NAG) and the N-acetylmuramic acid (NAM) of the peptidoglycan layer in the bacterial cell walls. Such a mechanism of action of lysozyme is limited to Gram-positive bacteria (**Figure 5**).

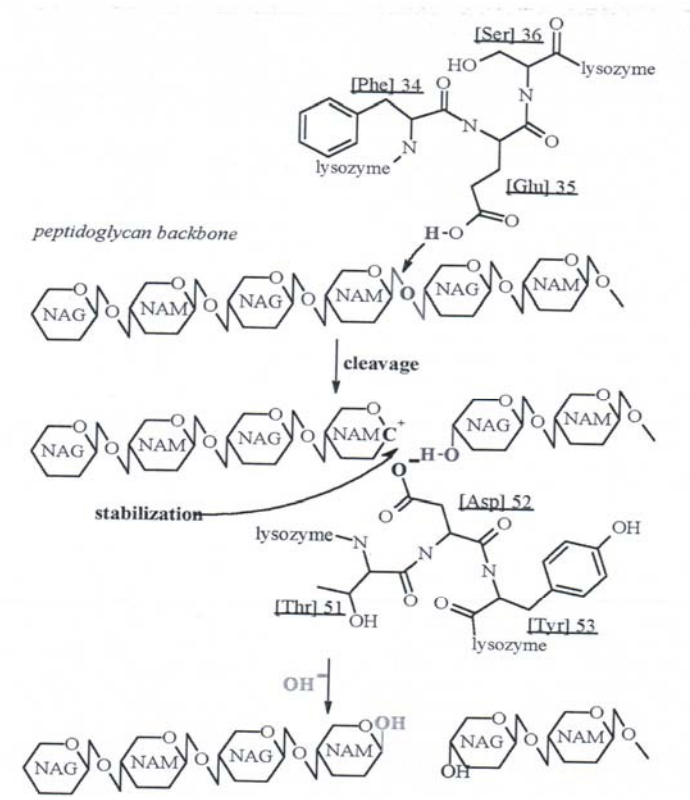


Figure 5. Mechanism of action of lysozyme (Massachalack and Michelis 2003).

In order for lysozyme to be effective against Gram-negative bacteria, it must overcome this outer membrane permeability barrier. One of the possibilities that this barrier could be circumvented is by equipping the lysozyme molecule with a hydrophobic carrier, which could mediate its interaction and insertion into membrane. This would facilitate its delivery into the site of its action which successively would lead to damage of the cytoplasmic membrane. Another mechanism of the bactericidal action proposed for lysozyme is independent of its enzymatic activity but attributed mainly to its cationic and hydrophobic properties. This mechanism of action is supported by the fact that denaturated lysozyme lacking enzymatic activity is still able to inhibit the bacterial growth (Ibrahim *et al.* 2001, Touch *et al.* 2004, Gorbenko *et al.* 2007).

3.3 Application of lysozyme in food

Although lysozyme, as naturally occurring antimicrobial, is widely applied to food systems for its preservative properties, its use in the food industry is limited. The most known lysozyme application is preventing late blowing of semi-hard and hard cheeses by inhibiting growth of *Clostridium tyrobutyricum* (Proctor and Cunningham 1988, Pellegrino and Tirelli 2000). Lysozyme lyses the cell wall of the vegetative form of *Cl. tyrobutyricum* through the

enzymatic cleavage and consequently control clostridial growth and butyric acid fermentation during the maturation of cheeses, in particular those made from pressed and cooked curds, e.g., Swiss cheese, Parmesan, Edam, Gouda, and Cheddar. There are some limitations to effective use of lysozyme such as resistance of particular clostridia spores, sensitivity of starter cultures and high number of spores (Bogovič-Matijašić *et al.* 2007). Lysozyme in combination with Na_2-EDTA (ethylenediaminetetraacetic disodium salt) inhibit the growth of spoilage microorganisms such as coliforms and *Pseudomonas* spp., without affecting the lactic acid bacteria and could be use to prolong the shelf life of mozzarella cheese (Sinigaglia *et al.* 2008).

Lysozyme in combination with other biological preservatives such as: nisin, organic acid or essential oils prevent growth of *Listeria monocytogenes* on meat surfaces and meat product (Losso *et al.* 2000).

The Japanesse have been the largest users of lysozyme in practical application. They have patented process using lysozyme as a preservative on fresh fruits, vegetables, tofu bean curd, seafoods, meats and sake (Proctor and Cunningham 1988).

Besides, lysozyme is used as a preservative in wine, in order to control malolactic fermentation (Proctor and Cunningham 1988, Tirelli and De Noni 2007).

4. Combined antimicrobial systems

Traditionally, it was common to use only one chemical antimicrobial agent in food product for preservation purpose. In recent years, the use of combined agent in a single food system has become more frequent.

The use of combined agents theoretically provides a greater spectrum of activity with an increasing antimicrobial action against pathogenic or spoilage organisms (Parish and Davidson 1993). Combined antimicrobial agents have been extensively used in pharmaceutical industry and the methods have been developed to determine types of interactions between two antimicrobials (Barry 1976). Combined studies are conducted to determine if specific types of interactions occurs between the two combined antimicrobials. The terms additive, antagonistic and synergistic are used to describe possible antimictobial interactions (Parish and Davidson 1993).

Additives effects occurs when the antimicrobial activity of a compound is neither enhanced nor reduced, while in the presence of another agents (Davidson and Parish 1989).

Synergism refers to an enhancement of overall antimicrobial activity of one component when in the presence of a second antimicrobial agent (Davidson and Parish 1989).

Antagonism occurs when microbial growth is observed in areas, where individual agents would be present in inhibitory concentration (Barry 1976).

Antimicrobial activity could be determined to measure minimal inhibitory concentration (MIC) (Davison and Parish 1989). Interpretation of MIC results for combined testing is conducted with isobolograms. Isobologram construction can be simplified using fraction inhibitory concentration (FIC), which are the MIC normalized to unity. The FIC is the concentration of a compound needed to inhibit growth, when combined with a known amount of a second antimicrobial compound. It is calculated as the ratio MIC of the compound, when combined with a second compound, divided by the MIC of the first compound alone. The FIC of two compounds in an inhibitory combination can be adding to a give a total FIC index. An FIC index near 1 indicates additive, whereas <1 indicates synergy and >1 antagonism (Hall *et al.* 1983, Davidson and Parish 1989, Parish and Davidson 1993). Many publications have been published on combined effect nisin lysozyme and third components as EDTA, weak acid organic and their salts.

The mixture of nisin and lysozyme demonstrated synergy against food spoilage by lactobacilli, Gram-positive bacteria, because they reinforce each other mechanisms of bacterial killing (Chung and Hancock 2000).

Synergy was observed through measurements of kinetics of bacterial killing of *Lactobacillus curvatus* strain 854 and by scanning electron microscopy as a consequent change in optical density at 600 nm (Chung and Hancock 2000). Lysozyme at 500 $\mu g.mL^{-1}$ killed very slowly (1 log decrease in colony forming units in 3 h), whereas nisin at 500 $\mu g.mL^{-1}$ was more effective (4 $\log_{10}$ decrease in 2 h). However combination of nisin 125 $\mu g.mL^{-1}$ lysozyme 375 $\mu g.mL^{-1}$ showed a nearly identical level of kill to 500 $\mu g.mL^{-1}$ nisin after 60 min, but a much stronger kill after 2 h (8 $\log_{10}$ of killing) on strength MRS media (Chung and Hancock 2000). Furthermore, the same mixture nisin and lysozyme was more active against *Lactobacillus sake* strain 6 than *L. curvatus* 845 (Chung and Hancock 2000).

Nattress and Baker (2001) demonstrated that the 3:1 mixture of lysozyme and nisin showed synergy on sterile pork and pure bactetrial cultures. Nisin, lysozyme and mixtures of the two at weight to weight ratios of 1:1 nisin/lysozyme and 1:3 nisin/lysozyme were used at concentrations of 2500, 5000 and 10.000 $\mu g.L^{-1}$ to attain estimated concentrations of antimicrobial on cores of pork fat and lean tissue of 65, 130 and 260 $\mu g/cm^2$, respectively. Both lysozyme and nisin alone as well as mixtures of the two effectively inhibited *Brochothrix thermosphacta* B2 at 250 μg/ml in APT broth for 10 days at 2°C. In the presence of 500 $\mu g.mL^{-1}$ lysozyme, *B. thermosphacta* B2 grew after 12 days of incubation. Only 125 μg of antimicrobial/ml, an estimated surface concentration of 130 $\mu g/cm^2$ of each of the antimicrobials, was required to inhibit *B. thermosphacta* B2 for 27 days at 2°C in pork juice. In APT broth and in pork juice,

lysozyme didn't show antimicrobial activity against *Carnobacterium* sp. 845 at concentrations of 500 and 1000 $\mu g.L^{-1}$, respectively. Nisin and mixtures of the two antimicrobials inhibited *Carnobacterium sp.* 845 with 3 log population units lower than untreated samples after 26 and 27 days incubation for APT and pork juice, respectively. The antimicrobial effect was concentration dependent. On lean pork tissue, numbers of *Carnobacterium* sp. 845 were the lowest, when 260 $\mu g/cm^2$ of a 1:3 (w/w) ratio of nisin to lysozyme was introduced to the cores (Nattress and Baker 2001).

Mangalasary *et al.* (2007) presented that the application of 2 $mg.mL^{-1}$ nisin and 10 $mg.mL^{-1}$ lysozyme in combination at the temperature 62.5 and 65°C were effective in reducing the time required for a targeted log reduction in *Listeria monocytogenes* populations on the ready-to-eat (RTE) bologna surface. The nisin- lysozyme combination required 23 % less time at 62.5ºC and 31% les time at 65ºC for 4 log reduction on BHI agar medium. Lysozyme alone did not enhance antilisterial activity in combination with heat. Also the same combination of $2mg.mL^{-1}$ nisin and 10 $mg.mL^{-1}$ lysozyme in package pasteurization of vacuum package RTE low fat turkey bologna caused the reduction of population *Listeria monocytogenes* (Mangalasary *et al.* 2008). Nisin and lysozyme treatments reduced of *L. monocytogenes* 1.4 $\log_{10}$ cfu/cm^2 population immediately after in-package pasteurization. There was an additive inhibitory effect of nisin and lysozyme, because in-package pasteurized bologna combined, lysozyme or nisin had 3.4 and 3.2 $\log_{10}$ cfu/cm^2 *L. monocytogenes* populations, respectively, compared to 4.1 for control (Mangalasary *et al.* 2008).

Chung and Hancock (2000) probed by scanning electron microscopy that nisin had not apparent effect on cell morphology other than increasing surface ruffling. However, lysozyme treatment caused the production of small balls of material on cells surface. The combination nisin 37.5 $\mu g.mL^{-1}$ and lysozyme 112.5 $\mu g.mL^{-1}$ caused a perturbation of cell morphology including apparent holes or craters in the cell surface. Moreover the same authors proposed that mechanism of synergy could increase cell lysis. Conversely, nisin may inhibit energy depending processes that repair lysozyme damage (Chung and Hancock 2000). Nisin has an effect immediately on the bacteria and lysozyme extended the effect during the storage (Nattress and Baker 2001). Mangalasary *et al* (2007) reported that a combination of nisin and lysozyme is helpful for reducing the tailing of survivor curves for high pressure treated bacterial populations, because this combination reduce the fraction of cells and these treatment is compared with the use of nisin or lysozyme alone.

The combination nisin and lysozyme is beneficial for food systems even in the presence of high salt conditions which inhibited the activity of lysozyme. The benefit becomes more obvious

when consider financial cost, because lysozyme is three-fold cheaper than nisin (Chung and Hancock 2000).

Etylenediaminetetraacetic acid (EDTA) enhanced the activity of nisin, lysozyme and monolaurin against Gram negatives microorganisms (Hughey and Johnson 1987, Stevens *et al.* 1991, Branen and Davidson 2004). EDTA is a chelating agent used in a wide variety of food product (21 CRF 172.135) to prevent oxidation and other deteriorative reactions catalyzed by metal ions. It has also antimicrobial activity and potential the activity of antimicrobials agent, antibiotics against Gram negative bacteria.

Branen and Davidson (2004) reported that low concentration of EDTA acted synergistically with nisin and lysozyme against *L. monocytogenes* and *E. coli*, but not aginst *Salmonella* Enteritidis or *P. fluorescens* in tryptic soy broth (TSB). EDTA at very low concentration (7.8 and 15.6 $\mu g.mL^{-1}$) synergistically enhanced the activity of nisin against *L. monocytogenes* by decreasing the nisin MIC 50% to 3.9 $\mu g.mL^{-1}$. *E. coli* treated with 31.3 and 313 $\mu g.mL^{-1}$ nisin had an increase lag time and induced a 40-50% decreased in OD_{620nm} at stationary phase (Branen and Davidson 2004).

EDTA enhanced lysozyme activity against *L. monocytogenes* and *E. coli* in microbiological media as well as food products (Hughey and Johnson 1987, Hughey *et al.* 1989, Branen and Davidson 2004). EDTA in combination with lysozyme has little or no effect on *Salmonella* Enteritidis or *P. fluorescens* (Payne *et al.* 1994, Branen and Davidson 2004). Combinations of EDTA and lysozyme were bactericidal to *E. coli* and the EDTA MIC decreased by 50% in the presence of 25 and 125 $\mu g\ m.L^{-1}$ lysozyme (Branen and Davidson 2004). Lysozyme alone inhibited growth of *L. monocytogenes* Scott A, but did not inhibit growth of the other *L. monocytogenes* strains in BHI broth (Hughey and Johnson 1987). Payne *et al.* (1994) reported that EDTA (1000 $\mu g\ m.L^{-1}$) enhanced inhibition of *E. coli* O157:H7 and *L. monocytogenes* by lysozyme (100–200 $\mu g\ m.L^{-1}$) in UHT milk.

Gill and Holley (2000) indicate that the combination of 500 $mg.kg^{-1}$ nisin 1500 $mg.kg^{-1}$ lysozyme and 500 $mg.kg^{-1}$ EDTA restrict the growth of *B. thermosphacta, E.coli* O157:H7, *Lactobacillus curvatus* in ham and bologna and also *Listeria monocytogenes* and *Leuconostoc mesenteroides* in bologna in selective et non selective medium. On both ham and bologna samples treated with lysozyme, nisin and EDTA, no *B. thermosphacta* were recovered (<1.81 log $cfu.cm^2$), up to and including 4 weeks of storage. On ham, the numbers of *E. coli* O157:H7 remained constant at approximately 4 log $cfu.cm^2$ on treated sample. *E. coli* O157:H7 population on treated

bologna dropped by approximately 1 log cfu.cm^2 over the first 3 weeks of storage before suddenly rising 8.33 log cfu.cm^2 by the fourth week. *Lb. curvatus* population level on ham was 1 log cfu.cm^{-2} at weeks 1 and 2, which increased to 2 log cfu.cm^2 at week 3. On bologna the difference in population levels was 2 log cfu.cm^2 at weeks 1, 2 and 3. The population of *L. monocytogenes* was at least 1 log cfu.cm^2 lower on bologna and no difference were observed on ham during two weeks of incubation (Gill and Holley 2000).

EDTA permeabilizes the outer membrane by releasing of LPS, and therefore it allowed nisin and lysozyme to reach the cytoplasmic membrane. The difference in outer membrane or lipopolysaccharide structure in *Salmonella* Enteritidis or *P. fluorescens* affect the resistance these bacteria to nisin in combination with EDTA. However the addition of increasing amounts of EDTA did not enhance the activity of nisin, because the EDTA could chelates the cations in growth medium and it might inhibit nisin activity (Branen and Davidson 2004).

Nisin in combination with lactic acid or its salts derivatives presented synergistic interaction. Scannell *et al.* (1997), Samelis *et al.* (2005) and Geornaras *et al.* (2006a,b) showed totally reduction of *L. monocytogenes* to 3 log cfu.mL^{-1} following treatment with nisin 500 IU.m.L^{-1} with 2.5-3 g.100mL^{-1} acetic and lactic acid or their salts in meat product. The synergistic effect between nisin 120-180 IU.kg^{-1}and 18 kg sodium lactate is beneficial to fish product in inhibiting the growth of *L. monocytogenes* to 3.8 log cfu.g^{-1} (Nykanen *et al.* 2000).

McEntire *et al.* (2003) obtained that lactate salts as potassium, calcium and magnesium do not show any synergy with nisin. However synergy with nisin was observed in combination with Zn lactate and Al chloride. Grower *et al.* (2004) speculated that synergy is a result from chelating effect of lactate. McEntire *et al.* (2003) proposed that metal action could be responsible for a synergy with nisin.

5. Food pathogens

5.1. Bacillus ssp.

Food poisoning due to *Bacillus* ssp. have been described since the beginning of XIX century, but in the early 1950s, after the taxonomy of *Bacillus*, *Bacillus cereus* was recognized on important food poisoning bacteria world wild. Food poisoning due to *B. subtilis* and *B.*

licheniformis have been summarised after the UK incidents from 1975 to 1986 on meat, pastry, fish and vegetable products (Kramer and Gilgert 1989).

Three classification scheme of *Bacillus* ssp is proposed: Krasi'lkov (1949), Prèvot (1961) and (Gibson and Gordon 1974). Krasil'nikov's scheme depends on the extent to witch the rod is swollen by endospore. Prèvot arranged the organisms info four genera: *Bacillus*, *Bacteridium*, *Inoninatus* and *Clostridium*. The clafissifacation of Gordon is based on a meticoulous study of 1134 strains.

The genus *Bacillus* is large, comprising more than 60 species that are mostly saprophytes, widely distributed in nature, spreading from soil to water, plants, and animals. The genus shows a great diversity of strains and species. The organisms are Gram-positive or Gram-variable spore-forming bacilli, mostly catalase- positive, that may be motile by peritrichous flagella. Most strains are mesophiles, but some are psychrothrophs and thermophiles. *Bacillus* contains strict aerobes (*B. megaterium*), as well as facultative anaerobes (e.g., *B. cereus, B. licheniformis*). The vegetative cells are rods ranging from 0.5-1.2 mm to 2.5-10 mm, and the endospores are in the central or paracentral, subterminal or terminal position. Survival of the organism results from the resistance of the spores to divers conditions (Dahl *et al.* 1999).

Bacillus spp. is found in a wide range of habitats, and includes species possessing environmental, industrial, and clinical significance (Drobniewski, 1993). *B. licheniformis* has also been associated with septicemia, peritonitis, ophthalmitis, and food poisoning in humans, as well as with bovine toxemia and abortions (Salkinoja-Salonen *et al.* 1999). High numbers or *B. subtilis* and *B. licheniformis* in foods may cause a mild form of foodborne illness such as diarrhoea or vomiting (Kramer and Gilbert 1989).

B. cereus causes illness by two types of enterotoxins, which can be produced in foods (Varnam and Evans, 1991, Granun and Lund 1997). Cooked rice dishes and other farinaceous foods are most commonly associated with illness caused by the emetic toxin (Kramer and Gilbert, 1989) where rice is cooked in bulk and allowed to cool slowly, permitting outgrowth of surviving spores (Lund, 1990). The diarrhoeal toxin appears to be produced during exponential growth either in the food or in the small intestine and has a molecular weight of 38 000-46000 kg/mol (Kramer and Gilbert, 1989). It is heat-labile, being inactivated by heating at 56 ºC for 5 min (Turnbull, 1976) and is more pH-labile than the emetic toxin, since it is inactivated by pH values of > 11 .O and < 4.0. Foods implicated include vegetables (Fernández e*t al.* 2002, Valero *et al.* 2003), meats (Smith *et al.* 2004, Pirhonen *et al.* 2005) and dairy products (Svensson *et al.* 2006, 2007).

Psychrophilic strains of *B. subtilis*, *B. circulans*, and *B. coagulans*, isolated from milk can growth at 0-1ºC and had characteristics typical for psychrophilic bacteria (Shehata *et al.* 1971). *B.*

cereus can be divided according to its growth temperature into psychrotrophic and mesophilic strains. Psychrotrophic strains growth at 5°C and relatively rapidly at 10°C while mesophilic strains fail to grow below 8°C and grow slowly at 10°C (Sutherland *et al.* 1996). Mesophilic strains grow at 37°C and higher and can survive refrigerator temperature without growth (Adams and Moss 2000). The minimum and maximum pH ranges from 5 to 8.5, with optimal 7.4 for *B. subtilis*, B. *licheniformis* and *B. pumilus* (Stolp and Starr 1981). However pH range for a *B. cereus* has been reported to be between pH 4.9 and 9.3 (Garcia- Arribas *et al* 1990). *B. cereus, B. subtilis, B. licheniformis, B. pumilus* and *B. megaterium* can gwroth in aw condition from 0.950 to 0.995 (Braun and Sutheland 2004).

Bacillus species are common soil inhabitants and may frequently contaminate foods, including dairy products, meats, infant-foods, rice dishes, vegetables, spices and cereals (Jacquette and Beuchat 1998, Christiansson *et al.* 1999). Some *Bacillus* species were found *in* different food product: *Bacillus cereus* in Port Salut Argentino cheese, honey and *Bacillus subtilis* in rye flour in Argentina (Iurlina *et al.* 2006). *B. cereus*, *B. coagulans* and *B. mycoides*, *B. subtilis, B. licheniformis, B. pumilis* and *B. stearothermophilus* contaminated the Sardinian dairy product (Cosentino *et al.* 1997)

Bacillus subtilis, B. licheniformis, B. megaterium and *B. cereus* cause spoilage of bread by rope formation. Rope is characterized by a sweet fruity odour, often described as resembling over ripe pineapples, together with patchy discolouration and softening of the loaf crumb (Thomson *et al.* 1993, Rosenkvist and Hansen 1995, Sorokulova *et al.* 2003) The origins of the *Bacillus* species are reported to be raw materials and bakery equipment (Bailey and Von Holy 1993). All types of flour, especially wheat flour, are contaminated with *Bacillus* spores as a result of soil contamination (Watanabe and Hayano 1993, Pandey and Palni 1997), cultivation and processing methods (Voysey 1989).

Spoilage of milk by *Bacillus* species result in enzyme production, especially proteases and lipases (Frank, 1997). For example, *B. cereus* produces a chymosin-like protease enzyme which is responsible for degradation of milk casein, resulting in coagulation (sweet curdling) and finally a bitter-tasting product (Frank, 1997). *B cereus* reportedly also produces phospholipase C which degrades fat globule membranes aggregation in cream (Frank 1997). Many *Bacillus* species have been isolated from milk, including *B. brevis, B. circulans, B. lentus, B. licheniformis, B. mycoides, B.polymyxa, B. pumilus, B. subtilis* and *B. thuringiensis* (García-Armesto and Sutherland 1997).

Bacillus ssp genera in food should not be ignored, because of the spore-forming species may reside in the surface of vegetables, in the environment or in equipment, affecting the end product quality (Fangio *et al.* 2010).

5.2. Listeria monocytogenes

Listeria monocytogenes was originally isolated by E.G.D Murray from laboratory rabbits in 1926 (Murray *et al.* 1926). Although the microorganisms has been used as a model for the study of intercellular parasitism for decades, *L. monocytogenes* was not considered a significant animals pathogens until the 1970s and early 1980s, when it was recognizes as a major foodborne human pathogens (Mead *et al.* 1999).

The genus *Listeria* is placed in the *Clostridium* sub branch of Gram-positive bacteria based upon the low G + C content of its genome. There are six species currently recognized: *Listeria monocytogenes, Listeria innocua, Listeria ivanovii, Listeria seeligeri, Listeria welshimeri* and *Listeria grayi* (Gasonov *et al.* 2005, Hain *et al.* 2007). Only two species of the genus are generally considered to be pathogenic, *L. monocytogenes* in humans and *L. ivanovii* in mammals. However, there were some reports of *L. seeligeri* and *L. ivanovii* causing illness in humans (Gasanov *et al*. 2005).

Listeria monocytogenes is the aetiological agent of the potentially life-threatening illness listeriosis which preferentially affects patients with impaired immune defences, leading to meningitis, meningoencephalitis, fetus infections, abortions, perinatal infections and gastroenteritis. It initially enters the human host via contaminated foods, penetrates the intestinal cell lining, and translocates into the liver, spleen, lymph nodes, brain, and, in pregnant women, the placenta. Although incidence is relatively low, listeriosis has a very high mortality rate. The transmission of infectious doses of *L. monocytogenes* to the human host often depends on its ability to grow in diverse environments, including refrigerated foods. In fact, the infectious dose in humans is relatively high and this makes post-contamination multiplication in food products initially contaminated at low levels necessary for the onset of disease in humans (Vázquez – Boland *et al.* 2001)

The nutritional requirements of *Listearie* are typical for others Gram positive bacteria. They growth well in common media such as brain heart infusion, trypticase soy and tryptose broths. Growth of *L. monocytogenes* in culture media has been observed at pH 4.4 in less than 7 days at 30°C (George *et al.* 1988), at pH 4.5 in tryptose broth at 19°C (Buchanan and Klawitter 1990) and at pH 4.66 in 60 days at 30°C (Colburn *et al.* 1990). When a tryptic soy broth was adjusted with various acid, minimum pH for *L. monocytogenes* was shows to be a function of the acid employed. At the same pH antimicrobial activity was acetic acid>lactic acid> citric acid >

malic acid > HCl (Parish and Higgins 1989). The minimum growth temperature on trypticase soy agar of 78 strains of *L. monocytogenes* was found to be 1.1±0.3°C, with a range of 0.5-3.0°C (Junttila *et al.* 1988). Bajard *et al.* (1996) indicated existence of a so-called "change temperature", occurring between 10 and 15ºC, below which *L. monocytogenes* grown faster than one would expect. The maximum growth of *L. monocytogenes* is around 42-45C (Bajard *et al.* 1996). *L. monocytogenes* is able to grow in the range of a_W from 0.888 to 0.997 in tryptic soy broth (Koutsoumanis and Sofos 2005), from 0.950 to 0.965 under exposed to various conditions: the osmotic solutes (NaCl or KCl), organic acid (acetic acid) and base (NaOH or KOH) (Cheroutre-Vialette *et al.* 1998).

The foods most frequently associated with contamination *L. monocytogenes* have been identified as RTE foods, smoked fish and other fishery product, meat product and cheese (Lianou *et al.*2007).

L. monocytogenes poses a microbiological risk in processed foods containing pork meats. The bacterium is able to adapt to hurdles encountered during the manufacturing and conservation process (Jay 1996, Rhoades *et al.* 2009). Processed meat products could be contaminated by *L. monocytogenes* at several stages: either the raw ingredients (Nørrung *et al.* 1999) are contaminated and the manufacturing process is insufficient to sterilize the product (Selby *et al.* 2006, Pal *et al.* 2008, Naidoo and Lindsay 2010) or by contact with unclean surfaces or people (Chasseignaux *et al.* 2001, 2002). Giovannacci *et al.* (1999) pointed out the persistence of *L. monocytogenes* strains over a year for two pork processing plants and for 3 years in a grinder.

L. monocytogenes is effectively inactivated by commercial heat treatments (pasteurization) used in the dairy industry. The food processing environment seems to represent a major source of finished product contamination. *L. monocytogenes* has been reported to be regularly isolated from food and dairy processing environments (Pritchard *et al.* 1995). *L. monocytogenes* can be transmitted by the consumption of raw milk (Rudolf and Scherer 2001, Linton *et al.* 2008*)* and other milk products, homemade milk products (Arslan and Őzdemir 2008) and by asymptomatic personnel handling of such products (Potter *et al.* 1984). *Listeria monocytogenes* causes problems in cheese industry. Cheese could be contaminated at any stage from farm to table, since *L. monocytogenes* is endogenous to farm and cheese processing environments and it shows ability to grow at chilling temperatures and to tolerate salt and low pH (Gameiro *et al.* 2007). Growth and survival of *L. monocytogenes* in cheeses is determined by the nature and activity of starter cultures, the rate and extent of acidification, the length, temperature and humidity of ripening, and the packaging and storage conditions (Morgan *et al.* 2001). Some incidences have been found in soft-white-fresh cheeses and light cheeses based on the substitution

of lipid components by proteins (Tsiotsias *et al.* 2002). Timbo *et al.* (2010) observed genetic diversity of *L. monocytogenes* isolates and genetic relatedness among strains recovered from imported cheese products coming to the United States from Mexico, Italy, Israel, Portugal, Colombia, Greece, and Spain.

Foods considered as high-risk for *L. monocytogenes* include ready-to-eat (RTE) foods and products that require long-term refrigerated storage. The presence of *L. monocytogenes* was found in the smoked fish 12% (Meloni *et al*. 2009) and between 10 to 20% (Gnanou Besse *et al.* 2004). Ready to eat fish products are more frequently contaminated with *L. monocytogenes* and contain the highest proportion of samples with >100 cfu/g than other ready to eat food categories (Jemmi *et al.* 2002).

The European Community derective on milk and milk based product specifies zero tolerance for soft cheeses and absorbance of the organism in 1 g of other product. France requires no *L. monocytogenes* in 25 g samples of foods for a at risk individuals (Jay *et al.* 2005a).

Regulation 2073/2005 categorizes ready-to-eat **(**RTE) foods into those that are able to support the growth of *L. monocytogenes* and those that are not. For RTE foods that are able to support the growth of *L. monocytogenes*, the new regulation demands the absence of the pathogen (in 25g) before the food has left the immediate control of the food business operator, who has produced it, but allows for up to 100 cfu/g for products placed on the market during their shelf-life. The 100 cfu/g limit is applied throughout the shelf life of marketed RTE foods unable to support *L. monocytogenes* growth (Angelidis *et al.* 2010).

L. monocytogenes strains from food and food-processing environments are susceptible to the antibiotics commonly used in veterinary and human listeriosis treatment. Considering that *L. monocytogenes* is slowly becoming antibiotic resistant (Conter *et al.* 2009, Rahimi *et al.* 2010) or bacteriocins resistant (Gandhi and Chikindas 2007) surveillance of emerging antimicrobial resistance of this pathogen is important to ensure effective treatment of human listeriosis.

5.3. Staphylococcus aureus

Staphylococcal food-poisoning or food intoxication syndrome was first studied in 1894 by J. Denys and later in 1914 by M.A. Barber, who produced in himself the signs and symptoms of the disease by consuming milk that had been contaminated with a culture of *Staphylococcus aureus.* The capacity of some strains of *S. aureus* to produce food poisoning was proved

conclusively in 1930 by G. M. Dack *et al.*(1930) who showed that the symptoms could be produced by feeding culture filtrates of *S. aureus.*

Staphylococcus genus is ubiquitously distributed in nature, with some species inhabiting specific ecological niches. *Staphylococci* are found living naturally on the skin and mucous membranes of warm-blooded animals and humans, but are also isolated from a wide range of foodstuffs such as meat, cheese and milk, and from environmental sources such as soil, sand, air and water (Kloos and Schleifer 1986).

Staphylococci are Gram-positive spherical bacteria that occur in microscopic clusters resembling bunches of grapes. They are facultative anaerobic, nutritionally undemanding and catalase-positive. According to the current List of Bacterial Names with Standing in Nomenclature (Euzéby 2004) rRNA sequence studies, the genera *Staphylococcus* and *Micrococcus* belong to the Gram-positive bacteria with a low DNA G + C content. They are closely related to *Bacilli* and other Gram-positive bacteria with low DNA G + C content such as *Enterococci*, *Streptococci*, *Lactobacilli* and *Listeria* (Garrity and Holt 2001).

Staphylococcus aureus growth occurs over the range 7- 47.8°C and enterotoxins are produced between10 and 46°C, with the optimum between 40ºC and 45ºC. *S. aureus* is relatively salts tolerant, it can grow in 7-10% concentrations, and some strains can grow in 20%. *S. aureus* can grow over the range 4.0-9.8 pH, but its optimum is in the range 6-7 pH. *Staphylococci* is able to grow at values lower than that any of other nonhalophilic bacteria. Growth has been demonstrated as low as 0.83 under otherwise ideal conditions and 0.86 is recognized minimum aw (Jay *et al.* 2005b). Most staphylococci species are coagulase-negative. Exceptions are *S. aureus, S. intermedius, S. delphini, S.schleiferi* subsp. *coagulans, S. lutrae* and some strains of *S. hyicus* (Kloos and Bannerman 1995).

S. aureus causes various infections such as simple abscesses, sepsis and toxinoses in food toxic shock syndrome. The symptoms of food poisoning in humans are mainly due to the secretion of emetic and pyrogenic toxinsnamed staphylococcal enterotoxins (SEs). These toxins are single-chain proteins, and today, at least those designated SEA to SE, SEG to SER, and SEU have been identified (Balaban and Rasooly 2000, Dinges *et al.* 2000). *S. aureus* is the mucous membrane of the nasopharynx, where it exists as a persistent or transient member of the normal microbiota without causing any symptoms. Organisms present in the nose may contaminate the hands; nasal carriers can thus become skin carriers, and these microorganisms can therefore be easily transmitted into manually handled foods. Some *S. aureus* excrete SEs can cause poisoning after the ingestion of contaminated food. It is also applied to cooked food due to the thermoresistance of the SEs (Balaban and Rasooly 2000). Staphylococcal food-poisoning (SFP) is

caused by staphylococcal enterotoxins (SEs) produced during massive growth of *S. aureus* in food. SFP is a prevalent cause of foodborne disease worldwide (Jablonski and Bohach 2001).

S. aureus is commonly found in milk and dairy products, particularly in cheese made either from raw or pasteurized milk (Coveney *et al.* 1994). *S. aureus* is present on the skin and mucosae of food-producing animal reservoirs, such as ruminants and it are frequently associated to subclinical mastitis leading to contamination of milk and previous dairy product (Jablonski and Bohach 2001). In France *S. aureus* is reported as the most frequent pathogen involved in food-borne diseases associated with dairy products and especially with raw milk cheeses (de Bouyser *et al.* 2001). It is generally accepted that SE production constitutes a risk, when *S. aureus* bacteria exceed a threshold of 10^5 *S. aureus* CFU/g of cheese during manufacture. During the cheese-making process, natural staphylococcal contamination is minor in the total microbial population. The initial *S. aureus* contamination is usually below 10^3 CFU/ml of raw milk, while bacterial starters are inoculated at least at 10^6 CFU/ml of milk (Dunquenne *et al.* 2010).

The presence of *S. aureus* was founded in ready-to-eat (RTE) and perishable foods, such as raw pork or smoked ham (Atanassova *et al.* 2001, Mataragas *et al.* 2008), poultry product (de Silvia Malheiros *et al.* 2010) or fish (Simon and Sanjeev 2007). The occurrence of enterotoxigenic *S. aureus* in ready-to-eat food product has been reported from different parts of the world including South East Asian countries like Taiwan, South Korea, Thailand, Vietman (Oh *et al.* 2007, Chiang *et al.* 2008, Houng *et al.* 2010).

Staphylococci can be expected to exist, at least in low numbers in all food products, that are of animal origin or in those that are handled directly by humans, unless heat-processing steps are applied to effect their destruction (Jay *et al.* 2005b).

II PACKAGING

Packaging science is a socio-scientific discipline which operates in society to ensure delivery of goods to the ultimate consumer of those goods in the best condition intended for their use. The system of product, packaging and distribution, within three main environments physical, atmospheric and human. In each of these environments, packaging performs three functions: protection, utility and communication. Packaging is also a domain between producer and consumer whose aim is to guarantee a product with a fixed qualitative standard from the producer and at the same time offers to the consumer a product which corresponds to the illustrated standards, in terms of hygiene, organoleptic and nutritional characteristics and quality of use (Meroni 2000). Consumers demands for more environmentally friendly packaging techniques, and for fresh, minimally processed foods with naturally preserved quality, are putting new demands on food packaging (Ahvenainen and Hurme 1997; Ahvenainen 2003, Marsh *et al.* 2007). Food companies have also placed a greater emphasis on increasing product shelf-life and product safety (Ackermann *et al.* 1995). Modern food packaging technologies include modified atmosphere packaging, active packaging and smart packaging, the main purpose of which is to enhance food safety and quality in as natural way as possible (Hotchkiss 1995).

1. Biodegradable packaging

The plastic has become overemphasized in the modern society. It has been widely applied in food and merchandise packaging, agriculture mulching, gas, liquors separation and medical technologies. The commodity plastics such as: polyethylene (PE), polypropylene (PP), polystyrene (PS), poly (vinyl chloride) (PVC) in a variety of forms such as: flexible bags, sachets, rigid containers have revolutionized the packaging industry (Mohanty *et al.* 2000). The plastic packaging is not easily recycled, these items end up, and they can last forever and never degrade in landfills. Many countries are faced with a decrease in landfill space, especially in densely populated areas. So finding new landfills for consumer and industrial waste may become more difficult in the future (Comstock *et al.* 2004). Biodegradable materials offer a possible alternative to the traditional non-biodegradable polymers, especially in short life-time application, where their recycling is difficult and not economical (Avella *et al.* 2005, Alves *et al.* 2006).

The term ‘‘biodegradable’’ materials is used to describe those materials which can be degraded by the enzymatic action of living organisms, such as bacteria, yeasts, fungi and the ultimate end-products of the degradation process, these being CO_2, H_2O, and biomass under aerobic conditions and hydrocarbons, methane and biomass under anaerobic conditions (Doi and Fukuda 1994). The objectives in the development of biodegradable packaging are renewable and potentially more sustainable sources of raw materials and integrated waste management approaches to reduce landfill (Davis and Song 2006). The raw material (**Figure 6**) are essentially derived from agriculture feedstock’s or marine food processing industry wastes and therefore it capitalizes on the natural resources preconservation with an underpinning on environmentally friendly and safe atmosphere (Tharanathan 2003).

Some biodegradable materials based or derived from starch such as Master – Bi™ (www.materbi.com), Nature Works™ Polylactide (www.cargilldow.com), Bioska™ (www.plastiroll.fi), Bioplast™ (www.biotec.be), Solanyl™ (www.biopolymer.net), Potatopac™ (www.potatoes.com), Greenfil™ (www.greenlightint.co.uk) and Eco-Foam™ (www.eco-foam.com) have been as commercialized and available. Despite higher cost compared with the traditional plastic counterpart, many have found increasing commercial applications in packaging (Davis and Song 2006).

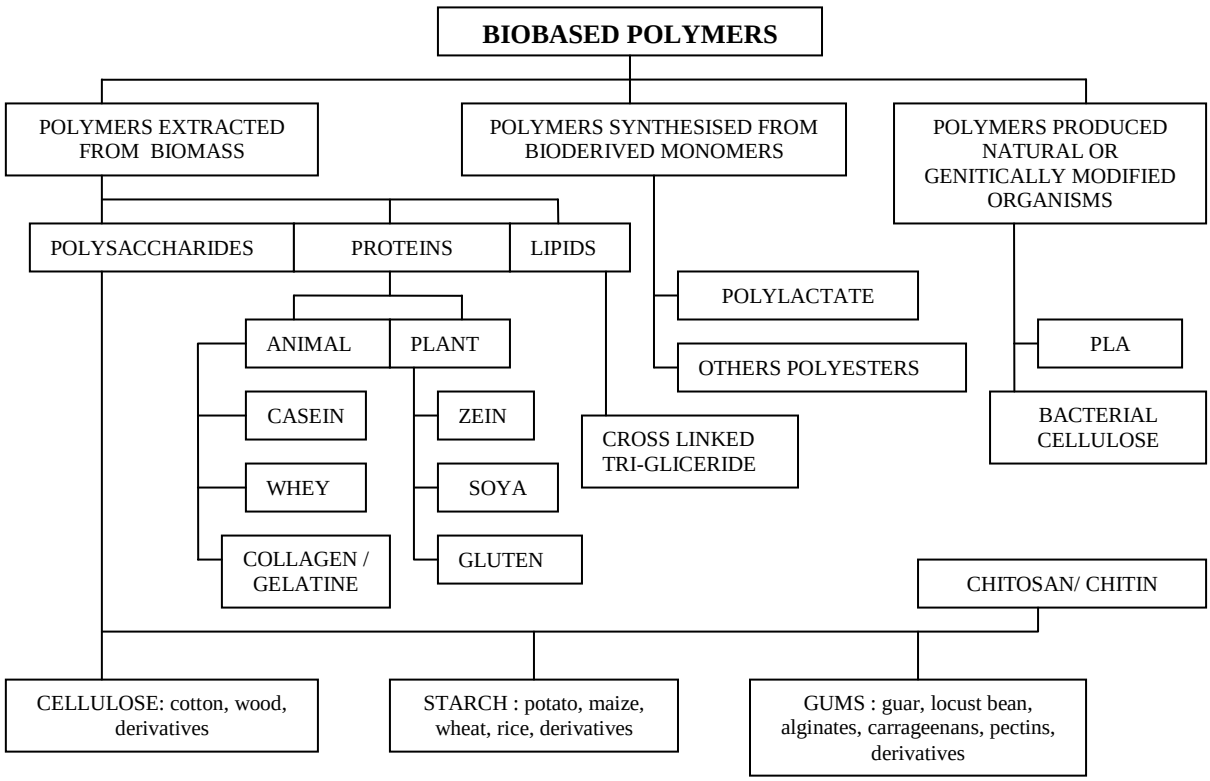


Figure 6. Natural biopolymers used in biodegradable packaging films (Weber *et al.* 2002).

The biologically based packaging must remain stable without changes of mechanical and or barriers properties physical and functional properly during storage. The most important parameters for controlling stability of biologically based packaging are appropriate: water activity, pH, oxygen, storage times and temperature (Petersen *et al.* 1999).

However using the biodegradable food packaging has been limited due to their poor barrier properties and weak mechanical properties. The others problems associated with the renewable biopolymers are threefold: performance, processing, and cost. The performance and processing problems caused by polymers extracted directly from biomass cellulose (Catia 2001), starch (Weiping *et al.* 2006), proteins (Cuq *et al.* 1998) are mainly due to their hydrophilic character. For this reason the natural polymers were blended (Long and Li 2006) or compete with others synthetic polymers or chemically modified by with the aim for extending their applications in different industrial fields as packaging, agriculture, hygiene and cutlery (Le Digabel and Avérous 2006). But modification or production of their derivatives is costly and difficult (Petersen, *et al.* 1999). Biodegradable packaging like the conventional packaging provide the protection of food, maintaining its sensory quality, safety and communication to consumer (Robertson 1993).

Packaging technologies are being implemented to play an important role in food preservation strategies, environmental impact and the consumer demands has led to new packaging development. Various term for new packaging can be find in the literature as: active, smart, interactive, biodegradable, intelligent packaging.

2. Paper and paperboard packaging

The use of paper and paperboards for food packaging dates back to the 17th century with accelerated usage in the later part of the 19th century (Kirwan 2003). Paper is widely used in food packaging applications and is generally more appreciated by consumers due to their advantages compared to plastic packaging (Song *et al.* 2000). Paper is inexpensive and it's manufactured from renewable and recyclables sources. Paper has a long successful history performing as filter, barrier media and can be even functional as sterile packaging. Paper is biodegradable (Pelton 2009) and offers a mechanical strength and flexibility, but is highly hygroscopic. It's a lack of barrier properties requires treatments to improve the surface properties (Andersson 2008). To improve prevent or minimize the loss of physical strength and to improve the water barrier property, the surface of paperboard can be coated (Dury-Burn *et al.* 2006, Parker *et al.* 2006, Triantafyllou *et al.* 2007, Han *et al.* 2010, Mascheroni *et al.* 2010).

Paper and paperboards are commonly used in corrugated boxes, milk cartons, folding cartons, bags and sacks, and wrapping paper. Tissue paper, paper plates, and cups are other examples of paper and paperboard products (Marsh and Bugusu 2007). Paper and paperboard packaging is used over a wide temperature range, from frozen food storage to the high temperatures boiling water and heating in microva and conventional radiant heat ovens (Kirwan 2003).

2.1 Raw materials

Paper is a network of natural cellulosic fibres made up of microfibrils which are composed of long chain cellulose ($C_6H_{10}O_5$) molecules in a crystalline structure (**Figure 7**). Cellulose fibres for papermaking are micro porous with a pore size ranging from 0.1 to 3mm. (Haggkvist *et al.* 1998). Since cellulose is hydroscopic, due to the OH sites in the basic unit of cellulose, paper can absorb water from the environment or from the food (Duny –Burn *et al.* 2006). The pulp fiber is produced from wood chips by acid or alkaline hydrolysis in which lignin in wood pulp is dissolved and removed by washing to leave only cellulose fibers.

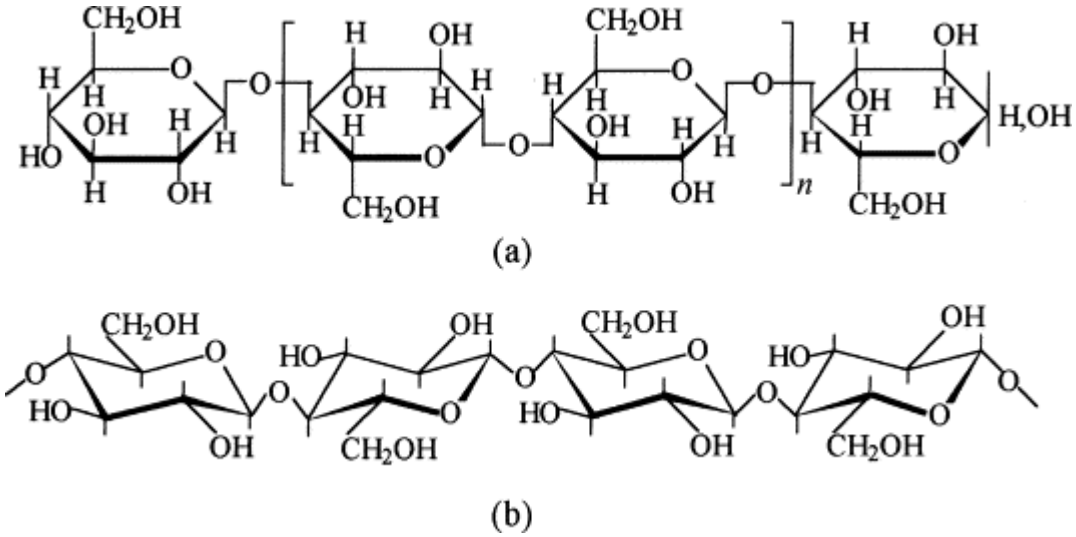


Figure 7. Cellulose structure: (a) Haworth formula; (b) chair formula (Bikales and Segal 1971).

2.2 Paper manufacture

The principal steps of papermaking include stock preparation, forming to create a uniform wet web, pressing to remove liquid water, drying to evaporate most of the remaining water and consolidate the product, calendaring to reduce the sheet thickness and improve the surface smoothness, and reeling to collect the dry product.

- Forming is the critical step of the papermaking process. The objective of forming is to drain water from a dilute fiber suspension deposited on a forming table, and to create a uniform fiber mat that has a sufficient consistency to be passed to the press section of the paper machine. Forming has a strong influence on a range of paper properties. Forming sets the fiber orientation in the sheet, which will influence the directionality of paper properties.
- Pressing is water removal from the wet web. Pressing also has an important influence on the finished product properties. The network of wet fibres leaving the former is bulky and contains a large volume of air. The product obtained by drying an unpressed, is deficient in wet web has a low density, strength, and smoothness, and is poorly suited for use as a printing base or packaging product. Only some hygienic tissue papers are made from unpressed webs.
- In drying, the web is heated and moisture is removed by evaporation. The high energy requirements, it makes the most expensive part of the paper machine to operate, and paper is dried only when a large portion of water is removed by drainage, suction, and pressing. The web leaves the press section and enters the dryer section of a paper machine at moisture content around 60%. The final moisture content after drying is 10% or less.
- Improving paper smoothness is calendering for printing and writing papers, and also for some packaging grades of paper and board. The dry web leaving the dryer section is bulky and has a rough surface. At the end of the paper machine, the dried and calendered sheet is reeled onto a large-diameter spool to produce large reels of paper called parent or jumbo rolls.
- The basic principles of reeling and winding are similar. During reeling, the spool of paper is pressed against a drum to obtain the desired roll density (Poirier *et al.* 2001).

2.3 Type of papers used in packaging

A wide range papers are commercially available to meet market needs resulting from the choice of fibres, blanched, unbleached, verging, recycled and modified at the stock preparation stages (Kirwan 2003). To illustrate various type papers, some examples are described at Table 3**.**

Table 3. Example of type paper commercialised on market (Kirwan 2003).

Type paper	Characteristic
Basic paper	• Structured cellulose and blanching agent (ex.Rocal)
Wet strength paper	• at least 30% of dry strength during water saturation • urea formaldehyde and melanine formaldehyde adding at the stock • resistant to water absorption
Microcreping	• invisible crimp into paper during drying by Clupak process • ability to withstand dynamic stress • hydration fibres at the stock preparation stage
Greaseproof Glassine	• fibres almost gelatinous • supercalendered greaseproof paper • very dense sheet with high smooth and glossy
Vegetable parchment	• passing trough a bath sulphuric acid • high grease resistance and wet strength
Tissues	• low and chloride and sulphate residues • lamination to aluminium foil or incorporation long fibre for a strong permeable sheet at low grammage
Paper labels	• machine glazed, machine finished or calendared Kraft papers (100% sulphate chemical pulp)
Sac kraft Impregnated paper	• bleached kraft • wax impregnation or fluorocarbon treatment • greasy fat resistance
Laminating papers	• lamination to aluminium foil and extrusion coated PE

Overall paper and board is a naturally renewable product, which does not pollute the environment in the course of manufacture and use. It is a recyclable and biodegradable material for packaging.

3. Active packaging

The first use of active packaging was proposed by Labuza, who defined active packaging as range of technologies, which represent the board lines between active, intelligent packaging (Labuza and Breene 1989**).** Active packaging systems improve quality and safety food, it could be divided into three categories: absorbers or scavengers, releasing systems and others systems (**Table 4**)**.** Absorbing or scavenging systems removed the undesired compounds such as: oxygen, carbon dioxide, ethylene, excessive water or taints. Releasing systems actively add or emit compounds to food or into head space of package such as: carbon dioxide, antioxidants, and preservatives. Others systems may have miscellaneous tasks such as: self-heating, self cooling and preservation. Depending on the psychical forms 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 (Rooney 1995, Floros *et al.* 1997, Meroni 2000, Ozdemir and Floros 2004).

Table 4. Example of some active packaging systems (Ozdemir and Floros 2004).

Type of active packaging systems	Substance used and mode of action
oxygen absorbing	enzymatic systems (glucose oxidase, alcohol oxidase, ethanol vapour); chemical systems (powdered iron oxide, ascorbic acid oxidation, catalytic conversion of oxygen)
carbon dioxide absorbing or emitting	iron powder -calcium hydroxide, ferrous carbonate-metal halide
moisture absorbing	silica gel, propylene, glycol
ethlylene absorbing	silica gel -potassium permanganate, silicon dioxide powder, zeolite, ozone
etanol emitting	encapsulated ethanol
antimicrobial releasing	sorbate, benzoate, antibiotics, silver-zeolite
antioxidant releasing	BHA, ascorbic acid, tocopherol
flavour absorbing	baking soda
flavour releasing	many food flavours
colour containing	various food colours
anti-fogging and anti-sticking	biaxially oriented vinylon
light absorbing or regulating	UV blocking agents
monitoring	time temperature indicators
temperature controlling	non- woven micro perforated plastic
gas permeable	perforated or micro porous films
microwave susceptors	methalized thermoplastics
insect repellent	low toxicity fumigants- pyrethrins, permethrin

4. Antimicrobial packaging

Antimicrobial packaging is one of the many applications of active packaging (Floros *et al.* 1997). Antimicrobial packaging is a promising and rapidly emerging biotechnology in which antimicrobial agents are incorporated into or coated onto food packaging materials to prolong the shelf life of packaged foods. The purposes of antimicrobial active packaging are the preservation of the food from microbial spoilage and hazardous foodborne microorganisms (Han 2000, Han 2003, Suppakul *et al.* 2003). Many preservatives such as: organic acids, fungicides, bacteriocins, proteins, enzymes, inorganic gases, silver substitute zeolite have been incorporated in packaging materials to provide antimicrobial activity (**Table 5**). Combinations of more than one antimicrobial incorporated into packaging have also been investigated (Vermeiren *et al.* 1999, Cutter 2002, Quintavalla and Vicini 2002, Cagri *et al.* 2004).

Table 5. Example of potential antimicrobial agents for antimicrobial food packaging systems

Classification	Antimicrobial agents
organic acid	acetic acid, benzoic acid, lactic acid, citric acid, malic acid, propionic acid, sorbic acid,
acid salt	potassium, sorbate, benzoate sorbate
fatty acid	lauric acid, palmitoelic acid
bacteriocin	nisin, pediocin, lacticin, subtilin
chelating agent	EDTA, citrate, lactoferrin
enzyme	lysozyme, lactoperoxidase, glucose oxidase
metals	silver, copper, zinconium
antibiotic	natamycin
fungicide	benomyl, imazalil, sulfur dioxide
sanitasing gas	ozone, chlorine dioxide, carbon monodioxide, carbon dioxide
sanitaser	triclosan
polysaccharide	chitosan
phelonic	catechin, cresol, hydroquinone
plant volatile	allyl isothiocynate, cinnamaldehyde, eugenol, thymol, carvacol
plants/ spice extract	grape seed extract, grapefruit seed extract, brassica erucic acid oil, rosemary oils, oregano oil
probiotic	lactic acid bacteria

A variety reviews and different types of antimicrobial packaging, antimicrobial agents, food applications are available at **Table 6** (Vermeiner, *et al.* 1999, Han 2000, Appendini and Hotchkiss 2002, Suppakul *et al.* 2003, Cagri *et al.* 2004, Cha and Chinnan 2004, Cooksey 2005, Cutter 2006, Rolf 2007, Coma 2008).

Table 6. Some examples of antimicrobial packaging.

Antimicrobial agent	Type packaging	Target microorganisms	Reference
sodium lactate polylysine	whey protein isolate film	Lactic acid Bacteria	Zinoviadou *et al.* 2010
potassium sorbate sodium benzoate	HPMC lipid film	*Penicillium digitatum, Penicillium italicum*	Valencia-Chamorro *et al.* 2009
sodium lactate potassium sorbate sodium benzoate nisin	chitosan-coated plastic film	*Listeria monocytogenes*	Ye *et al.* 2008
sodium lactate sodium diacetate	alginate coatings	*Listeria monocytogenes*	Neetoo *et al.* 2010
nisin potassium sorbate garlic oils	chitosan film	*Escherichia coli, Staphylococcus aureus, Salmonella* Typhimurium, *Bacillus cereus, Listeria monocytogenes*	Pranoto *et al.* 2005
nisin	cellulose film	*Listeria monocytogenes*	Nuygen *et al.* 2008
nisin	whey protein film	*Pseudomonas aeruginosa,Yarrowia lipolytica*	Pentado *et al.* 2010
natamicin malic acid		*Penicillium commune, Penicillium chrysogenum* *Listeria monocytogenes*	
nisin lactoferrin lysozyme	chitosan film	*Escherichia coli O157:H7, Listeria monocytogenes*	Brown *et al.* 2008
nisaplin	hydroxypropylmethcellulose HPMC	*Listeria* ssp., *Enterococcus* ssp., *Staphylococcus* ssp., *Bacillus* ssp.	Iram *et al.* 2010
pediocins	cellulose	*Listeria innocua, Salmonella* ssp.	Santiago Silva *et al.* 2009

Lysozyme	sodium caseinate film	*Micrococcus lysodeikticus, Staphylococcus aureus*	Mendes de Souza *et al.* 2010
Lysozyme	chitosan film	*Listeria monocytogenes, Escherichia coli, Pseudomonas fluorescens*	Duan *et al.* 2007
lysozyme nisin grape seed extract	Na-alginate [kappa]-carrageenan film	*Listeria monocytogenes, Escherichia coli, Salmonella* Enteriditis, *Staphylococcus aureus*	Cha *et al.* 2002
lysozyme albunim proteins disodium EDTA	zein fim	*Bacillus subtilis, Escherichia coli*	Gučbilimez *et al.* 2007
silver	chitosan lactate film	*Escherichia coli*	Tankhiwale and Bajpai 2010
chitosan	poly(vinyl alcohol) pectin film	*Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas, Candida albicans*	Tripathi *et al.* 2010
chitosan	chitosan film	*Aspergillus niger, Alternaria alternata, Rhizopus oryzae*	Ziani *et al.* 2009
chitosan -guanidine	paper	*Escherichia coli, Staphylococcus aureus*	Sun *et al.* 2010
chitosan potassium sorbate	sweet potato starch film	*Escherichia coli, Staphylococcus aureus*	Shen 2010
chitosan	HPMC film	*Aspergillus niger, Kocuria rhizophila*	Sebti *et al.* 2007
essential oils	chitosan film	*Listeria monocytogenes, Escherichia coliO157:H7*	Zivanovic *et al.* 2005
cinnamon, clove buld	apple film	*Escherichia coli O157:H7, Salmonella enterica, Listeria monocytogenes*	Du *et al.* 2008
Essential oils	milk protein film	*Escherichia coli O157:H7, Pseudomonas* spp.	Oussalah *et al.* 2004

4.1 Antimicrobial packaging system

According to Cooksey (2001), there are two basic categories of antimicrobial films. Antimicrobial agents may be incorporated in the non food parts of the packaging systems, which are the packages or the in package atmosphere. Antimicrobial agents can be incorporated directly in packaging materials in the form of films, over-coating on films, sheet, trays and containers or in the in-package space in the form of insert, sachets or pads. (**Figure 8**, Han 2003). 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 (Han 2000). The coating with a materials can release the antimicrobial agents onto the surface of the food, were a large portion of spoilage and contaminations occurs (Appendini and Hotchkiss 2002, Sebti *et al.* 2002, Buonocore *et al.* 2003). The antimicrobial agents may either be released through evaporation in the headspace (volatile substances) or migrate into the food (non-volatile additives) through diffusion. Antimicrobial packaging materials contact with the surface of the food if they are non-volatile, so the antimicrobial agents can diffuse to the surface, therefore, surface characteristics and diffusion kinetics become important. The theoretical advantage of volatile antimicrobials is that they can penetrate the bulk matrix of the food and that the polymer need not necessarily directly contact the product (Appendini and Hotchikss 2002).

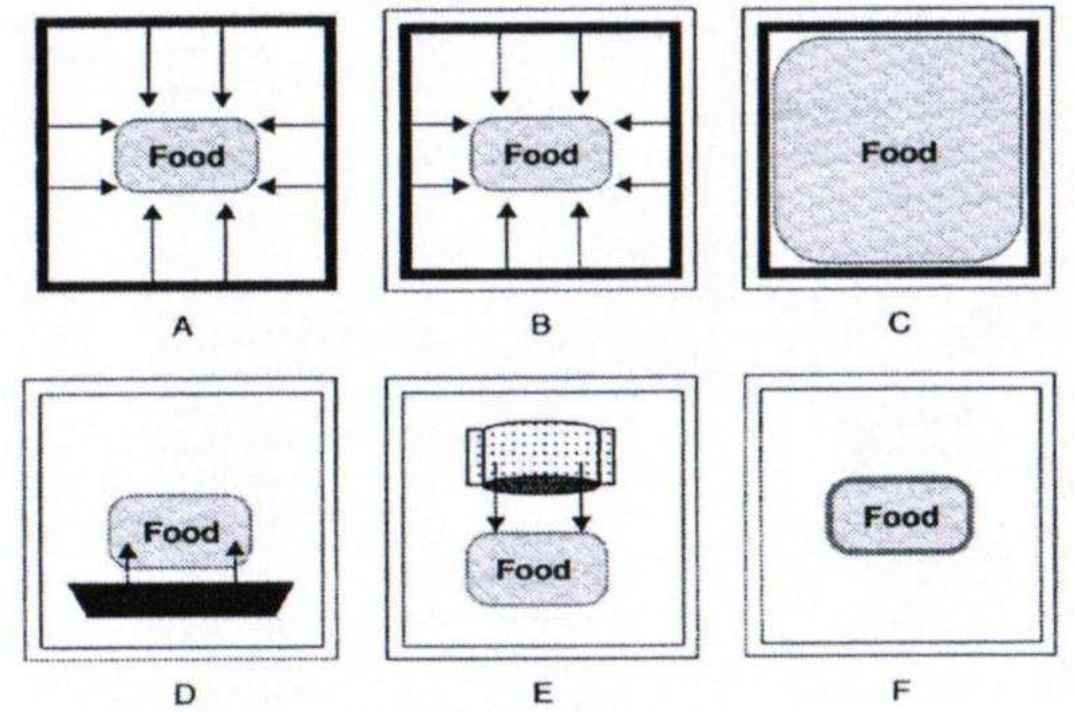


Figure 8. Possible ways to construct antimicrobial food packaging systems; A the use of antimicrobial packaging material; B antimicrobial coating on conventional packaging materials; C immobilization of antimicrobial agents in polymeric packaging materials; D the use of antimicrobial trays or pad; E the use of sachet or insert volatile antimicrobial agents; F antimicrobial edible coating on food (Han 2003).

The fundamental driving force in the transfer of components through a package system is the tendency to equilibrate the chemical potential. Mass transport through polymeric materials can be described as a multistep process. First, molecules collide with the polymer surface. Then they adsorb and dissolve into the polymer mass. In the polymer film, the molecules diffuse randomly as their own kinetic energy keeps them moving from vacancy to vacancy as the polymer chains move. The movement of the molecules depends on the availability of vacancies or 'holes' in the polymer film. These 'holes' are formed of vacancies in the polymer film. These 'holes' are formed as large chain segments of the polymer slide over each other due to thermal agitation. The random diffusion yields a net movement from the side of the polymer film that is in contact with a high concentration or partial pressure of permeant to the side that is in contact with a low concentration of permeant. The last step involves desorption and evaporation of the molecules from the surface of the film on the downstream side (Singh and Hedman 1993). Absorption involves the first two steps of this process, i.e. adsorption and diffusion, whereas permeation involves all three steps (Delassus 1997). Mass transfer processes between foods and packages are governed by both kinetics and thermodynamics (Arvanitoyannis and Bosnea 2004, Dury- Burn *et al.* 2007). The diffusion coefficient is a kinetic parameter that provides information on migration velocity, whereas solubility and partition coefficients are thermodynamic parameters that measure migrant transfer (Desobry 1998, Tehrany and Desobry 2004, 2007).

Several studies have reported the migration phenomena in food/packaging systems (Dole *et al.* 2006, Mauricio-Iglesias *et al.* 2009, De Abreu *et al.* 2010). In laboratory (LiBio) Mousavi *et al.* (1998a,b) developed a mathematical of migration of volatile compounds into packaged food via free space, Tehrany (2004), Tehrany and Desobry (2006) developed a method to calculate partition coefficient in food packaging systems and Desobry (1998) studied mass transfer in food system.

4.2 Factors affecting the effectiveness of antimicrobial packaging

Many factors should be considered in designing antimicrobial packaging systems beside the factors desirable above such as: antimicrobial agent characteristics, incorporation methods, permeation and evaporation. Extra factors include specific activity, resistance of microorganisms, controlled release, release mechanisms, chemical nature of food and antimicrobials storage and distribution conditions, film container casting process conditions, physical, chemical properties of

antimicrobial packaging materials, organoleptic characteristics and toxicity of antimicrobials and corresponding regulations (Han 2003).

The target microorganisms and the food composition must be considered in antimicrobial packaging (**Table 7)**. As with any antimicrobial, those to be incorporated into polymers have to be selected based on their spectrum of activity, mode of action, chemical composition, and the rate of growth and physiological state of the targeted microorganisms. The solubility of antmicrobial agent to the food is also a crucial factor (Han 2000, 2003). If the antimicrobial agent is compatible with the packaging materials, a significant amount of the agent may be incorporated into the packaging materials without any deterioration of its physical and mechanical integrality (Han and Floros 1997).

Table 7. Application of antimicrobial packaging against various food pathogens in different food systems (Cha and Chinnan 2004).

Food product	Antimicrobial agent	Target microorganism
Meat and fish product		
beef	pediocin, nisin, triclosan	*Listeria monocytogenes, Brochothrix thermosphata, Salmonella Typhimurium, Escherichia coli H157:H7, Bacillus subtilis*
ground beef	grape fruit seed extract	*Micrococcus flavus, Escherichia coli Staphylococcus aureus, Bacillus subtilis*
ham	lacticin 3147, nisin	*Lactococcus lacits*, *Listeria innocua*, *Staphylococcus aureus*
poultry	nisin	*Salmonella Typhimurium*
ham, bologne,pastrami	acetic acid, propionic acid	*Lactobacillus sakei, Serratia liquefaciens*
fresh broiler skin	nisin	*Salmonella Typhimurium*
Vegetable product		
strawberry	potassium sorbate, citric acid	aerobic, mesophilic, psychotrophic bacteria mold, yeast, coliforms
tomato	citric acid, acetic acid, sorbic acid, ethanol	*Salmonella Montevideo*
lettuce, soybean sprouts	grape fruit seed extract	*Escherichia coli, Staphylococcus aureus*
Milk and dairy product		
skim milk	nisin	*Lactobacillus curvatus*
cheddar cheese	lacticin 3147, nisin	*Lactobacillus lactis, Listeria innocua Staphylococcus aureus*
cheese	imazalil	*Penicillium sp., Aspergillus toxicarius*

The properties of a food as well as the extrinsic parameters determine the fate of microorganisms. Because microorganisms require water, carbon sources (sugars, alcohols) for energy, nitrogen (amino acids), B vitamins, related growth, factors, and minerals, the composition, water activity (aw), and oxidation-reduction potential (ORP) of food are important properties in determining their growth and proliferation. Additionally, extrinsic parameters such

as pH, relative humidity, temperature, and gaseous atmosphere will influence microbial growth (Cutter 2002).

The design of antimicrobial packaging systems requires controlled release technology and microbial growth kinetics. When the mass transfer rate an antimicrobial agent is faster than the growth kinetic of the target microorganisms, the antimicrobial agent will be depleted before the expected storage period. The packaging will be losing its antimicrobial activity because the package food has infinitive volume compared to the volume of packaged material and the amount of antimicrobial agent. Consequently the microorganism will start to grow following the depletion of antimicrobial agent. However, when the migration is too slow to control the growth of the microorganism, the microorganism can grow before the antimicrobial agent is release (Han 2000, 2003).

4.3 Edible films as an antimicrobial packaging

Edible films and coating, which contains antimicrobial agent are antimicrobial packaging, that effectively inhibit pathogenic and spoilage organisms in food. The use of edible films and coatings for a wide range of food products, including fresh and minimally processed vegetables and fruits, receives increasing interest. It improves product shelf-life and effectively inhibits pathogenic and spoilage organisms in food, may add to the texture and sensory characteristics, and is environmentally friendly. Their application relates to their function as internal moisture or solute barriers with heterogeneous foods, individual protection of food pieces, encapsulation of functional food additives (Guilbert *et al.* 1995, Guilbert *et al.* 1996, Cutter 2004, Kwhaldia *et al.* 2004, Lin and Zhao 2007, Olivias *et al.* 2008, Vargas *et al.* 2008).

4.4 Application of antimicrobial packaging in food

Antimicrobial polymers can be used in several food related applications including packaging (Hotkchiss 1995 a,b). One is to extend the shelf-life and promote safety by reducing the rate of growth of specific microorganisms by direct contact of the package with the surface of solid foods as: meats, cheese or in the bulk of liquids, milk or meat exudates. Second, antimicrobial packaging materials could be self-sterilizing or sanitizing. Such antimicrobial packaging materials reduce the potential for recontamination of processed products and simplify the treatment of materials in order to eliminate product contamination. Self-sterilizing packaging might eliminate the need for peroxide treatment in aseptic packaging. The self sanitizing might be

particularly useful for high acid products such as fruit juices. Antimicrobial polymers might also be used to cover surfaces of food processing equipment so that they self sanitize during use. Examples include filler gaskets, conveyers, gloves, garments, and other personal hygiene equipment (Appendini and Hotchkis 2002). Antimicrobial packaging films should be able to provide antimicrobial activity even during or after processing steps as: heat or pressure treatments and can exert antimicrobial activity on the few remaining microbes. Antimicrobial films could allow processing to be done at lower temperatures or pressures, thus reducing processing costs without compromising food safety (Rolf 2007).

The commercialization of active packaging systems requires evaluations of the many marketing aspects as: logistic, cost and consumer acceptance of this packaging which tends to be confused with the foodstuffs. Consumers acceptance is related to the conveniences and easiness of the use of this new packaging and any conflict with their culture and lifestyles (Meroni 2000).

Active packaging was firstly introduced in the market of Japan in the mind 1970s, and only in the mind 1990s was raised the attention of the industry in Europe and USA (Restuccia *et al.* 2010). Until 2004 in Europe there was a legislative lack for active and intelligent packaging, which decreased their penetration in the EU market. To face the problem Regalement 1935/2004/EC and more specifically Regalemention 450/2009/EC set new legal basis for their correct use, safety and marketing (Restuccia *et al.* 2010).

MATERIALS
&
METHODS

II. MATERIALS AND METHODS

1. MATERIALS

1.1 Antimicrobials

Nisin was used in the form of the commercial preparation (Nisaplin®, 2.5% pure, SIGMA®). Solution of nisin was prepared by dissolving Nisaplin® in 0.02 N HCl to assure complete solubilization. Subsequently, 5 N NaOH was added until a pH of 6.5 and a final concentration of 10 $g.l^{-1}$ of nisin was obtained. Solution of nisin was prepared on day of the experiment.

Lysozyme from egg hen egg white, Fluka Biochemika, white, crystalline power) was prepared in distilled water to obtain a concentration of 10 $g.l^{-1}$. Solution of lysozyme was prepared on day of the experiment.

Lactic acid was prepared from a 90% L-lactic acid stock solution (Acros Organics®) dissolved in sterile distilled water. Solution of lactic acid was prepared on day of the experiment.

1.2 Bacterial strains and culture conditions

Bacillus brevis CIP 5268, *B. cereus* CIP 6624, *B. coagulans* CIP 6625, *B. licheniformis* CIP 52.71, *B. macerans* CIP 6619, *B. polymixa* CIP 6622, *B. stearothermophilus* CIP 675, *B. thermoacidurans* CIP 526, *Staphylococcus aureus* CIP 677, *S. aureus* CIP 7625, *S. aureus* CIP 4.83, *S. aureus* CIP 5710, *Listeria innocua* CIP 125111, *L. ivanovii* CIP 12510, *L. monocytogenes* CIP 82110, *L. monocytogenes* CIP 7831, *Micrococcus luteus* CIP A270 were obtained from the Institut Pasteur Collection (CIP) Paris, France, *B. megaterium* ATCC 9885, *B. subtilis* ATCC 6633 were obtained from the American Type Culture Collection (ATCC) Mannassas, USA and *L. seeligerii* SLCC 3954 was obtained from the Special *Listeria* Culture Collection (SLCC), University of Wurzburg, Germany.

All strains were stored in TSBYE medium supplemented with glycerol (10%) at - 30°C and propagated twice before use.

1.3 Medium

TSAYE- Tween medium, which consisted of (per liter): 6g of Yeast Bacto™ Extract (Becton, Dickinson and Company, Le Pont de Claix, France), 30g of Broth Trypcase Soya (Biokar Diagnostics, Beauvais, France), 12g of Agar Bacteriological (Biokar Diagnostics,

Beauvais, France), 1% of polyoxyethylene sorbitan monooleat Tween 80 (Merck, Germany) was mixed in 1L of distilled water, sterilized for 15 min at 121°C.

TSBYE- Tween medium, which consisted of (per liter): 12g of Yeast Bacto™ Extract (Becton, Dickinson and Company, Le Pont de Claix, France), 60g of Broth Trypcase Soya (Biokar Diagnostics, Beauvais, France), 1% of polyoxyethylene sorbitan monooleat Tween 80 (Merck, Germany) was mixed in 1L of distilled water, sterilized for 15 min at 121°C.

Agarose gels were prepared at 2 %, 3%, 5% (w agarose/w distilled water). The mixture was heated to the dilution agar, under continuous mechanical agitation. Perti dishes were filled with 10 ml the agar (this volume corresponds to thickness of 1 mm agarose gel), then allowed to solidify at the room temperature and then held at 4°C for 3h to allow to full solidification.

1.4 Cellulose support

Paper (Rocal 400, 37 $g.m^2$, Ahlstrom Pont-eveque, France) possess following properties (**Table 8**).

Table 8. Characteristic of Rocal paper.

Characteristic	Unity	Medium value	Protocol standard
Grammage	$g.m^2$	37±2.12	ISO 536
Thickness	µm	33±1	ISO 534
Moisture	%	6±0.7	ISO 287
Mechanical			
Tensile strength SM	$KN.m^{-1}$	3.09±0.15	ISO 1924
Tensile strength ST	$KN.m^{-1}$	2.22±0.17	ISO 1924
Elongation SM	%	1.48±0.11	ISO 1924
Elongation ST	%	5.48±0.39	ISO 1924
Tearing strength SM	mN	201±11	NF Q 03-011
Tearing strength ST	mN	200±9	NF Q 03-011
Bursting strength	kPA	116±6	NF Q 03-011
Optical			
Brightness EL	%	86±5.65	ISO 2470
Opacity photovolt	%	70.5±3.53	ISO 2470
Opacity after paraf	%	62.5±3.53	ISO 2470
Permeabilility			
Water vapour permeability	$g.\mu m.m^2.j^{-1}.k.Pa^{-1}$	868±10	NF H 00-030
Oxygen permeability	$g.\mu m.m^2.j^{-1}.k.Pa^{-1}$	825±15	Laboratory Method (Desobry and Hardy 1997)

2. METHODS

2.1 Determination of sensitivity nisin & lysozyme & lactic acid alone against *Bacillus, Listeria and Staphylococcus aureus* strains

Minimum Inhibitory Concentration is defined as the lowest concentration of the agent that inhibits visible growth. The agar and broth dilution assays were used. The inhibitory compound is serially diluted. In agar dilution methods, the antimicrobial is placed in a well. The compound diffuses through the agar, and inhibition activity is indicated by a zone of no growth around a well (Barry 1986). In broth dilution assays the inhibitory compound is distributed in a nutrient broth, which then is inoculated with a single strain of microorganism. The absence of turbidity is considered a compound activity (Lorian 1986).

Agar diffusion technique was described by Tamer and Fowler (1964) was used to determine the MIC of nisin, lysozyme and lactic acid against different tested strains. For antibacterial activity detection, 1% of polyoxyethylene sorbitan monooleat Tween 80 (Merck, Germany) was added to TSAYE medium. TSAYE-Tween medium was cooled to about 40°C and inoculated with 1% of the 24 h culture (~ 10^9 cfu.mL^{-1}) of each tested strains. Sterile Petri dishes were filled with 12 ml the agar media, allowed to solidify at the room temperature and then held at 4°C for 3 h to allow full solidification. Following the solidification on each plate wells were bored. After placing of 20µl of each nisin and lysozyme and lactic acid solution into a well, the agar plates were stored at 4°C for 24 h to allow diffusion of solutions and then incubated at 30°C *Bacillus* strains and 37°C *Listeria* and *Staphylococcus* strains until zones inhibitions were evident. The inhibitions zone diameters were measured with aid of a graduated ruler. Each selected microorganisms and antimicrobials were replicated three times.

Broth dilution technique was used (Ericsson and Sherries 2007). Sterile polysterene 96-well microplate (Nunc, Roskilde, Denmark) were filled with 100µl of distilled water. Then 100µl of nisin, lysozyme or lactic acid were added to the first well and of a row and two-fold dilutions were made and mixed three times to thoroughly mix the solution between each dilution. A log phase culture of *Bacillus*, *Listeria* and *Staphylococcus* tested strains was adjusted to the absorbance of OD 0.02 (at 660_{nm}) per ml, then diluted in TSBYE -Tween medium. The 100µl of diluted culture was dispended into each well. Microplates were mixed during 5 min and incubated at 30°C *Bacillus* strains and 37°C *Listeria* and *Staphylococcus* for 24 and 48 h with the

absorbance at 660 nm reading being taking every 0, 2, 4, 6 and 24 h with a microplate reader. Triplicate wells were used for each treatment and each experiment was repeated at least twice.

2.2 Determination of interactions between nisin & lysozyme, nisin & lactic acid and nisin & lysozyme & lactic acid against *Listeria monocytogenes* CIP 82.110 and *Staphylococus aureus* CIP 4.83 and nisin & lysozyme against *Bacillus licheniformis* CIP 52.71 and *Bacillus subtilis* ATCC 6633

A checkerboarder microassay was used to evaluate all combination of lysozyme and lactic acid with nisin studies (Barry 1976). Concentration of each antimicrobial used against *L. monocytogenes* and *S. aureus* were following ($g.L^{-1}$): nisin 0.5; 0.25; 0.125; 0.06; 0.03 and lysozyme ($g.L^{-1}$) :0.06; 0.03; 0.0015; 0.0075; 0.0035 and lactic acid (%) 0.5; 1% respectively. Concentration of each antimicrobial used against *Bacillus* strains were following ($g.L^{-1}$): nisin and lysozyme ($g.L^{-1}$) :0.039; 0.078; 0.156; 0.312; 0.625; 1.25 respectively. Microplates were used in combination studies. For each test, a total volume 200 µl was used consisting of 100 µl (10^7 $cfu.mL^{-1}$) of *L. monocytogenes* or *S. aureus* diluted in TSBYE-Tween broth and 50 µl of each antimicrobial. Microplates were mixed during 5 min, incubated at 30°C for *Bacillus* strains and 37ºC for *L. monocytogenes* and *S. aureus* for 24 h with the absorbance 660 nm reading being taking every 0, 2, 4, 6 and 24 h. Triplicate wells were used for each treatment and each experiment was repeated at least three times.

2.3 Evaluation of the Fractional Inhibitory Concentration (FIC)

Combined studies are conducted to determine if specific types of interactions occur between the two combined antimicrobials. MIC of the individual antimicrobials and all of the combination were plotted to a form of isobologram (Parish and Davidson 1993). Fractional inhibitory concentration (FIC) for an individual antimicrobial agent is the ratio of the concentration in an inhibitory combination with a second agent to the concentration of the concentration by itself (Barry, 1976). The FIC is calculated as the ratio of the MIC of a compound when combined a second one compound divided by the MIC of the first compound alone: $FIC_{A} = MIC_{A \text{ with } B} / MIC_{A}$. The FIC of each antimicrobial in a combination were added to give a total FIC index: $FIC_{index} = FIC_{A} + FIC_{B}$

Calculation of FIC_{index} can be an indicative of interactions between of antimicrobial. As defined by Barry (Barry 1976) can be occur an additive, synergistic or antagonism effect. Additive effect occur when the antimicrobial activity of a compound is neither enhanced nor reduced while in the presence of another agent. Synergism refers to an enhancement of overall antimicrobial activity of compound when in the presence of a second antimicrobial agent.

Antagonism occurs when the antimicrobial activity of one compound is reduced when in presence of a second agent. If a FIC_{index} is < 1 the interaction is synergistic, if a FIC_{index} = 1 the interaction is additive and an FIC_{index} is > 1 antagonistic (Parish and Davidson 1993).

2.4 Effect of antimicrobials: nisin & lysozyme & lactic acid alone or in the mixture on the inhibition of *Listeria* monocytogenes CIP 82.110 and *Staphylococcus aureus* CIP 4.83 using an experimental design

An experimental design using a Doehlert (Doehlert, 1970) matrix was performed in order to evaluation of mixture composition with nisin, lysozyme and lactic acid (**Table 9**) on inhibition *L. monocytogenes* and *S. aureus*. Proportions of mixture are indicated as percentage and the sum of the proportions of the these three ingredients is unity:

% Nisin + % Lysozyme + % Lactic acid = 10%

The factorial growth (response) expressed as $\log_{10}$ cfu.mL^{-1} can be predicted in all experimental regions according to the following equation:

$$Log_{10} = \beta 1\ N + \beta 2\ L + \beta 3\ LA + \beta 12\ N\&L + \beta\ 13\ N\&LA + \beta 23\ L\&LA + \beta\ 123\ N\&L\&LA$$

where β1 determines nisin, β2 lysozyme; β3 lactic acid ; β12 the mixture nisin and lysozyme; β13 the mixture nisin and lysozyme ; β23 the mixture lysozyme and lactic acid and β123, the mixture nisin, lysozyme and lactic acid. Data analysis, ANOVA, and polynomial regressions were performed using the NEMROD® software (LPRAI, Marseille, France; Mathieu and Phan-Tan-Luu, 1997).

Table 9: Experimental design for three variables nisin, lysozyme and lactic acid according to (Doehlert 1970) uniform shell design.

Nº experiment	Abbreviation	Nisin concentration ($g.L^{-1}$)	Lysozyme Concentration ($g.L^{-1}$)	Lactic Acid Concentration (%)
0	Control	0	0	0
1	N	0.25	0	0
2	L	0	0.015	0
3	LA	0	0	0.5
4	N&L	0.125	0.0075	0
5	N&LA	0.125	0	0.25
6	L&LA	0.0000	0.0075	0.25
7	$N_{1/3}\&L_{1/3}\&LA_{1/3}$	0.00825	0.00495	0.165
8	$N_{2/3}L_{1/3}\&LA_{1/3}$	0.165	0.0025	0.08
9	$N_{1/3}\&L_{2/3}\&LA_{1/3}$	0.04	0.0099	0.08
10	$N_{1/3}\&L_{1/3}\&LA_{2/3}$	0.04	0.0025	0.33

Enumeration studies of these 10 mixtures nisin, lysozyme and lactic acid were done to determine effectiveness of combination these antimicrobial molecules against *Listeria monocytogenes* CIP 82.110 and *Staphylococcus aureus* CIP 4.83 with two initial inoculum level

of 10^4 and 10^7 cfu.mL^1. The mixtures were prepared to contain the inoculum: *L. monocytogenes*, *S. aureus* lysozyme, nisin and lactic acid respectively were added to TSBYE with Tween broth, then were inoculated at 37°C and sample were taken at 6 and 24 h. Samples 10 fold serially were diluted with solution of tryptone salt. Surviving and injured *L. monocytogenes*, *S. aureus* were enumerated on TSAYE + Tween medium with use of a spiral plating system (Don Whitley Scientific Limited, West, Yorkshire, Uk). Plates were incubated for 24h at 37°C .

2.5 Impact of nisin & lysozyme on cell membrane of *Listeria monocytogenes* CIP 82.110

The influence of nisin and lysozyme on potassium efflux and membrane potential (ΔΨ) in *L. monocytogenes* were determined.

2.5.1 Measurement of membrane potential (ΔΨ)

Nigericin and valinomycin (Sigma-Aldrich) were prepared in chloroform or in dimethylsulfoxide, respectively, to a concentration of 10 mM. Cells were harvested in the log growth phase (O.D 660=0.6), washed twice with ice-cold 50 mM 4-2-hydroxyethyl- 1 piperazineethanesulfonic acid (HEPES) buffer containing 0.6 mM of KCl, glucose 0.2%, adjusted at pH 7.0 with KOH 40%, resuspended in the same buffer to 1/100 of their initial volume, and stored on ice.

The ΔΨ was qualitatively measured with the fluorescent probe 3,3′dipropylthiadicarbocyanine iodide [(DiSC3(5), Sigma-Aldrich]. Glucose-energized cells (to a final population of 2.5×108 cfu mL^{-1}) were added to quartz cuvette containing HEPES buffer and DiSC3(5) (5 μM). Then, after 3 min of incubation with valinomycin (1.5 nM), which dissipates the ΔY, nisin and lysozyme (1× MIC) or nigericin (1.5 nM) were added. Fluorescence measurements were performed with a spectrofluorometer FLX (Safas-Monaco, Monaco) at 622 and 670 nm for excitation and emission, respectively (Herranz *et al.* 2001).

2.5.2 Effect of nisin and lysozyme on potassium efflux

Potassium leakage was determined as described by Denyer and Hugo (1991). Stationary-phase cells *L. monocytogenes* were harvested by centrifugation (10,000×g at 4°C, 15 min). The pellets were washed three times with tryptone-salt (TS; 0.9%) supplemented with glucose (1%). Cells were resuspended in TS-glucose to a final population of 2.5×108 cfu mL−1. Nisin, lysozyme and the same volume of buffer without the peptide were added to a final concentration corresponding to 0.5× MIC and MIC. Cells were incubated with nisin and lysozyme for 60 min at 30°C. The cells were harvested

by centrifugation (10,000×g at 4°C, 15 min), and the concentration of the potassium of the supernatant was determined with the atomic absorption spectrophotometer 1100 (Perkin-Elmer, Courtaboeuf, France).

2.6 Effectiveness of antibacterial activity on the paper with nisin & lysozyme & lactic acid

Paper were tested for antimicrobial activity against *Bacillus. licheniformis* CIP 52.71, *Listeria monocytogenes* CIP 82.110 and *Staphylococcus aureus* CIP 4.83. Following mixtures were coated onto paper: combination of nisin 0.625 $g.L^{-1}$ and lysozyme (1.25 $g.L^{-1}$) against *B. licheniformis* and combination nisin 0,165 $g.L^{-1}$, lysozyme 0,0025 $g.L^{-1}$ and lactic acid 0,08% or nisin 0,125 $g.L^{-1}$ and lactic acid 0,25%. The coating of 20 µl mixtures was manually on one side of the paper 1cm*1 cm using a pipette. Paper was dried by exposure to ambient temperature and placed in a Petri dish with TSAYE medium, inoculated with 1% of the 24 h culture (~ 10^9 $CFU.mL^{-1}$) of each tested strains. After incubation at 37ºC for 24 h, antimicrobial activity was observed as a zone of inhibition of the indicator organism around the paper. The inhibitions zone diameters were measured with aid of a graduated ruler.

2.7 HPLC active compounds assay

Quantitative determination of nisin into the water solution was made by slighty modifying the method proposed by Liu Hansen (1990) and lysozyme by Liao (2001). Nisin and lysozyme release kinetics were evaluated by HPLC method. A 50 and 10µl samples of lysozyme (initial-untreated, acetone, ethanol) and nisin (initial-untreated, acetone, ethanol) was applied to the C_{18} column at 25°C and eluted at the flow rate of 1,5 ml min^{-1} in a gradient of acetonitrile in aqueous 0,1% trifluoroacetic acid (TFA) using the procedure summarized in Table 10, detected at a wavelength of 220 nm and 254 nm and injection volume 10 and 50µl wavelength. The samples before the HPLC analysis were filtered using the filter with a pore 0,22 µm in diameter Millex® GP, Milipore.

Table 10. The mobile phase composition.

Gradient	water	acetonitrile 0.1% TFA
0-10	80	20
10-20	60	40
20-30	40	60
30-40	20	80

2.8 Purification of nisin and lysozyme

2.8.1 Purification from whey proteins by using the extraction acetone

First 6 volumes of acetone was added to 1 volume of nisin and lysozyme standard solutions at the temperature 20ºC. The suspensions were precipitated for 4 h at 20ºC. After precipitation, the suspensions immediately were centrifuged at 11000 rpm for 20 min at 4ºC (Centrifuge model eppendorf 5804R). The supernatants were washed with 6 volumes acetone at –20ºC and centrifuged 11000 rpm for 20 min at 4ºC. The supernatants were dried at the room temperature and then dissolved in phosphate buffer. The purify nisin and lysozyme samples were analyzed in HPLC.

2.8.2 Purification from whey proteins by using the extraction ethanol

The 6 volumes of 25 % ethanol (vol/vol) diluted with distilled water was added to 1 volume of nisin and lysozyme standard solutions and stirred during 2 h at the room temperature. The suspensions were centrifuged at 1500 rpm for 5 min at the room temperature (Centrifuge Firlabo). The supernatants were dissolved in 25 % (vol/vol) ethanol. The purify nisin and lysozyme samples were analyzed in HPLC.

2.9 Nisin quantification by BCA protein method

The calorimetric assay using bicinchoninic acid (BCA) as test reagent is useful for quantitative protein determinations due to its high sensitivity, ease, and tolerance to various contaminations present in biological samples or added during purification (Schoel *et al.* 1995).

The BCA Protein Assay combines reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The reaction that leads to BCA color formation is strongly influenced by four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) in the amino acid sequence of the protein. However, unlike the Coomassie dye-binding methods, the universal peptide backbone also contributes to color formation, helping to minimize variability caused by protein compositional differences. (Krohn 2002).

The BCA Protein Assay Kit (Pierce, Bonn, Germany) was used according to the manufacturer's instructions using the microplate procedure. 0.2 ml BCA working reagent were mixed with 25 µl of the sample, placed at 37°C for 30 min. The absorbance was read at 490 nm. Absorbance 490 nm was converted to nisin concentration using a nisin equilibrium curve established from gels containing known nisin quantities. Nisin standard solutions of concentration 0.15, 0.3, 0.6, 1.25 2.5 mg.mL^{-1}. The Figure 9 presented nisin quantification, based on bicinchoninic acid assay.

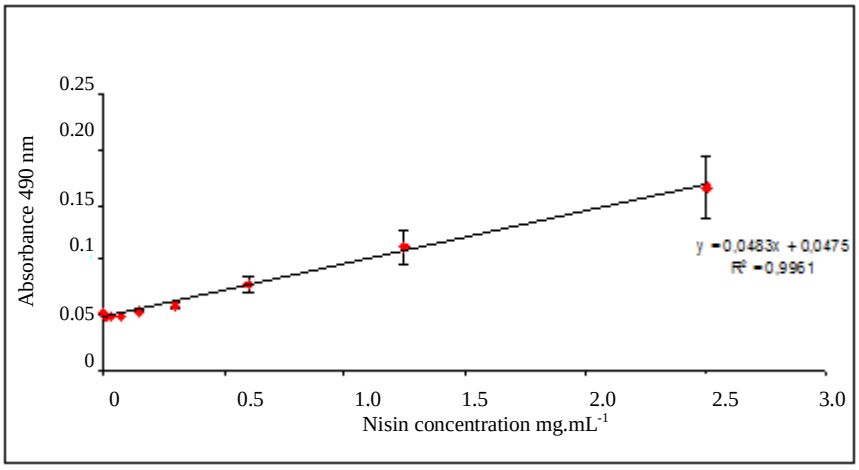


Figure 9. Standard curve of determination nisin by BCA protein assay.

2.10 Incorporation nisin onto paper for diffusion evaluation

The piece of paper (63,5 cm^2) with 0.75 mL^{-1} of nisin at the concentration of 20 g.L^{-1} was placed onto Petri dish with the 2 and 5% agarose gel, Petri dish was coated with paraffin and parafilm layers, to prevent the distortion of the gel during handling, to laterally water-proof and to limit gel dehydration during diffusion. The diffusion experiments were performed at temperature 37°C for 24 h and repeated at least three times. Nisin extracted from agarose gel was quantified using the BCA method protein assay and quantified using nisin equilibrium curves previously established from gels containing known nisin concentrations**.**

2.11 Diffusion test

Two diffusion tests were performed in extraction nisin from agarose gel. The bottom surface of the agarose gel was placed on a nisin at the concentration 20 g.L^{-1} incorporated into paper (63,5 cm^2 and fixed to assure an interfacial contact with agarose gel. The diffusion experiments were performed for 5 days at 37 ºC After 24, 48, 72 and 110 h of incubation at 37 ºC, 2% and 5% agarose gel were cut into disc (Ø 3cm; approximately weight 1g) small pieces, placed

in tube, immersed in 4 ml HCl 0,1M solution, then stored at 20ºC for 5 h. The loyphilization were used before quantification nisin by BCA protein assay. Samples were loyphilizated at -57ºC during 18 h (Loyphilisateur Christ Aplha 1-2, Germany), then were diluted in 0.2 ml of distilled water. A volume of 0.2 ml of solution BCA and 25 µl of sample with nisin were mixed and heated at 37ºC for 30 minutes. The absorbance at 490 nm was measured using multi mode microplate reader (Biotek Synergy HT, USA).

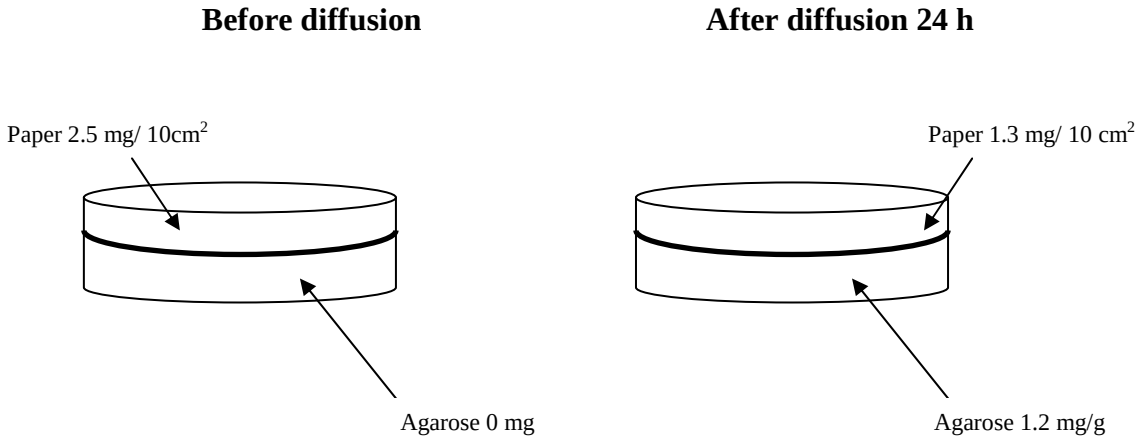


Figure 10. Theoretic evaluation of nisin quantity before and after 24 h of diffusion in paper and agarose.

To evaluate nisin diffusion from cellulose support to the gel, the evolution of nisin quantity into cellulose during diffusion time and nisin concentration in agarose during the migration was calculated..

The absorbance 490 nm was converted to nisin concentration using an equilibrium curve established from nisin determination by BCA protein method. The following regression equation was used.

$$y= 0.0483x + 0.0475$$

where y was absorbance at 490 nm and x was nisin concentration evaluated in BCA protein method.

RESULTS
&
DISCUSSION

III. Results and Discussion

The results section of the present thesis is composed of four parts.

In the first part, antibacterial activity of nisin and lysozyme, used alone or in combination was studied against some *Bacillus* ssp.

The objective of the second part was to examine the antimicrobial activity of nisin, lysozyme and lactic acid alone or in combination against *L. monocytoge*nes CIP 82.110 and *S. aureus* CIP 4.83.

Last, the optimized mixture of nisin, lysozyme and lactic acid was incorporated onto paper packaging and antimicrobial effectiveness was determined.

The purpose of the diffusion part was to try out and see the diffusion of nisin incorporated in a cellulose support such as paper.

III 1. Antibacterial activity of nisin & lysozyme used alone or in combination against *Bacillus* strains

The objective of these experiments was to determine, the most effective mixture containing nisin and lysozyme, which was able to inhibit population of *Bacillus* strains. The purpose of these studies was twofold. First, the model strain was selected by determination of Minimum Inhibitory Concentration (MIC). Second, the analysis was carried out in order to evaluate the interactions between nisin and lysozyme. Agar diffusion and broth dilution methods were applied.

1. Determination of the sensitivity of *Bacillus* strains to nisin and lysozyme

Sensitivity to nisin and lysozyme against a great diversity of *Bacillus* strains was determined by the agar diffusion method in order to select a model strain (**Figure 11**).

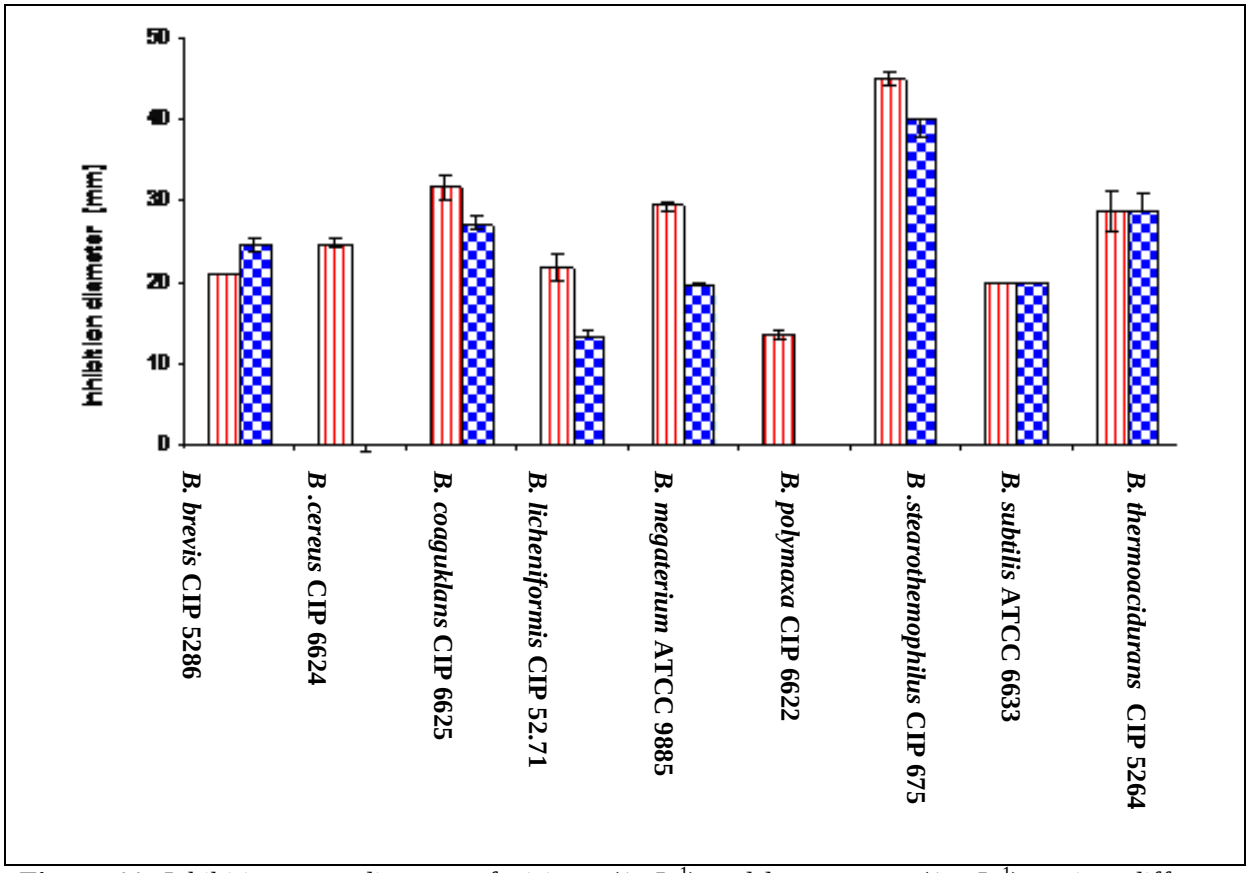


Figure 11. Inhibition zone diameter of nisin □ (1g.L^{-1}) and lysozyme ■ (1 g.L^{-1}) against different *Bacillus* strains in TSAYE+ 1% Tween medium after incubation at 30°C for 24 h.

Bacillus ssp. strains were sensitive to nisin, with inhibition diameter varying from 13 to 45 mm. The lowest sensitive strain was *B. polymyxa* CIP 6622 (inhibition diameter of 13 mm), whereas the most sensitive strain was *B. stearothermophilus* CIP 67.5 (inhibition diameter 45 mm). *B. cereus* CIP 6624 and *B. polymyxa* CIP 6622 were resistant to lysozyme at concentration tested. Others *Bacillus* strains were inhibited by lysozyme, with inhibition diameter varying from 13 to 40 mm. The most sensitive strain was *B. stearothermophilus* CIP 67.5 (inhibition diameter 40 mm), whereas the less sensitive was *B. licheniformis* CIP 52.71 (inhibition diameter 13 mm). *Bacillus megaterium* ATCC 9885 and *B. licheniformis* CIP 52.71 seemed to be the most sensitive among the dairy spoilage strains.

2. Determination of the Minimum Inhibitory Concentration (MIC) on agar medium.

MIC values determined against all *Bacillus* strains varied from less than 0.02 to over 1.25 g.L^{-1} for nisin and lysozyme (**Table 11**). Among tested strains *B. coagulans* CIP 6625, *B. megaterium* ATCC 9885 and *B. stearothermophilus* CIP 675 were the most vulnerable to nisin and lysozyme. They had the lowest MIC <0.02 g.L^{-1}. Nisin 0.625 g.L^{-1} was a MIC for a majority

of strains. *B. polymaxa* CIP 6622 had the highest MIC for nisin 1.25 g L^{-1}. The lowest inhibition diameter has already been seen for this strain (**Figure 11**). The MIC ranges of lysozyme were relatively higher than those of nisin and intermediary MIC value from 0.039 (*B. thermoacidurans CIP 5264*) to 0.156 g.L^{-1} (*B. subtilis* ATCC 6633). *Bacillus cereus* CIP 6625 was less sensitive to lysozyme than other strains and had the highest MIC up 1.25 g.L^{-1}, and was resistant to concentration of 1 g.L^{-1}, which has already been tested.

Table 11. Minimum inhibitory concentration (MIC) of nisin and lysozyme (expressed in g.L^{-1}) determined on trypticase soy agar (TSA-YE) at 30°C after 24 h of incubation.

Bacillus species	Strains designations	Nisin MIC (g. L^{-1})	Lysozyme MIC (g. L^{-1})
B. brevis	CIP 5286	0.625	0.039
B. cereus	CIP 6624	0.625	> 1.25
B. coagulans	CIP 6625	< 0.02	< 0.02
B. licheniformis	CIP 52.71	0.625	1.25
B. megaterium	ATCC 9885	< 0.02	< 0.02
B. polymyxa	CIP 6622	1.25	1.25
B. stearothermophilus	CIP 675	< 0.02	< 0.02
B. subtilis	ATCC 6633	0.625	0.156
B. thermoacidurans	CIP 5264	0.039	1.25

The minimal concentration of 1.25 g.L^{-1} lysozyme was necessary to inhibit *B. polymaxa* and *B. thermoacidurans*, therefore lysozyme at concentration of 1 g.L^{-1} did not present inhibition diameters at Figure 11.

Nisin can inhibit outgrowth of *Bacillus* spores in agar media (Jarvis, 1967, Hurst *et al.* 1981, Roberts and Hoover, 1996) and the nisin MIC values (0.625 g.L^{-1}) against *B. licheniformis* CIP 52.71 or *B.subtilis* ATCC 6633 were in agreement with previous studies obtained by Pol and Smid (1999) where similar nisin MIC value against *B. cereus* (0.625 g.L^{-1}) were determined. Our results confirmed that *B. coagulans* and *B. sterothermophilus* were very nisin sensitive (MIC nisin<0.02 g.L^{-1}) as already noticed by Tramer (1964). In contrast with Gould and Hurst (1962), it has not been observed that *B. subtlis* was less resistant to nisin than *B. cereus.* The most sensitive *Bacillus* strains tested in this study were *B. coagulans* and *B. sterothermophilus*. It was reported that *B. cereus* strains are among the least sensitive *Bacillus* against different antimicrobials including nisin and lysozyme (Pirtitijarvi *et al.* 2001).

Lysozyme inhibits all *Bacillus* ssp.. These results are the same as reported by Abdou *et al.* (2007). However the conditions of experiments were different. Abdou *et al.* (2007) worked with lysozyme peptide powder, produced by partial enzymatic hydrolysis from native lysozyme using

pepsin enzyme. Lysozyme peptide powder at the concentration of 100 µg.mL^{-1} completely inhibited *B. subtilis, B. licheniformis, B. pumilus, B. mycoides, B. coagulans, B. amyloliquefaciens, B. megaterium, B. polymexa, and B. macerans*. Meanwhile, *B. cereus* and *B. stearothermophilus* showed slight resistance.

All *Bacillus* ssp. strains tested were inhibited by the antimicrobial agents. Among the tested microorganisms, *Bacillus subtilis* ATCC 6633 and *Bacillus licheniformis* CIP 52.71 were selected for further analysis for several reasons:

- Nisin inhibited growth of these two strains with MIC 0.625 g.L^{-1}. At this concentration all *Bacillus* strains would be inhibited except *B. polymyxa* CIP 6622.
- These two strains showed different sensitivity to lysozyme. *Bacillus* subtilis ATCC 6633 was more sensitive (MIC 0.156 g.L^{-1}) to lysozyme than *B. licheniformis* CIP 52.71 (MIC 1.25 g.L^{-1}). The objective of this study was to determine the interactions between nisin and lysozyme. It seemed very interesting to choose two strains with different sensitivity to lysozyme.

3. Factors affecting the Minimum Inhibitory Concentration

MIC value is influenced by a number of factors, including inoculum density, agar composition medium, pH, incubation time and temperature (Barry 1986).

The objective of this study was to evaluate the effect of initial cell numbers and physiological state of target bacteria, standard solution concentration and also storage on antimicrobial activity.

The *Bacillus* inoculum size of 10^5 and 10^6 cfu.mL^{-1} didn't affect the MIC values for nisin as well as lysozyme, which was constant at 0.25 or 1.25 g.L^{-1} respectively (**Table 12**).

Table 12. Effect of inoculum size on nisin and lysozyme MIC values against *B. licheniformis* CIP 52.71 in TSBYE medium at 30ºC after 24 h of incubation.

Inoculum size (cfu.mL^{-1})	Nisin MIC (g.L^{-1})	Lysozyme MIC (g.L^{-1})
1.9 10^6	0.25	1.25
1.9 10^5	0.25	1.25

The nisin MIC against *B. licheniformis* CIP 52.71 was 0.25 g.L^{-1} and to lysozyme was 1.25 g.L^{-1} for all inoculum size. However, higher concentration of suspension allowed to obtain a clear radius of the inhibition zone (data no shown).

The nisin MIC value differed from the previous one (**Table 11**). It could be meant that other parameters such as physiological state of bacteria, standard solution concentration or solubility might interact with MIC values.

The MIC depended on the physiological state of bacteria (**Table 13**). Nisin had less effect on vegetative cells (MIC 0.625 $g.L^{-1}$) than on spore (MIC< 0.313 $g.L^{-1}$). Nevertheless lysozyme appeared to be more active on vegetative cells (MIC 0.156 $g.L^{-1}$) than on spore-former (MIC 0.625 $g.L^{-1}$). This could be explained by the mechanism action of lysozyme which is depended of its muramidase activity and lysozyme lyses or dissolves bacterial cell wall (Jolles and Jolles 1984).

Table 13. Influence of physiological state of *Bacillus subtilis* ATCC 6633 on nisin and lysozyme MIC values in TSBYE medium at 30ºC after 24 of incubation.

Physiological state	Nisin MIC ($g.L^{-1}$)	Lysozyme MIC ($g.L^{-1}$)
Vegetative cells	0.625	0.156
Spore	< 0.313	0.625

The antimicrobial activity of nisin and lysozyme was affected by the standard solution concentration (**Table 14**). The different standard solutions concentration, which contained 5, 2.5 and 1.25 $g.L^{-1}$of nisin or lysozyme showed variance in MIC. The nisin MIC values against *B. subtilis* ATCC 6633, varied from 0.625 to 0.313 $g.L^{-1}$ (one dilution, no significant). The Lysozyme MIC values decreased proportionally to standard solutions concentration at intervals between 0.078 and 0.02 $g.L^{-1}$.

Table 14. Effect of standard solution concentration on nisin and lysozyme MIC values against *Bacillus subtilis* CIP 6633 in TSBYE medium at 30ºC after 24 of incubation.

	Initial nisin concentration ($g.L^{-1}$)			Initial lysozyme concentration ($g.L^{-1}$)		
	5	2.5	1.25	5	2.5	1.25
MIC ($g.L^{-1}$)	0.625	0.313	0.625	< 0.078	< 0.039	< 0.02

These results suggested some problems in solubilisation of lysozyme when the initial concentration is too high. Lysozyme is less stable in distilled water than in alcohol or sugar solutions (Robson 1988). In order to ovoid this problem, standard solution of 1.25 $g.L^{-1}$ would be used for further experiments.

The MIC ranges of nisin and lysozyme varied during conservation of the standard solutions at 4°C during 4 days (**Table 15**).

Table 15. Effect of storage of the standard solution of nisin and lysozyme at 4°C during 0 and 4 days on MIC values against selected *Bacillus* strains

Strains	Nisin MIC ($g.L^{-1}$)		Lysozyme MIC ($g.L^{-1}$)	
	day 0	day 4	day 0	day 4
B. licheniformis CIP 52.71	0.625	1.25	2.5	0.313
B. subtilis ATCC 6633	0.625	1.25	< 0.156	0.625

The nisin MIC value increased significantly against *B. licheniformis* CIP 52.71 and *B. subtilis* ATCC 6633 after 4 days of storage at 4°C. However lysozyme demonstrated significant difference in MIC value against *B. licheniformis* CIP 52.71 and *B. subtilis* ATCC 6633 during storage time. Surprisingly lysozyme MIC value decreased against *B. licheniformis* CIP 52.71 and increased against *B. subtilis* ATCC 6633 after 4 days at 4°C. This indicate modification of activity during storage at 4°C.

In the present study nisin (0.25 - 0.625 $g.L^{-1}$) and lysozyme (0.156 - 1.25 $g.L^{-1}$) MIC values varied among experiments. Differences in MIC values were observed between agar diffusion method and liquid medium. These contrasting results might be due to difference in experimental conditions including initial inoculum level, strain variation, duration of exposure, concentration of antimicrobial and growth phase as already mentioned by Branen and Davidson (2004). Beauchat *et al.* (1997) stated that the inhibitory effect of nisin was greater on vegetative cell of *B. cereus* than on its spores. Although working cultures were prepared in the same way, some variations in physiological stage of the culture could occur and could explain great variability observed among our results, as reported by Beauchat *et al.* (1997) and Meghrous *et al.* (1999).

Physiological state of bacteria and preparation of standard solution influence nisin and lysozyme MIC values. Vegetative cells were more sensitive to lysozyme (MIC 0.156 $g.L^{-1}$) than nisin (0.625 $g.L^{-1}$). Nisin at the concentration two times lower (< 0.313 $g.L^{-1}$) than lysozyme (0.625 $g.L^{-1}$) inhibited spore.

Moreover susceptibility of strains is greatly affected by cultural conditions. Bell and de Lacy (1985) showed that nisin is less effective against outgrowth of *B. licheniformirs* spores, when the salt is present in the media. Salt appears to have a sporidical action on nisin by interfering with nisin adsorption onto the spores (Bell and de Lacy 1985). Black *et al.* (2008) observed resistance of *B. subtilis* 8872 after nisin treatment at 20 h and 40 ºC. Jaquette and Beauchat (1998) observed that the combined effects of pH, nisin and temperature influence the

growth and survival of *Bacillus cereus*. The effectiveness of nisin at concentration 1µg.mL^{-1} in controlling the growth of *B. cereus* was more pronounced at 8ºC than 15ºC and the pH approximately 5.5 (Jaquette and Beuchat 1998).

Our results showed that lysozyme has only a bacteriostatic effect in TSBYE broth against *Bacillus* species. Lysozyme is known to induce spore germination by hydrolyzing peptidoglycan of the spore cortex, but most intact bacterial spores are protected from lysozyme by outer membranes covering the cortex (Gould 1995). Spore strains are known to have naturally leaky coats and are therefore lysozyme sensitive, but in most cases, lysozyme is only capable of hydrolysing the peptidoglycan of the spore cortex if the overlying coat is first made leaky (Raso 1998).

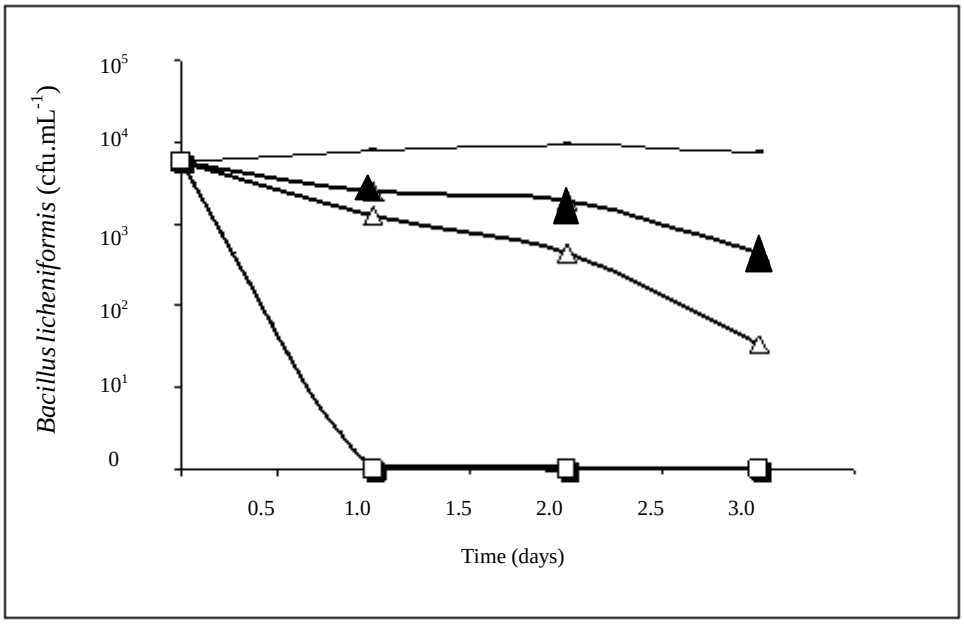
Nisin and lysozyme solutions were prepared extemporaneously, the same day of the experiments. Standard solution of 1.25 g.L^{-1} and first dilution would be used for further experiments in order to avoid variation nisin and lysozyme MIC. Standard solution demonstrated less influence on nisin MIC (0.625-1.25 g.L^{-1}) than on lysozyme MIC (0.02 – 2.5 g.L^{-1}).

4. Effect of nisin & lysozyme on growth of *Bacillus* strains in liquid medium

Nisin at the concentration of 0.625 or 0.3125 g.L^{-1}, inhibited *B. licheniformis* CIP 52.71 and *B. subtilis* ATCC 6633 at least 3 days of incubation at 30ºC (**Figures 12 A&B**). A bactericid effect induced a reduction of respectively 4 and 3 $\log_{10}$ cfu.mL^{-1} in one day. The concentrations of 0.3125 g.L^{-1} of nisin corresponded to the MIC for these strains in liquid medium in the conditions tested. It had been already observed at table 12, 13 and 14.

Lysozyme reduced partially the growth of *B. licheniformis* CIP 52.71 with 1 or 2 $\log_{10}$ cfu.mL^{-1} of reduction in 3 days, depending on the concentration tested. Lysozyme had essentially a bacteriostatic effect against *B. subtilis* ATCC 6633 in broth medium. It had already been seen on table 14 and 15.

A



B

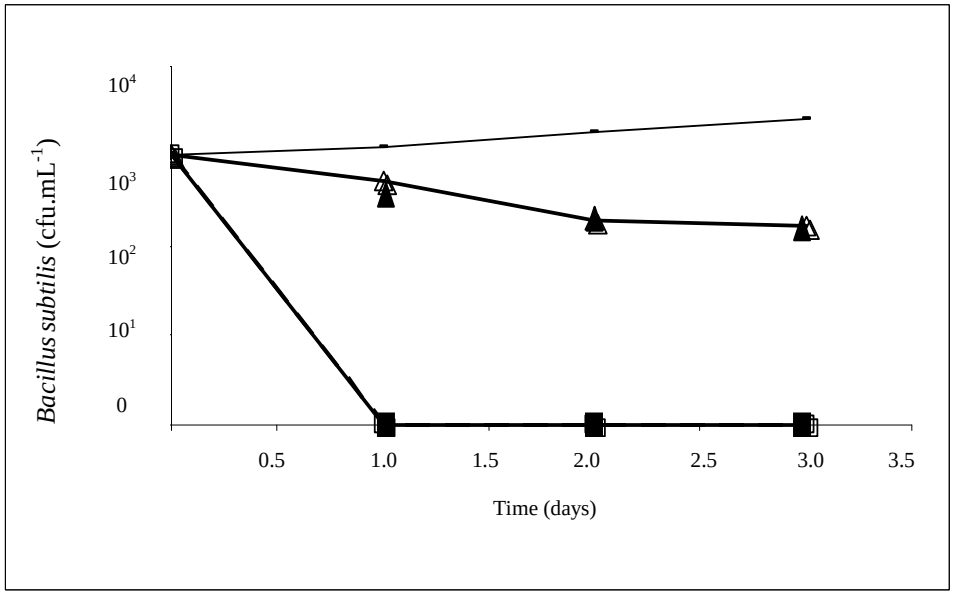


Figure 12. Effect of nisin and lysozyme of *B. licheniformis* CIP 52.71 (A) and *B. subtilis* ATCC 6633 (B) growth during 3 days at 30ºC in TSBYE - Tween broth in control (-) in presence of nisin 0.625 $g.L^{-1}$ (□), nisin 0.3125 $g.L^{-1}$ (■) lysozyme 1.25 $g.L^{-1}$ (Δ) and lysozyme 0.625 $g.L^{-1}$ (▲).

Our data showed that nisin alone presented a significant effectiveness to inhibit *Bacillus* strains for a long time when used at concentration higher than MIC values.

In liquid medium nisin MIC was decreased from 0.625 $g.L^{-1}$ to 0.3125 $g.L^{-1}$ for *B. licheniformis* CIP 52.71 and *B subtilis* ATCC 6633. Nevertheless *B. subtilis* ATCC 6633 and *B. licheniformis* CIP 52.71 were sensitive to lysozyme in liquid medium, with a bactericid or bacteriostatic effect observed for concentration of 0.625 $g.L^{-1}$.

5. Evaluation of antimicrobial interactions between nisin & lysozyme against *Bacillus licheniformis* CIP 52.71 and *Bacillus subtilis* ATCC 6633

The objective of this study was to determine possible interactions between nisin and lysozyme against the two selected strains.

Three types of interactions can occur between nisin and lysozyme: synergy, antagonism or additive effect (Barry 1976). Synergism is indicated by inhibition of growth in areas where individual agent would be present in sub-inhibitory concentrations. Antagonism occurs when microbial growth is observed in areas where individual agents would be presented in inhibitory concentrations (Barry 1976). Additives effects occurs when the antimicrobial activity of a compound is neither enhanced nor reduced, while in the presence of another agents (Davidson and Parish 1989).

The presence of nisin, or lysozyme, simultaneously with the other inhibitor didn't modify the growth of the two strains tested, when nisin concentration was lower than its MIC (0.3125 $g.L^{-1}$) and lysozyme concentration was lower than its MIC (1.25 $g.L^{-1}$) (**Table 16** and **17**).

Table 16. Effect of different concentrations of nisin and lysozyme or their combinations on the inhibition of *B. subtilis* ATCC 6633 after 24 hours at 30 ºC in TSBYE. In bold character MIC value.

B. subtilis ATCC 6633		Lysozyme concentration ($g.L^{-1}$)							
		0	0.039	0.078	0.156	0.3125	0.625	**1.25**	2.5
Nisin concentration [$g.L^{-1}$]	0	+[a]	+	+	+	+	+	+	+
	0.0195	+	+	+	+	+	+	+	+
	0.039	+	+	+	+	+	+	+	-[b c]
	0.078	+	+	+	+	+	+	+	+
	0.156	+	-[c]	+	+	+	+	+	-[c]
	0.3125	-	-	-	-	-	-	-	-
	0.625	-	-	-	-	-	-	-	-
	1.25	-	-	-	-	-	-	-	-

[a] No Inhibition; [b] Inhibition [c] Value abhorrent

No interactions were observed between nisin and lysozyme after 24 hours at 30ºC in broth medium. The absorbance reading did not allow to see the bacteriostatic effect of lysozyme and to explain the growth, which was observed with lysozyme 1.25 $g.L^{-1}$. The MIC nisin was a little different from other manipulation. It corresponded only to one dilution, which was not too significant.

Table 17. Effect of different concentrations of nisin and lysozyme or their combinations on the inhibition of *B. licheniformis* CIP 52.71 after 24 hours at 30ºC in TSBYE. In bold character MIC value.

B. licheniformis CIP 52.71^T		Lysozyme concentration (g.L^{-1})							
		0	0.039	0.078	0.156	0.3125	0.625	**1.25**	2.5
Nisin concentration [g.L^{-1}]	0	+	+[a]	+	+	+	+	+	+
	0.0195	+	+	+	+	+	+	+	+
	0.039	+	+	+	+	+	+	+	+
	0.078	+	+	+	+	+	+	+	+
	0.156	+	+	+	+	+	+	+	+
	0.3125	+	+	+	+	+	+	+	-[b]
	0.625	-	-	-	-	-	-	-	-
	1.25	-	-	-	-	-	-	-	-

[a] No Inhibition; [b] Inhibition

The combination of nisin and lysozyme did not modify MIC values of nisin and lysozyme alone. Under aplied conditions, no interaction between nisin and lysozyme occurred.

Combination of nisin with lysozyme at lower concentration than MIC values presented low significant effectiveness to inhibit *Bacillus* strains for a long time and no synergistic or additive effects have been demonstrated. However lysozyme combining with nisin reduced *B. cereus* vegetative cells in the last part of the exposure period, and suggested an increasing lifetime of the created pores (Pol and Smid 1999). The higher effectiveness could be achieved in inhibiting tested microorganisms, when heat treatments were applied. Nisin and high pressure treatment present synergism and can inhibit *B. subtilis* spores in milk (Black *et al*. 2008). Jaquette and Beuchat (1998) observed the combined effects of pH, nisin and temperature on growth and survival of *B. cereus*.

The present thesis is focusing on the improvement of the paper wrapping materials by adding nisin and lysozyme in combination and on the determination of the synergism, which can occur between these antimicrobial agents.

The results of experiments on *Bacillus* strains showed that the purpose may be difficult to achieve, and this fact has been confirmed by many different experiments and methods. So, this model with *Bacillus* strains has been replaced by *Listeria monocytogenes* CIP 82.110 and *Staphylococcus aureus* CIP 4.83 strains (Gill and Holley 2000, Mangalasary *et al.* 2007, 2008). Lactic acid as the third antimicrobial agent was chosen for further analysis with *L. monocytogenes* and *S. aureus* (Nykanen *e al.* 2000, Georgnaras *et al.* 2006a,b).

III 2. Antibacterial activity of nisin & lysozyme & lactic acid used alone or in combination against *Listeria monocytogenes* CIP 82.110.

The objective of these experiments was to determine and optimize combination of nisin, lysozyme and lactic acid to inhibit population of *Listeria* strains.

A sensitive *Listeria* strain was selected by determination of MIC. Interactions occurring between nisin and lysozyme, nisin and lactic acid, lysozyme and lactic acid were investigated by FIC determination. A Doelhert experimental design was applied to test the interactions between the three inhibitors and to determine the most antibacterial combination.

1. Determination of the Minimum Inhibitory Concentration (MIC) of nisin & lysozyme & lactic acid against several *Listeria* strains

The preliminary experiments were conducted to determine the MIC values of nisin, lysozyme and lactic acid in agar (TSYAE) and broth (TSBYE) medium against *Listeria* strains in order to select a model strain (**Table 18**).

Table 18. Minimum inhibitory concentration (MIC) of nisin, lysozyme and lactic acid, on different *Listeria* strains determined on trypticase soy agar (TSAYE) and trypticase soy broth (TSBYE) at 37°C after 24 h of incubation.

Listeria strains	Agar diffusion technique			Broth diffusion technique		
	Nisin ($g.L^{-1}$)	Lysozyme ($g.L^{-1}$)	Lactic acid (%)	Nisin ($g.L^{-1}$)	Lysozyme ($g.L^{-1}$)	Lactic acid (%)
L. grayi CIP 7818	nd	nd	nd	1	>1	1
L. innocua CIP 125111	0.5	>1	>1	1	>1	>1
L. ivanovii LMA 94	nd	nd	nd	>1	>1	1
L. ivanovii CIP 12510	0.25	>1	>1	0.25	>1	1
L. monocytogenes CIP 82110	0.5	0.06	>1	0.5	0.5	0.25
L. monocytogenes CIP 7831	0.125	0.06	>1	1	>1	1
L. seeligerii SLCC 3954	1	0.015	>1	0.25	>1	0.5

nd not determined

The MICs determined for inhibition of growth by nisin, lysozyme and lactic acid showed that the MICs were different for agar and broth dilution assays.

Nisin inhibited *Listeria* strains in the range of MIC from 0.125 to over 1 $g.L^{-1}$. In agar diffusion technique, the lowest nisin MIC (0.125 $g.L^{-1}$) was observed for *Listeria monocytogenes* CIP 7831, the highest (1 $g.L^{-1}$) was obtained with *Listeria seeligerii* SLCC 3945. However *Listeria seeligerii* SLCC 3945 and *Listeria ivanovii* CIP 12510 (MIC 0.25$g.L^{-1}$) were the most sensitive strains in broth medium.

MIC variations were observed between *L. monocytogenes* CIP 82.110 and *L. monocytogenes* CIP 7831, two strains of the same species. *L. monocytogenes* CIP 7831 was more sensitive to nisin in solid medium (0.125 $g.L^{-1}$) and less sensitive in liquid medium (1.0 $g.L^{-1}$).

The results of MIC study demonstrated that *Listeria* species showed great variation in sensitivity to nisin, with the range of MIC from 0.125 to over 1 $g.L^{-1}$. The nisin MIC values in agar dilution assays were lower than broth diffusion assay. The lowest nisin MIC (0.125 $g.L^{-1}$) was observed for *Listeria monocytogenes* CIP 7831 and the highest nisin MIC 1 $g.L^{-1}$ was for *Listeria seeligerii* SLCC 3945 in agar diffusion technique. Benkerroum and Sandine (1988), presented that in agar plate, growth inhibition of 10^4 $cfu.mL^{-1}$ required 7.4 10^2 $IU.mL^{-1}$ (*L. monocytogenes* ATCC 7644) or 1.2 10^5 $IU.mL^{-1}$ (*L. monocytogenes* V7) nisin under the same conditions in TSA plate. *L. monocytogenes* ATCC 7644 needed only 1.9 $IU.mL^{-1}$ nisin to be inhibited or *L. monocytogenes* V7 is inhibited by 3.4 $10^3 IU.mL^{-1}$. Similar Moltagh *et. al.* (1991) presented diverse viability loss for *Listeria* strains in tryptic soy broth for nisin purified and prepared in laboratory by growing *L. lactic* subsp. *lactic* ATCC 11454 in broth. In the study the lowest MIC (740 $IU.mL^{-1}$) was obtained for *L. ivanovii*, and *L. monocytogenes* had the highest (1.18 10^5 $UI.mL^{-1}$).

The antibacterial activity of lysozyme was detected only by agar diffusion methods. The MIC values were higher in liquid than in solid medium except for one strain (*Listeria seeligerii* SLCC 3945). The lowest lysozyme MIC values in agar medium were observed against *Listeria seeligerii* SLCC 3945 (0.015 $g.L^{-1}$) and the highest MIC values were observed for *Listeria monocytogenes* CIP 82.110 and CIP 7831 (0.06 $g.L^{-1}$).

In contrast, Hughey and Johnson (1987) reported that lysozyme was not able to inhibit *Listeria monocytogenes Scott A* and *Listeria monocytogenes Ohio* strains on brain heart infusion agar, even when *Listeria* was inoculated into media containing 20 or 200 $mg.L^{-1}$ of lysozyme. No lysis occurred when lysozyme was injected into growth culture of *L. monocytogenes.* They suggested that two factors influenced or limited of the effectiveness of lysozyme. The peptidoglycan may be masked by other cell wall components. Cell wall lyses is more effective in culture slowed in growth rate by lowered temperature, because cell wall synthesis in rapidly growth culture probably exceed the rate of degradation by lysozyme. Johansen *et al.* (1994) showed that the antibacterial activity of lysozyme depended on the growth temperature of the cell in tryptic soy broth. *Listeria monocytogenes* Scott A cells grown at 5ºC were significantly more sensitive to lysozyme than cells grown at 25ºC and more sensitive than cells grown at 37ºC. Cells grown at 5ºC were quickly lysed with a concentration of 1.000 $IU.mL^{-1}$, which caused a

significant decrease in an absorbance of 0.6 (100 %) after 4h as compared to the control. For cell grown at 25 or 37ºC the same lysozyme concentration caused only an absorbance decrease after 4h of 0.24 (50%) or 0.06 (25%), respectively. The results suggested that antibacterial activity of lysozyme could be determined only by broth diffusion methods. Cunningham *et al.* (1991) showed that the evaluation of antibacterial activity of lysozyme is difficult to interpret. Lysozyme adhered to glass and lost its activity quickly when studied in Pyrex, polypropylene or polyethylene containers. It can be inactive by the presence of peptone, beef liver extract or boiled soyabean ingredients found in bacteriogical media (Nattress and Baker 2001, 2003). Chang and Carr (1971) observed that the enzymatic activity of lysozyme was strongly decreased by increasing salt concentration.

Antibacterial activity of lactic acid was more efficient in broth medium, with a MIC of 0.5% against *Listeria seeligerii* SLCC 3945 and *Listeria monocytogenes* CIP 82.110, whereas MIC values were higher than 1 $g.L^{-1}$ in agar medium.

The data showed that lactic acid was more efficient in broth dilution technique, with a MIC 0.5% against *Listeria seeligerii* SLCC 3945 and *Listeria monocytogenes* CIP 82.110. Ahmed and Marth (1990) showed that inhibition of *L. monocytogenes* by lactic acid was greatly affected by temperature of incubation and concentration of the acid. The bacterium proliferated in the presence of 0.5% lactic acid at 7, 13, 21, 35ºC and in the presence of 0.1% lactic acid at all temperatures except 7ºC. Gravesen *et al.* (2004) demonstrated that *Listeria monocytogenes* was more sensible to D-lactic acid which gave 0.6 - 2.2 log reduction than L- lactic.

Among the tested microorganisms, *Listeria monocytogenes* CIP 82.110 is the only strain inhibited by the three inhibitors in liquid medium and is selected for further analysis.

2. Effects of nisin & lysozyme & lactic acid on growth of *Listeria monocytogenes CIP 82.110*

The effects of different concentrations of nisin, lysozyme and lactic acid on growth kinetic of *L. monocytogenes* were determined (**Figure 13**).

The presence of 0.06 $g.L^{-1}$ nisin inhibited *L. monocytogenes* after 6 h incubation at 37 ºC (nisin A 0.134 and control A 0.188), indicating a rapid nisin effect on *L. monocytogenes* growth.

This short inhibitory effect was followed by regrowth, but population level after 24 h was lower than in the control culture for 0.125 to 0.5 $g.L^{-1}$ nisin. The maximal inhibitory effect was obtained with nisin 0.5 $g.L^{-1}$ with 60% reduction of absorbance after 24 h.

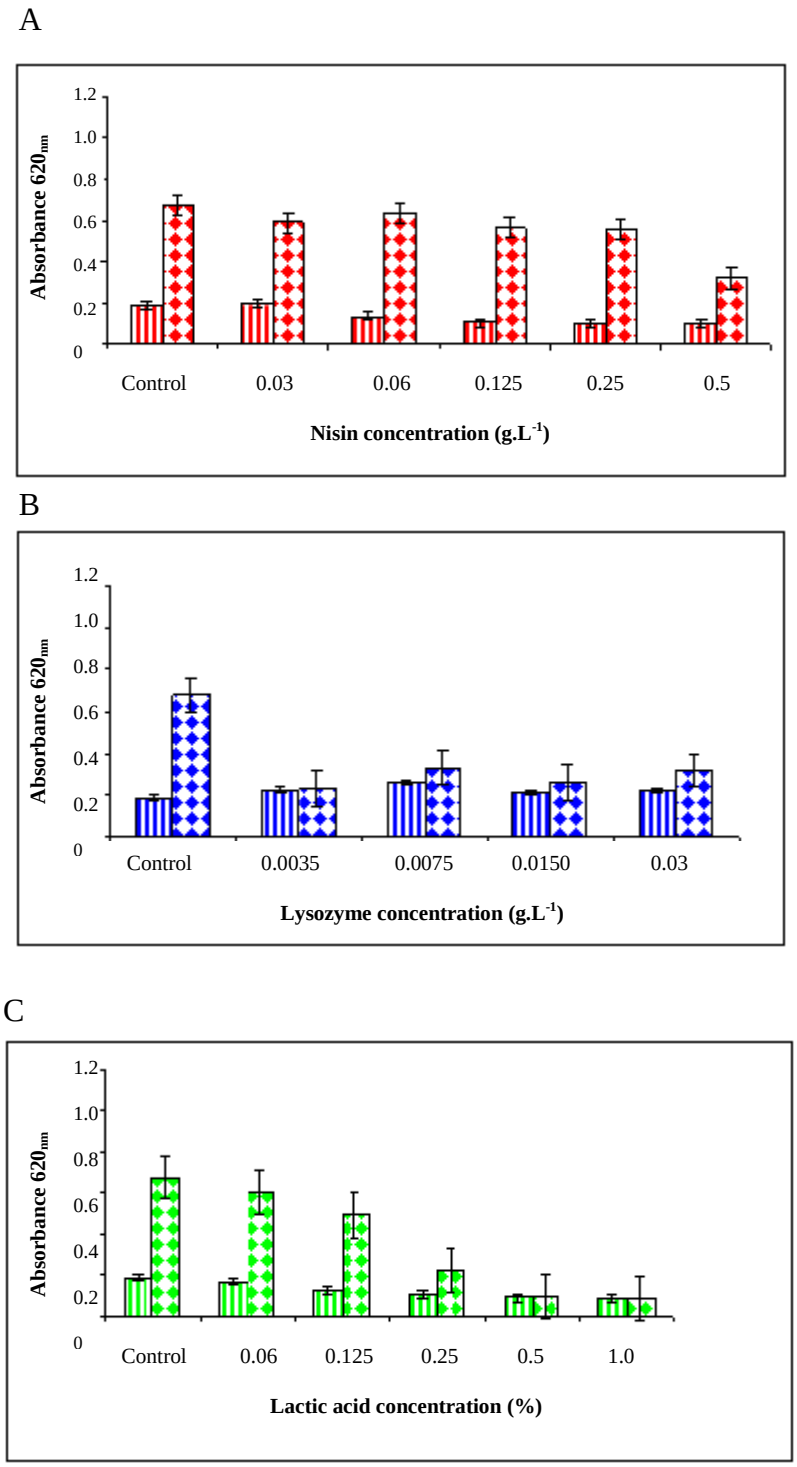


Figure 13. Growth kinetic of *Listeria monocytogenes* CIP 82.110 measured by $A_{620\ nm}$ in the presence of different concentrations of nisin (A), lysozyme (B), lactic acid (C) after 6 (II) and 24 h (♦) on trypticase soy broth at 37ºC.

The absorbance after 24 h obtained in the presence of lysozyme concentrations tested was similar at 6 h and 24 h and identical to the control at 6 h. This indicated a global bacteriostatic effect of lysozyme against *L. monocytogenes* over 24 h. This effect was obtained for concentration ranging from 0.0035 to 0.03 $g.L^{-1}$ and was quite similar for all of these concentrations.

Lactic acid at concentration of 0.25% inhibited significantly the growth of *L. monocytogenes* after 6 h and 24 h of incubation with 91% of absorbance reduction in 24 h. Moreover lactic acid 0.5% induced a 99% reduction in population level after 6 and 24 h of incubation, compared to the control.

Although nisin at concentration of 0.06 $g.L^{-1}$ had quickly inhibited the growth of *L. monocytogenes* for 6 h of incubation at 37ºC, a regrowth of *L. monocytogenes* occurred after 24 h of incubation.

Lysozyme in the range of concentration 0.0035 to 0.03 $g.L^{-1}$ had a bacteriostatic effect.

Lactic acid 0.25% concentration completely inhibited *L. monocytogenes* growth for 6 h of incubation. No regrowth of *L. monocytogenes* has been observed for 24 h of incubation.

3. Interactions between nisin & lysozyme, nisin & lactic acid and lysozyme & lactic acid on *Listeria monocytogenes* CIP 82110

The objective of the study was to determine possible interactions between nisin and lysozyme, nisin and lactic acid, lysozyme and lactic acid against *L. monocytogenes*.

The selected concentrations of nisin corresponded to the MIC value at 6 h of incubation (0.06 $g.L^{-1}$), to 2xMIC (0.125 $g.L^{-1}$) and MIC/2 (0.03 $g.L^{-1}$).

Lysozyme was used to at bacteriostatic concentrations 0.0035 and 0.03 $g.L^{-1}$ and higher concentration of 0.06 $g.L^{-1}$ previously tested.

Lactic acid was used at inhibitory levels of 0.5 and 1%.

The evaluation of interactions between two inhibitors was studied by growth kinetics in presence of combination of two inhibitors, one in fixed concentration and the other one in variable concentrations, and also by FIC determination.

To determine FIC index, various concentrations of two inhibitors and all combination of these concentrations were tested in liquid medium and Fractional Inhibitory Concentration (FIC) were calculated. FIC is the concentration of compound needed to inhibit growth, when combined with second antibacterial compounds (Parish and Davidson 1993). Inhibition data was expressed in FIC (Hall *et al.* 1983). FIC nisin = (MIC of nisin with MIC lysozyme)/MIC nisin. The FIC index was calculated with FICs for individual antibacterial agents: FICindex = FICnisin + FIClysozyme. A FICindex near 1 indicates additivity, <1 synergy, and >1 antagonism of the inhibitory combination.

3.1 Evaluation of antibacterial interaction between nisin & lysozyme

Effectiveness of combination lysozyme 0.0035 $g.L^{-1}$ with different nisin concentrations was evaluated (**Figure 14**).

Nisin alone was ineffective in reducing *L. monocytogenes* because the concentrations which have been chosen were lower than MIC (0.5 $g.L^{-1}$). Nisin at 0.125 $g.L^{-1}$ decreased only population after 24 h (A 0.656), comparative to control (A 0.674). Lysozyme at 0.0035 $g.L^{-1}$ confirmed its bacteriostatic effect at the concentration tested, and the absorbance of population was 0.229 at 24 h of incubation. The antibacterial activity of nisin and lysozyme induced maximal absorbance reduction of 50%, compared to the control. The antibacterial activity was lower than in presence of lysozyme alone. This combination was tested only one time, so it was impossible to calculate standard deviations.

No synergistic or additive effects between these two inhibitors were observed.

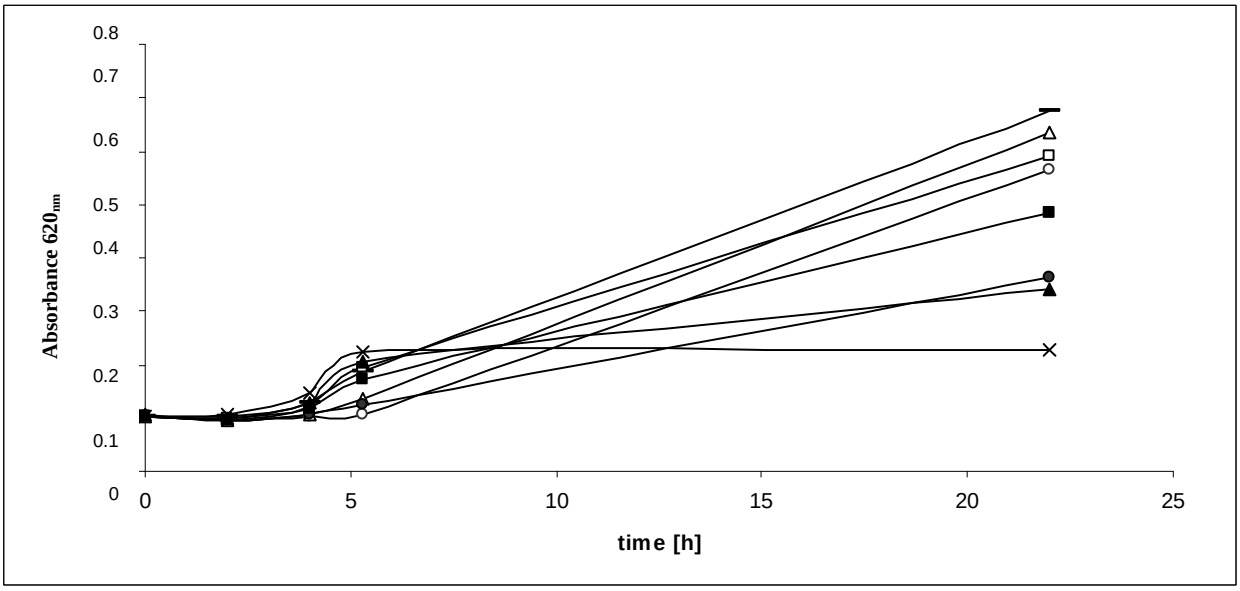


Figure 14. Kinetic of *L. monocytogenes* at 37ºC in TSBYE - Tween broth in control (-), solution nisin 0.03 $g.L^{-1}$ (Δ), nisin 0.06 $g.L^{-1}$ (□), nisin 0.125 $g.L^{-1}$ (o), nisin 0.03 $g.L^{-1}$ and lysozyme 0.0035 $g.L^{-1}$ (▲), nisin 0.06 $g.L^{-1}$ and lysozyme 0.0035 $g.L^{-1}$ (■) , nisin 0.125 $g.L^{-1}$ and lysozyme 0.0035 $g.L^{-1}$ (●) and lysozyme (x) 0.0035 $g.L^{-1}$ during 24 h incubation.

Antibacterial efficiency of various combinations nisin and lysozyme was tested to determine FIC index (**Table 19**). Inhibition observed in presence of nisin and lysozyme was due to lysozyme. The synergy and additive interactions were not observed between nisin and lysozyme at 37ºC, after 24 h incubation in TSBYE medium.

Table 19. FIC calculation for nisin and lysozyme against *Listeria monocytogenes* at 37ºC, after 24 h incubation in TSBYE medium (- no inhibition, + inhibition).

	FIC nisin	*0*	*0.06*	*0.12*	*0.25*
FIC Lysozyme	Lysozyme concentrations ($g.L^{-1}$)	Nisin concentrations (g. L^{-1})			
		0	0.03	0.06	0.125
0	0	-	-	-	-
0.25	0.0035	+	+	+	+
1	0.015	+	+	+	+
4	0.06	+	+	+	+

FIC index was over 1, so antagonism was observed between nisin and lysozyme.

3.2 Evaluation of antibacterial interaction between nisin & lactic acid

Effectiveness of combinations of 0.5 % lactic acid with different nisin concentrations was evaluated against *L. monocytogenes* (**Figure 15**).

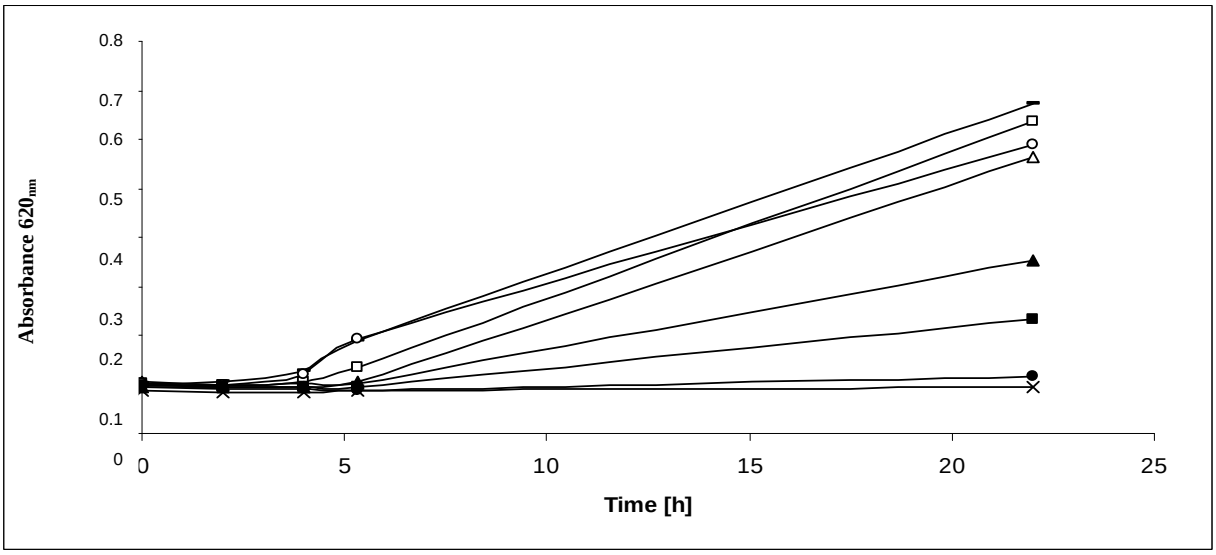


Figure 15. Kinetic of *L. monocytogenes* at 37ºC in TSBYE - Tween broth in; control (-). solution nisin 0.03 $g.L^{-1}$ (Δ), nisin 0.06 $g.L^{-1}$ (□), nisin 0.125 $g.L^{-1}$ (o), nisin 0.03 $g.L^{-1}$ and lactic acid 0.5% (▲), nisin 0.06 $g.L^{-1}$ and lactic acid 0.5% (■), nisin 0.125 $g.L^{-1}$ and lactic acid 0.5%(●) and lactic acid 0.5% (x) during 24 h incubation.

Nisin alone was ineffective in reducing *L. monocytogenes*, because the concentrations which have been chosen were lower than MIC (0.5 $g.L^{-1}$). Lactic acid 0.5% totally inhibited the population with a reduction of absorbance to 0.093 at 24 h of incubation. The antibacterial activity of nisin and lactic acid was maximal with 0.125 $g.L^{-1}$ nisin and 0.5% lactic acid, with 96% in

absorbance reduction, compared to the control. The antibacterial activity of 0.06 and 0.03 $g.L^{-1}$ nisin combined with 0.5% lactic acid was lower than in presence of lactic acid alone. This result indicated that the presence of nisin decreased the activity of lactic acid against *L. monocytogenes.* This combination was tested only one time, so it was impossible to calculate standard deviations.

No synergistic or additive effects between these two inhibitors were observed.

The type of interactive effects between nisin and lactic acid against *L. monocytogenes* was evaluated by FIC index determination (**Table 20)**. There were not synergistic combinations and additive effect between nisin and lactic acid inhibitory to *L. monocytogenes* at 37ºC, during 24 h.

Table 20. FIC calculation of nisin and lactic acid against *L. monocytogenes* at 37ºC, after 24 h incubation in TSBYE medium (- no inhibition, +inhibition).

FIC Lactic acid	*FIC nisin*	*0*	*0.06*	*0.12*	*0.25*
	Lactic acid concentration	Nisin concentrations ($g.L^{-1}$)			
	(%)	0	0.03	0.06	0.125
0	0	-	-	-	-
1	0.5	+	+	+	+
2	1	+	+	+	+

FIC index was >1, so it meant that antagonism interactions occurred between nisin and lactic acid.

3.3 Evaluation of antibacterial interaction between lysozyme & lactic acid

Effectiveness of combinations of 0.5 % lactic acid with different lysozyme concentrations a was evaluated against *L. monocytogenes* (**Figure 16**).

Lysozyme alone at 0.0035, 0.015 and 0.06 $g.L^{-1}$ had an antibacterial activity against *L. monocytogenes* with a stable absorbance of 0.239. Lactic acid at 0.5% totally inhibited the population with an absorbance of 0.093 at 24 h of incubation. The combination lysozyme and lactic acid decreased by 50% *L. monocytogenes* population with an absorbance of 0.337, higher than that obtained with lactic acid alone. This combination was tested only one time, so it was impossible to calculate standard deviations.

No synergistic or additive effects between these two inhibitors were observed.

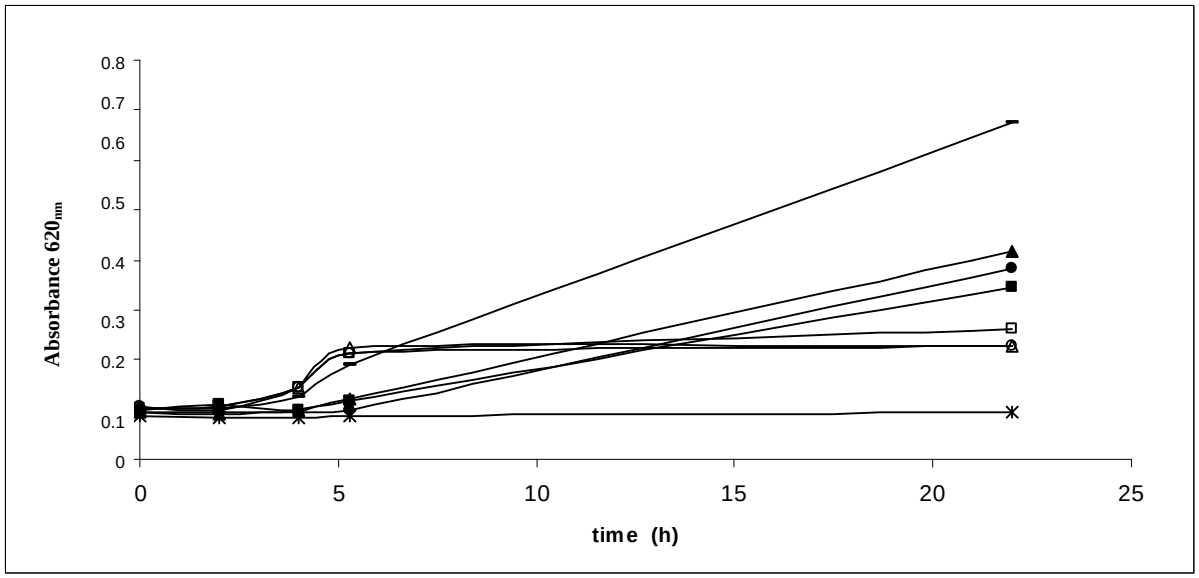


Figure 16. Kinetic of *L. monocytogenes* at 37ºC in TSBYE - Tween broth in control (-), solutions: lysozyme 0,0035 g.L^{-1}(Δ), lysozyme 0,015 g.L^{-1}(□)lysozyme 0,06 g.L^{-1} (o), lysozyme 0.0035 g.L^{-1} and lactic acid 0.5% (▲), lysozyme 0.015 g.L^{-1} and lactic acid 0.5% (■), lysozyme 0.06 g.L^{-1} and lactic acid 0.5% (●),and lactic acid 0.5% (x) during 24 h incubation.

The results of FIC calculation of lysozyme and lactic acid were negative (**Table 21**). There were not synergistic combinations between lysozyme and lactic acid inhibitory to *Listeria monocytogenes* at 37ºC, during 24 h of incubation.

Table 21. FIC calculation of lysozyme and lactic acid against *L. monocytogenes* at 37ºC, after 24 h incubation in TSBYE medium (- no inhibition, +inhibition).

FIC Lactic acid	*FIC Lysozyme*	*0*	*0.25*	*1*	*4*
	Lactic Acid Cocentrations (%)	Lysozyme concentrations (g.L^{-1})			
		0	0.0035	0.015	0.06
0	0	-	+	+	+
1	0.5	+	+	+	+
2	1	+	+	+	+

FIC index was >1 and antagonism interactions were observed between lysozyme and lactic acid.

Antagonism interactions were observed between nisin and lysozyme, nisin and lactic acid, lysozyme and lactic acid during *L. monocytogenes* growth in TSBYE medium at 37ºC, under the condition tested.

The concentration of lactic acid was too higher and totally inhibited *L. monocytogenes* growth. In consequence effect of other antimicrobial agents could not be visualized when lactic acids is presented.

4.0 Doehlert experiment design in combined system nisin & lysozyme & lactic acid

In order to find appropriate mixture composition with nisin, lysozyme and lactic acid, an experimental design using the Doehlert matrix (Doehlert, 1970) was used. The mathematical tools were chosen to replace the traditional kinetics experiments to study the interactions between nisin, lysozyme and lactic acid and to optimize their combinations. The main objective was to obtain the maximum of inhibition with minimal concentration of each inhibitor. Furthermore the maximum information and precision could be obtained from reduced numbers of experiments.

For this study, a serial mixture of nisin, lysozyme and lactic acid were formulated (**Table 22**). Nisin with 0.25 $g.L^{-1}$, lysozyme with 0.0035 $g.L^{-1}$ and lactic acid 0.5% concentrations were used in combination to test efficacy of mixture with concentration of each inhibitor lower than their MIC values.

Table 22. Experimental design for three variables nisin, lysozyme, lactic acid according to Doehlert uniform shell design.

Nº experiment	Abbreviation	Nisin concentration ($g.L^{-1}$)	Lysozyme Concentration ($g.L^{-1}$)	Lactic Acid Concentration (%)
0	Control	0	0	0
1	N	0.25	0	0
2	L	0	0.015	0
3	LA	0	0	0.5
4	$N_{1/2}$&$L_{1/2}$	0.125	0.0075	0
5	$N_{1/2}$&$LA_{1/2}$	0.125	0	0.25
6	$L_{1/2}$&$LA_{1/2}$	0	0.0075	0.25
7	$N_{1/3}$&$L_{1/3}$&$LA_{1/3}$	0.00825	0.00495	0.165
8	$N_{4/6}L_{1/6}$&$LA_{1/6}$	0.165	0.0025	0.08
9	$N_{1/6}$&$L_{4/6}$&$LA_{1/6}$	0.04	0.0099	0.08
10	$N_{1/6}$&$L_{1/6}$&$LA_{4/6}$	0.04	0.0025	0.33

To compare the results of this study with the precedent one, an initial populations level of 10^7 $cfu.mL^{-1}$ *L. monocytogenes* culture was incubated at 37ºC in the presence of different inhibitors. Numeration were performed after 24 h of incubation (**Table 23**). Nisin conserved its antibacterial effect with a reduction of 4.8 log $cfu_{10}.mL^{-1}$, compared to control (8.9 $\log_{10}$ $cfu.mL^{-1}$) at 24 h of incubation at 37ºC, as already obtained in Figure 12. Lysozyme had not effect at 24 h and lactic acid was bactericide at 24 h, with a reduction in population of 3.1 log $cfu.mL^{-1.}$

Combination nisin and lactic acid reduced *L. monocytogenes* population to 3.0 $\log_{10}$ $cfu.mL^{-1}$ at 24 h of incubation. This reduction in *L. monocytogenes* population was more effective than nisin and lactic acid alone and it could demonstrated the synergy between these two inhibitors at the concentration tested.

Table 23. Survival of population level of *L. monocytogenes* at initial inoculum level of 10^7cfu.ml^{-1} at 24 h at 37ºC in presence of different concentrations of nisin, lysozyme, lactic acid and their combinations.

Mixture	*L. monocytogenes* ($\log_{10}$ cfu.mL^{-1})
	24 h
Control	8.9
N	4.1
L	9.1
LA	5.8
$N_{1/2}$&$L_{1/2}$	9.4
$N_{1/2}$&$LA_{1/2}$	3.0
$L_{1/2}$&$LA_{1/2}$	7.7
$N_{1/3}$&$L_{1/3}$&$LA_{1/3}$	9.6
$N_{4/6}L_{1/6}$&$LA_{1/6}$	7.9
$N_{1/6}$&$L_{4/6}$&$LA_{1/6}$	8.5
$N_{1/6}$&$L_{1/6}$&$LA_{4/6}$	8.0

Others combinations did not inhibit *L. monocytogenes* growth (populations level were similar to the control differences lesser than 1 log).

Similar experiments were performed with an initial population level of 10^4cfu.mL^{-1}. Numerations were performed after 6 h and 24 h of incubation. A significant reductions of *L. monocytogenes* population was observed at 6 h and 24 h of incubation at 37ºC **(Table 24)**.

Table 24. Survival of population *L. monocytogenes* at initial inoculum level of 10^4 cfu.mL^{-1} after 6 and 24 h at 37ºC in presence of different concentrations of nisin, lysozyme, lactic acid and their combinations.

Mixture	*L. monocytogenes* ($\log_{10}$ cfu.mL^{-1}) after	
	6 h	24 h
Control	5.4	6.7
N	2.1	4.2
L	5.2	6.3
LA	3.5	2.6
$N_{1/2}$&$L_{1/2}$	4.1	5.8
$N_{1/2}$&$LA_{1/2}$	2.1	4.0
$L_{1/2}$&$LA_{1/2}$	5.2	6.3
$N_{1/3}$&$L_{1/3}$&$LA_{1/3}$	4.1	5.8
$N_{4/6}L_{1/6}$&$LA_{1/6}$	2.5	3.8
$N_{1/6}$&$L_{4/6}$&$LA_{1/6}$	4.8	5.8
$N_{1/6}$&$L_{1/6}$&$LA_{4/6}$	2.7	2.2

Nisin 0.25 g.L^{-1} induced a bacteriostatic effect at 6 h with a reduction in *L. monocytogenes* population counts of 3.3 $\log_{10}$ cfu.mL^{-1}. However a regrowth was observed after 24 h, but a 1.5 $\log_{10}$ cfu.mL^{-1} reduction in *L. monocytogenes* was obtained, compared with control. Surprisingly, no influence of lysozyme on reduction of *L. monocytogenes* was observed. *L. monocytogenes*

population was similar to control after 6 h (5.2 $\log_{10}$ cfu.mL^{-1}) and 24 h (6.3 $\log_{10}$ cfu.mL^{-1}) of incubation. A bacteriostatic effect of lysozyme in previous experiments was reported and has been already presented in Figure 12. The difference in initial inoculum level (0.02 A-10^6 cfu.mL^{-1}), could influenced antimicrobial lysozyme activity. Lactic acid (0.5%) led to reduce the population of *Listeria* after 6 h (1.9 $\log_{10}$ cfu.mL^{-1}) and 24 h (4,1 $\log_{10}$ cfu.mL^{-1}) of incubation.

Nisin and lysozyme in combination decreased of *L. monocytogenes* population, but at low level in comparison to reduction obtained by nisin and lysozyme alone. Nevertheless combination nisin and lactic acid indicated synergy, because nisin at 0.125 g.L^{-1} concentration was smaller than MIC value (0.25 g.L^{-1}). This synergetic interaction couldn't be observed previously, because too high concentration of lactic acid 0.5 % was used in Figure 12.

Combinations nisin, lysozyme and lactic acid had different effect on *L. monocytogenes* inhibition. The $N_{4/6}L_{1/6}\&LA_{1/6}$ (3.8 $\log_{10}$ cfu.mL^{-1} reduction) and $N_{1/6}\&L_{1/6}\&LA_{4/6}$ (2.2 $\log_{10}$ cfu.mL^{-1} reduction) combinations were more effective than nisin (4.2 $\log_{10}$ cfu.mL^{-1} reduction), lysozyme (6.0 $\log_{10}$ cfu.mL^{-1} reduction) and lactic acid (2.6 $\log_{10}$ cfu.mL^{-1} reduction) alone. Nisin (0.165 g.L^{-1}) in combination $N_{4/6}L_{1/6}\&LA_{1/6}$ and lactic acid (0.335%) in combination $N_{1/6}\&L_{1/6}\&LA_{4/6}$ was used at high concentration, but these concentrations were lower than their MIC values in Table 18 and Figure 12. The synergy was observed between nisin, lysozyme and lactic acid.

Similar to Parente *et. al.* (1998), Ukuku and Shelef (1997), we have also observed that inoculum level might influence nisin antibacterial activity. When the inoculum was diluted to 10^4 cfu.mL^{-1}, significant reduction of *Listeria monocytogenes* CIP 82.110 has been obtained. Low level of *L. innocua* (10^3-10^4 cfu.mL^{-1}) and relative low doses of nisin (50- 80 IU.mL^{-1}) was able to prevent survival of *L. monocytogenes*, however higher concentration $\geq$ 250 IU.mL^{-1} of bacteriocins were required for *L. innocula* at a level up to 10^6 cfu.mL^{-1} (Parente *et. al* 1998). Benkerroum and Sandine (1988) reduced the population of different strains of *L. monocytogenes* to 10^4 cfu.mL^{-1} with MIC value ranging from 740 to 10^5 cfu.mL^{-1} in Trypticase soy agar (TSA) and 1.85 to 10^3 cfu.mL^{-1} in MRS. Ukuku and Shelef (1997) showed that the sensibility of *L. monocytogenes* depended on inoculum level in broth. When inocula of 10^3 and 10^4 cfu.mL^{-1} were treated with nisin, there were not survivals of *Listeria monocytogenes*. Harris *et al.* (1991) reduced the population of 10^9 cfu.mL^{-1} of three *Listeria monocytogenes* strains by 6 to 7 $\log_{10}$ with 10 µl.mL^{-1} of nisin. Mohamed (1984) obtained complete inhibition of *L. monocytogenes* with 32 or 256 IU.mL^{-1} of pure nisin using an inoculum level of 10^5 cfu.mL^{-1}.

Impact of factors and interactions between nisin, lysozyme and lactic acid could be evaluated by polynomial equations and visualised on iso-response curves (**Figure 17**).

The reponse was represented by the equation: $Log_{10} = \beta 1\ N + \beta 2\ L + \beta 3\ LA + \beta 12\ N\&L + \beta 13\ N\&LA + \beta 23\ L\&LA + \beta 123\ N\&L\&LA$

Indicator β1, β 2 and β3 are the coefficient of three factors alone (1: nisin, 2: lysozyme and 3: lactic acid). However β12 and β23 are the coefficient of the first level interaction and β123 is a coefficient of second level interactions.

The constants derived from the polynomial model are reported in Table 25.

Table 25. Coefficients calculated for inhibition of *L. monocytogenes* in trypticase soy broth for an inoculum level of 10^7 cfu.mL^{-1} after 24 h of incubation and after 6 h and 24 h of incubation for an inoculum level of 10^4 cfu.mL^{-1}.

Initial population level	10^7	10^4	
Inhibition time	24 h	6 h	24 h
ß1	4.33	2.08	4.13
ß 2	8.62	5.25	6.38
ß 3	6.06	3.31	2.20
ß 12	10.70	1.87	2.22
ß 13	- 6.81	3.21	1.46
ß 23	0.55	3.12	6.75
ß123	75.71	- 2.97	- 20.86
R^2	0.915	0.936	0.769
R^{2A}	0.745	0.808	0.306

The coefficient of correlation R^2 gave indications about the correlation between the model and experimental design. RA^2 is an adjusted coefficient of correlation. The coefficients of correlation were rather good of 0.936 and 0.915, or 0.769 thus the model was close to experimental values and iso reponse curves can be exploited.

L. monocytogenes isoreponse curves were visualized (**Figure 17**). With an initial population level of 10^7 cfu.mL^{-1} (**A**) a population level of $10^{7\text{-}9\text{-}}$ cfu.mL^{-1} was observed in entirety graph indicating no inhibitory effect, except in the presence of lactic acid and nisin which caused a decrease in *L. monocytogenes* population at 37ºC after 24 h of incubation (isopopulation curves of 5 log) (**A**).

For an initial population level of 10^4 cfu.mL^{-1} *L. monocytogenes* population level was identical in axis nisin-lactic acid (iso- response curve 2.00 log cfu.mL^{-1}) after 6 h of incubation (**Ba**), so that nisin and lactic acid alone or in combination had an antibacterial effect. The axis lysozyme-lactic acid, and lysozyme- nisin indicated increasing population in the presence of

lysozyme. Lysozyme did not reduce population of *L. monocytogenes*. Nisin, lysozyme and lactic acid simultaneously were marked in centre of graph. The synergistic interaction could be present between these agents, because iso-response curve indicated lower *L. monocytogenes* population. After 24 h of incubation at 37ºC (**Bb**) and in the presence of nisin and lactic acid alone or in combination, minimal population of *L. monocytogenes* was observe. Presence of lysozyme increased isopoulation curves, indicating an absence of inhibitory activity. In the centre of domain, in the presence of the three components, with a high proportion of nisin, an inhibitor effect is observed. The iso-population curves was near 4 of log cfu.mL^{-1}.

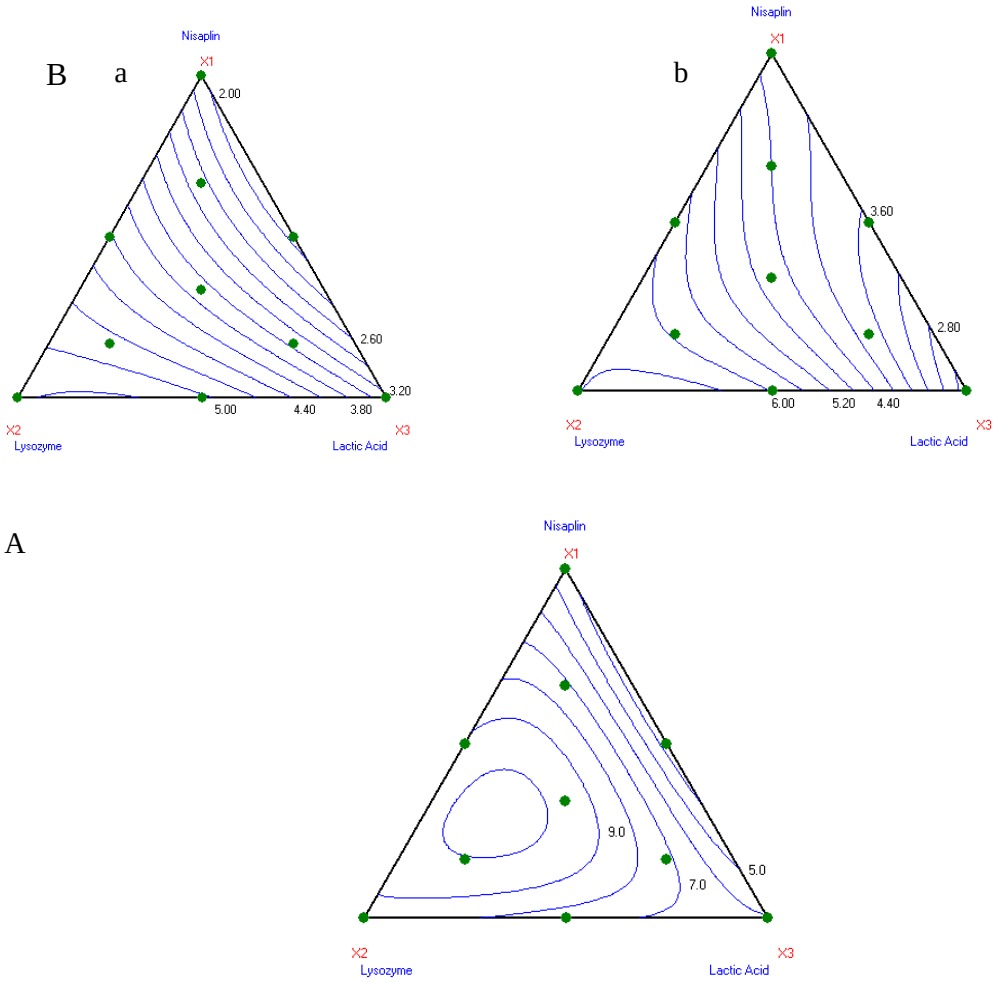


Figure 17. Response surface obtained with software analysis of the experimental design: Survival of *L. monocytogenes* in trypticase soy broth, in the presence of combinations of nisin, lysozyme and lactic acid, after 6 (a) and 24 (b) h and inoculum level 10^7 (A) and 10^4 (B) cfu.mL^{-1} at 37ºC.

In conclusion, we showed that the interactions and combinations between nisin, lysozyme and lactic acid against *Listeria monocytogenes* CIP 82.110 were significant. Antagonism interactions were observed between nisin- lysozyme, lysozyme- lactic acid during *L. monocytogenes* growth in TSBYE medium at 37ºC, under the condition tested. However synergetic interactions were proved between nisin – lactic acid and nisin-lysozyme - lactic

acid for special concentration determined of each inhibitors. The optimal combination were obtained by following mixtures: nisin 0.165 $g.L^{-1}$ - lysozyme 0.0025 $g.L^{-1}$ - lactic acid 0.08% , nisin 0.04 $g.L^{-1}$- lysozyme 0.0025 $g.L^{-1}$ - lactic acid 0.33% and nisin 0.125 $g.L^{-1}$ - lactic acid 0.25%.

Synergistic interaction between nisin and lysozyme were founded by other studies. The mixture of nisin and lysozyme N1: L3 (the two at weight to weight ratios of 1:1 nisin/lysozyme and 1:3 nisin/lysozyme were used at concentrations of 2500, 5000 and 10.000 $\mu g.L^{-1}$) demonstrated synergy against food spoilage by lactobacilli, Gram-positive bacteria, because they reinforce each other mechanisms of bacterial killing (Nattress and Baker 2001 2003). Also synergy was observed through measurements of kinetics of bacterial killing of *Lactobacillus curvatus* strain 854 and by scanning electron microscopy as a consequent change in optical density at 600 nm, which synergy had been observed in MIC assay (Chung and Hancock 2000). In contrast Gill and Holley (2000) did not observe any interactions between lysozyme and nisin against lactic acid bacteria. It could be a consequence of the different growth media used in the studies. Moreover Gill and Holley (2000) reported enhancement of the antibacterial effect of lysozyme and nisin, but MRS medium was diluted to be in deficient environment.

Masschalck *et al.* (2000) reported that a combination of nisin and lysozyme was helpful for reducing the tailing of survivor curves for high pressure treated bacterial populations, because this combination reduced the fraction of cells and this treatment was compared with the use of nisin or lysozyme alone. Mangalasary *et al.* (2007) presented that the application of nisin and lysozyme in combination of 1:5 at 62 and 65°C were effective in reducing the time required for a targeted log reduction in *Listeria monocytogenes* populations on the ready- to-eat (RTE) bologna surface.

III 3. Antibacterial activity of nisin & lysozyme & lactic acid used alone or in combination against *Staphylococcus aureus* CIP 4.83

The interactions between nisin-lysozyme- lactic acid were analyzed against *L. monocytogenes* CIP 82.110. It was interesting to see how this optimization could be applied to *S. aureus* population. Therefore, experiments have been realized with the same concentration of nisin, lysozyme and lactic acid than those used in previous studies with *L. monocytogenes*. The same experimental approach was used.

A sensitive *S. aureus* strain was selected by determination of MIC. Interactions between nisin and lysozyme, nisin and lactic acid, lysozyme and lactic acid were investigated by FIC determination. A Doelhert experimental design was applied to test the interactions between the three inhibitors and to determine the most antibacterial combination.

1. Determination the Minimum Inhibitory Concentration (MIC) nisin & lysozyme & lactic acid against *Staphylococcus aureus* strains.

The preliminary experiments were conducted to determine MIC value of nisin, lysozyme and lactic acid in agar (TSYAE) and broth (TSBYE) medium against *S. aureus* strains in order to select a model one (**Table 26**).

Table 26. Minimum inhibitory concentration (MIC) of nisin, lysozyme and lactic acid, expressed in ($g.L^{-1}$) on different *S. aureus* strains determined on trypticase soy agar (TSAYE) and trypticase soy broth (TSBYE) after 24 h at 37°C.

Staphylococcus aureus strains	Agar diffusion technique			Broth diffusion technique		
	Nisin ($g.L^{-1}$)	Lysozyme ($g.L^{-1}$)	Lactic acid (%)	Nisin ($g.L^{-1}$)	Lysozyme ($g.L^{-1}$)	Lactic acid (%)
S. aureus CIP 677	0,5	>1	>1	1	>1	1
S. aureus CIP 7625	1	>1	>1	>1	>1	1
S. aureus CIP 4.83	0.5	>1	>1	0.25	>1	0.25
S. aureus CIP 5710	1	>1	>1	0.25	>1	1

The MICs were different for agar and broth dilution assays for nisin and lactic acid. Nisin inhibited *S. aureus* strains in the range of MIC 0.25 to over 1 $g.L^{-1}$. The lowest nisin MIC (0.25 $g.L^{-1}$) was observed for *S. aureus* CIP 4.83 and CIP 5710, the highest nisin MIC over 1 $g.L^{-1}$ was for *S. aureus* CIP 7625 and CIP 5710 in agar diffusion technique. However *S. aureus* CIP 4.83 and CIP 5710 (MIC 0.25$g.L^{-1}$) were more sensitive to nisin in broth diffusion technique, with a MIC of 0.25 $g.L^{-1}$.

Lysozyme was not active against *S. aureus* strains at concentrations tested by the two methods.

Lactic acid inhibited all tested strains in liquid medium and *S. aureus* CIP 4.83 was the most sensitive strain, with a MIC of 0.25%. No inhibition occurred for concentration below 1% on agar medium.

The results of nisin MIC study against *S. aureus* showed the variation from 0.25 $g.L^{-1}$ to 1$g.L^{-1}$. Ray (1992) has observed similar results, because inhibition of *Staphylococcus aureus* required from 0.25 to 1.28 $g.L^{-1}$. Others authors suggested that the MICs of nisin against *S. aureus* Sa9 were respectively 0.3 $g.L^{-1}$ (Garcia *et al.* 2010a), 0.75 $g.L^{-1}$ (Martinez *et al.* 2008).

Staphylococcus aureus was resistant to antibacterial activity of lysozyme. This result was in agreement with Hughey Johnson (1987) and Pellergini *et al.* (1997). Under physiological conditions only a minority of Gram-positive bacteria were susceptible to lysozyme. It has been suggested that the main role of lysozyme is to participate in the removal of bacterial cell walls after the bacteria have been killed by antibacterial polypeptides present in egg albumin (Ibrahim *et al.* 2001). Amiri *et al.* (2008) presented that dextran conjugated lysozyme was not inhibitory against *S. aureus* in a culture media and cheese curd.

Among the tested microorganisms, *S. aureus* CIP 4.83 was the most sensitive to our antibacterial agents, so it was used for further analysis.

2. Effect of nisin & lysozyme & lactic acid on growth of *Staphylococcus aureus* CIP 4.83

The effect of different concentrations of nisin, lysozyme and lactic acid on growth kinetic of *S. aureus* were determined (**Figure 18**).

The presence of 0.03 $g.L^{-1}$ to 0.125 $g.L^{-1}$ nisin inhibited *S. aureus* (nisin A 0.121 and control A 0.234) at 6 h of incubation. This short inhibitory phase effect was followed by regrowth, with similar population than that of control. Nisin at 0.25 $g.L^{-1}$ significantly inhibited *S. aureus* after 24 h at 37ºC. The maximal inhibitory effect was obtained with nisin 0.5 $g.L^{-1}$ with a 99% reduction of absorbance after 24 h of incubation.

S. aureus was resistant to lysozyme even at high concentration of 1 $g.L^{-1}$. The absorbance values were similar to the control after 6 h and 24 h with different lysozyme concentrations.

Lactic acid at 0.125 % inhibited significantly the growth of *Staphylococcus aureus* CIP 4.83 after 6 h and 24 h of incubation with 85% reduction of absorbance at 24 h. Lactic acid at 0.5% induced after 24 h a 99% reduction in population level compared to the control.

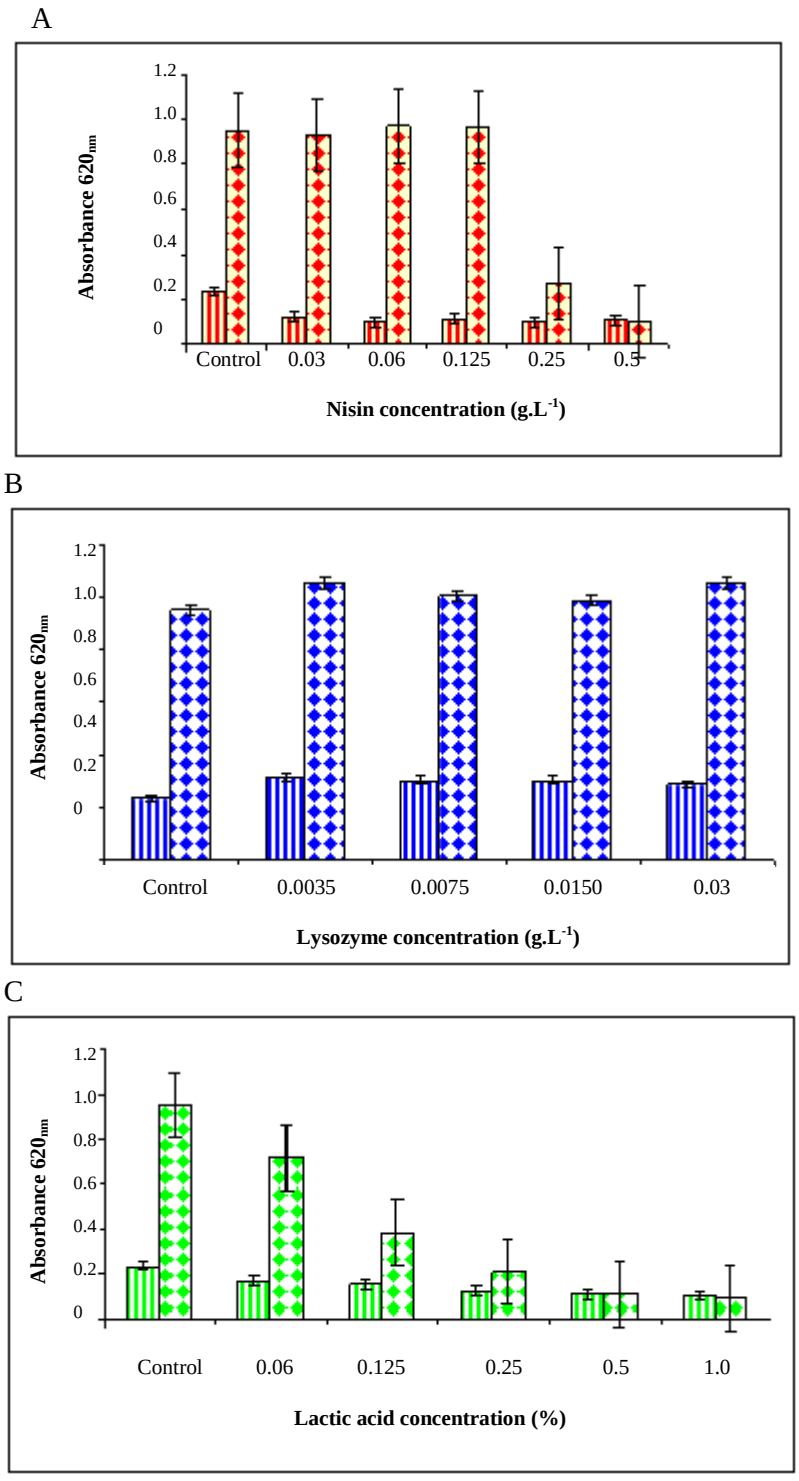


Figure 18. Growth kinetic of *S. aureus,* measured by A 620 $_{nm}$ in the presence different concentrations of nisin (A), lysozyme (B) and lactic acid (C) after 6(II) and 24 h (♦) in trypticase soy broth at 37ºC.

Although nisin 0.03 g.L^{-1} has quickly inhibited the growth of *S. aureus* at 37ºC after 6 h of incubation. A regrowth has been shown of *S. aureus* for 24 h of incubation

S. aureus was resistant to lysozyme, as it was reported in Table 26.

Lactic acid at 0.25% completely inhibited *S. aureus* growth at 6 h of incubation. No regrowth of *S. aureus* has been observed for 24 h of incubation.

3. Interactions between nisin- lysozyme, nisin & lactic acid and lysozyme & lactic acid on growth of *Staphylococcus aureus CIP 4.83*

The objective of the study was to determine possible interactions between nisin and lysozyme, nisin and lactic acid, lysozyme and lactic acid against *S. aureus*.

The inhibitors concentrations used were the same as than the one used in *L. monocytogenes* analysis. Nisin was used at 0.06g.L^{-1}, 0.125 g.L^{-1} and 0.03 g.L^{-1}. Although *S. aureus* was resistant on lysozyme, lysozyme was used at 0.0035 and 0.03 g.L^{-1} concentration to determine possible interactions. Lactic acid was used at inhibitory levels of 0.5 and 1%.

The evaluation of interactions between two inhibitors was studied by growth kinetics in presence of combination of two inhibitors, one at fixed concentration and the others one at variable concentrations, and also by FIC determination.

3.1 Evaluation of antibacterial interaction between nisin & lysozyme

Effectiveness of lysozyme 0.0035 g.L^{-1} with different combinations nisin and was evaluated against *S. aureus* (**Figure 19**).

Nisin alone was ineffective in reducing of *S. aureus*, because the concentration (0.125 g.L^{-1}), which have been chosen was lower than MIC (0.25 g.L^{-1}). Lysozyme at 0.0035 g.L^{-1} had not antibacterial activity in the concentration tested, the population had an absorbance of 1.052 at 24 h of incubation, similar to the control (0.145 A).

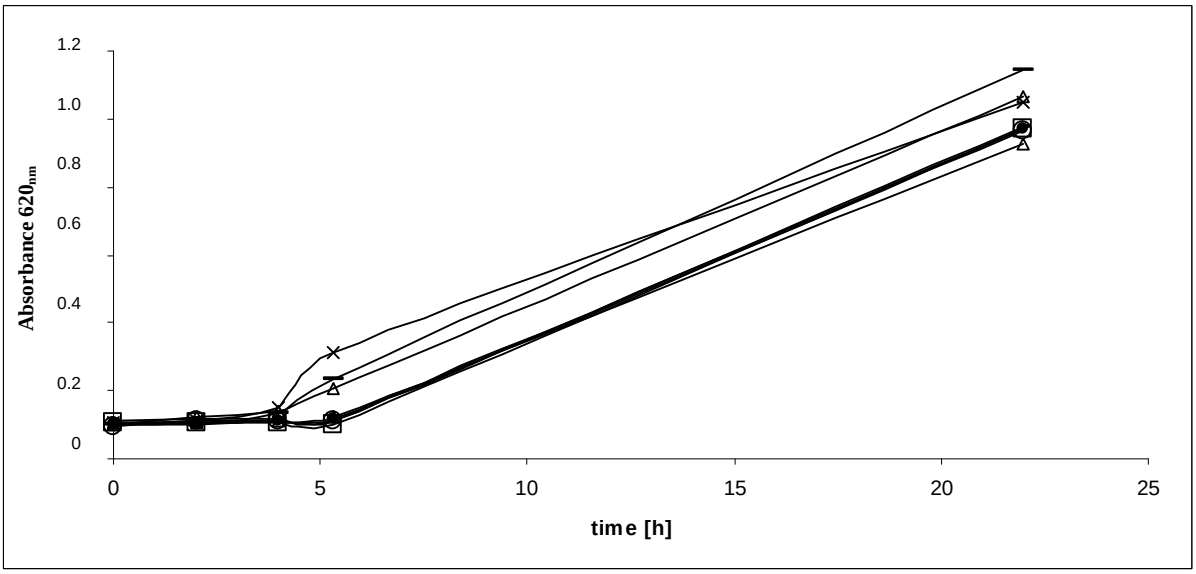


Figure 19. Kinetic of *S. aureus* at 37ºC in TSBYE - Tween broth in control (-), solution nisin 0.03 g.L^{-1} (Δ), nisin 0.06 g.L^{-1} (□), nisin 0.125 g.L^{-1} (o), nisin 0.03 g.L^{-1} and lysozyme 0.0035 g.L^{-1} (▲), nisin 0.06 g.L^{-1} and lysozyme 0.0035 g.L^{-1} (■) , nisin 0.125 g.L^{-1} and lysozyme 0.0035 g.L^{-1} (●), lysozyme 0.0035 g.L^{-1} (x) during 24 h incubation.

The antibacterial activity of nisin with 0.5 % lactic acid was 80% lower than in presence of lactic acid 0.5% alone. Moreover, the presence of nisin decreased the activity of lactic acid against *S. aureus.* This combination was tested only one time, so it was impossible to calculate standard deviations.

No synergistic or additive effects between these two inhibitors were observed.

The presence of a second inhibitor did not improve antibacterial efficacy. This combination was tested only one time, so it was impossible to calculate standard deviations.

No synergistic or additive effects between nisin and lysozyme were observed.

Antibacterial efficiency of various combinations of nisin and lysozyme was tested to determine FIC index (**Table 27**). Nisin and lysozyme didn't inhibit *L. monocytogenes* CIP 82.110 at the concentration tested. No synergy and additive were observed between nisin and lysozyme at 37ºC, after 24 h incubation in TSBYE medium.

Table 27. FIC calculation for nisin and lysozyme against *S. aureus* at 37ºC, after 24 h incubation in TSBYE medium (- no inhibition, + inhibition).

	FIC nisin	*0*	*0.06*	*0.12*	*0.25*
FIC lysozyme	Lysozyme concentrations ($g.L^{-1}$)	Nisin concentrations ($g.L^{-1}$)			
		0	0.03	0.06	0.125
0	0	-	-	-	-
0.25	0.0035	-	-	-	-
1	0.015	-	-	-	-
4	0.06	-	-	-	-

FIC index was over 1, antagonism was presented between nisin and lysozyme.

3.2 Evaluation of antibacterial interactions between nisin & lactic acid

Effectiveness combinations of lactic acid 0.5% with different nisin concentrations was evaluated against *S. aureus* (**Figure 20**).

Nisin alone was ineffective in reducing *S. aureus,* because the concentrations which have been chosen were lower than MIC (0.25 $g.L^{-1}$). Lactic acid at 0.5% totally inhibited the population with reduction of absorbance to 0.111, comparison in to absorbance 1.145 for the control at 24 h of incubation. The antibacterial activity of nisin with 0.5 % lactic acid was 80% lower than in presence of lactic acid 0.5% alone. Moreover, the presence of nisin decreased the activity of lactic

acid against *S. aureus.* This combination was tested only one time, so it was impossible to calculate standard deviations.

No synergistic or additive effects between these two inhibitors were observed.

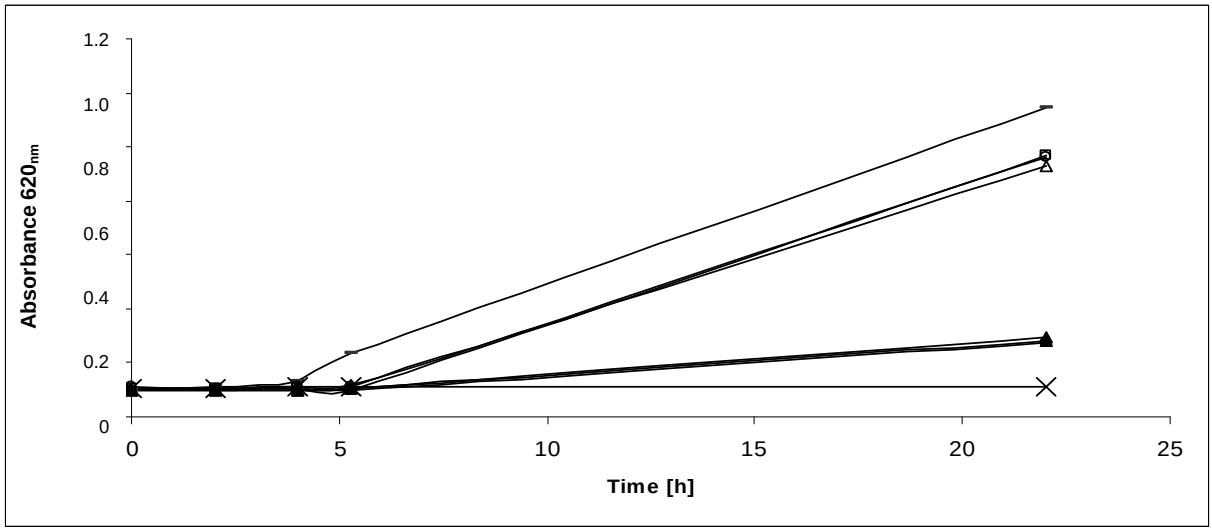


Figure 20. Kinetic of *S. aureus* at 37ºC in TSBYE - Tween broth in control (-), solution nisin 0.03 $g.L^{-1}$ (Δ), nisin 0.06 $g.L^{-1}$ (□), nisin 0.125 $g.L^{-1}$ (o), nisin 0.03 $g.L^{-1}$ and lactic acid 0.5% (▲), nisin 0.06 $g.L^{-1}$ and lactic acid 0.5% (■) , nisin 0.125 $g.L^{-1}$ and lactic acid 0.5% (●) and lactic acid 0.5% (x) during 24 h incubation.

The type of interactive effects between nisin and lactic acid against *S. aureus* was evaluated by FIC index determination(**Table 28**).

Table 28. FIC calculation of nisin and lactic acid against *S. aureus* at 37ºC, after 24 h incubation in TSBYE medium (- no inhibition, +inhibition).

FIC Lactic acid	*FIC nisin*	*0*	*0.06*	*0.12*	*0.25*
	Lactic acid Concentration	Nisin concentrations ($g.L^{-1}$)			
	(%)	0	0.03	0.06	0.125
0	0	-	-	-	-
1	0.5	+	+	+	+
2	1	+	+	+	+

The FIC calculations of nisin and lactic acid were negative. No synergistic or additive effects between nisin and lactic acid inhibitory to *S. aureus* at 37ºC, during 24 h were observed. FIC index was >1, it meant that antagonism interactions occurred between nisin and lactic acid.

3.3 Evaluation of antibacterial interactions between lysozyme & lactic acid

Effectiveness of combinations 0.5% lactic acid with different lysozyme concentrations was evaluated against *S. aureus* (**Figure 21**).

Lysozyme had no antibacterial activity against *S. aureus*, absorbance values were similar in the presence of lysozyme (0.983) and in the control (1.145) at 24 h of incubation. The antibacterial activity of lysozyme with 0.5 % lactic acid was 80% lower than in presence of lactic acid 0.5% alone. Lactic acid 0.5% totally inhibited (A 0.111) the population at 24 h of incubation. The presence of lysozyme decreased the activity of lactic acid against *S. aureus.* This combination was tested only one time, so it was impossible to calculate standard deviations.

No synergistic or additive effects between these two inhibitors were observed.

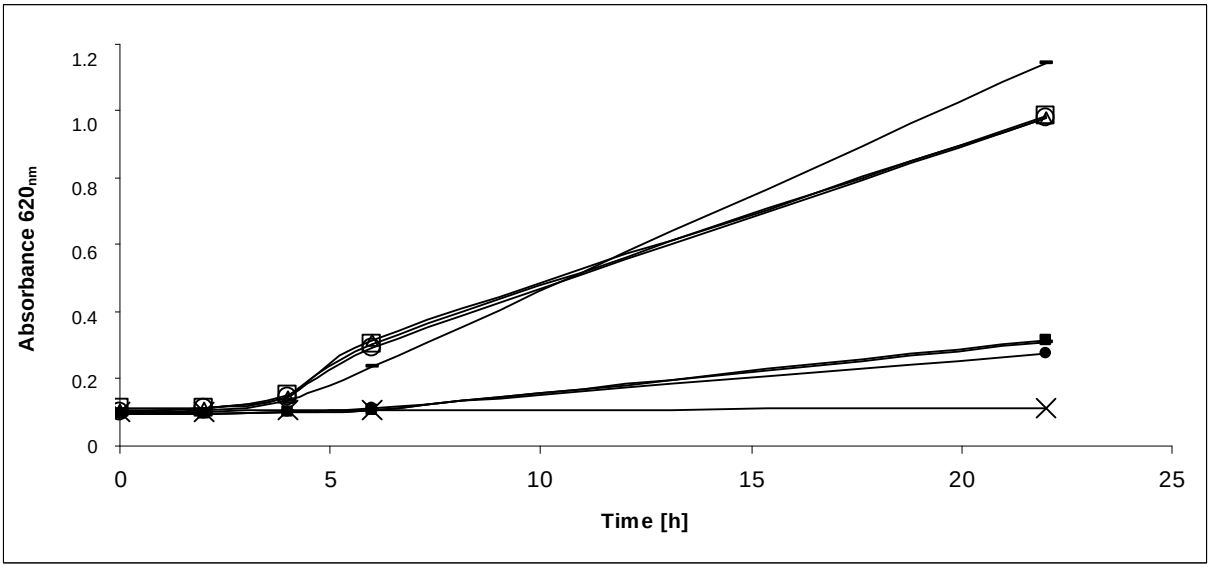


Figure 20. Kinetic of *S. aureus* at 37ºC in TSBYE - Tween broth on control (-), solutions: lysozyme 0.0035 g.L^{-1}(Δ), lysozyme 0.015 g.L^{-1}(□) lysozyme 0.06 g.L^{-1} (o), lysozyme 0.0035 g.L^{-1} and lactic acid 0.5% (▲), lysozyme 0.015 g.L^{-1} and lactic acid 0.5% (■), lysozyme 0.06 g.L^{-1} and lactic acid 0.5% (●) and lactic acid 0.5% (x) during 24 h incubation.

The type of interactive effects between nisin and lactic acid against *S. aureus* was evaluated by FIC index determination (**Table 29**).

Table 29. FIC calculation of lysozyme and lactic acid against *S. aureus* at 37ºC, after 24 h incubation in TSBYE medium (- no inhibition, +inhibition).

FIC Lactic acid	*FIC Lysozyme*	*0*	*0.25*	*1*	*4*
	Lactic Acid Cocentrations (%)	Lysozyme concentrations (g.L^{-1}) 0	0.0035	0.015	0.06
0	0	-	-	-	-
1	0.5	+	+	+	+
2	1	+	+	+	+

The FIC calculations of lysozyme and lactic acid were negative. No synergistic or additive between lysozyme and lactic acid were noticed against *S. aureus* at 37ºC during 24 h of incubation. FIC index was >1, antagonism interactions occurred between lysozyme and lactic acid.

Antagonism interactions were observed between nisin and lysozyme, nisin and lactic acid, lysozyme and lactic acid during inhibition *S. aureus* on TSBYE medium at 37ºC, under tested concentrations.

4. Doehlert experiment design in combined system nisin & lysozyme & lactic acid

The antibacterial activity of the various combinations of nisin, lysozyme and lactic acid (**Table 22**) against *S. aureus* were determined after 6 h and 24 h incubation at 37ºC for two initial inoculum level of 10^4 and 10^7 cfu.mL^{-1}, in the same conditions, which were previously used against *L. monocytogenes* CIP 82.110.

With an initial inoculum level of 10^7 cfu.mL^{-1}, an inhibitory effect lesser than 1 logwas obtained with all combination at 24 h of incubation (**Table 30**).

Table 30. Survival of population *S. aureus* at initial inoculum level of 10^7 cfu.mL^{-1} after 24 h at 37ºC in presence of different concentrations of nisin, lysozyme, lactic acid and their combinations

Mixture	*S. aureus* ($\log_{10}$ cfu.mL^{-1})
Control	9.7
N	4.2
L	8.6
LA	3.6
$N_{1/2}$&$L_{1/2}$	8.7
$N_{1/2}$&$LA_{1/2}$	8.6
$L_{1/2}$&$LA_{1/2}$	8.9
$N_{1/3}$&$L_{1/3}$&$LA_{1/3}$	8.6
$N_{4/6}L_{1/6}$&$LA_{1/6}$	8.8
$N_{1/6}$&$L_{4/6}$&$LA_{1/6}$	8.6
$N_{1/6}$&$L_{1/6}$&$LA_{4/6}$	8.9

Except for nisin and lactic acid alone, which inhibited *S. aureus* population with reduction of 5.5 and 6.1 $\log_{10}$ cfu.mL^{-1}, respectively (**Table 30)**.

Significant reductions of *S. aureus* population were observed at 6 h and 24 h of incubation with initial population level 10^4 cfu.mL^{-1} (**Table 31**). *S. aureus* population at 6 h of incubation was more or less inhibited by all of mixture, compared to control (7.9 $\log_{10}$ cfu.mL^{-1}). Nisin or lactic acid alone were the most effective and reduced respectively *S. aureus* population level by 6.9 and 4.8 $\log_{10}$ cfu.mL^{-1}. This inhibition was also observed after 24 h of incubation. Only combination $N_{1/2}$&$LA_{1/2}$ and $N_{1/6}$&$L_{4/6}$&$LA_{1/6}$ were efficient at 24 h with reduction of 5.3 $\log_{10}$

cfu.mL^{-1}, comparative to control. The synergy was observed between nisin- lactic acid and nisin-lysozyme-lactic acid, because low population level were obtained with minimal concentration of each inhibitors.

Table 31. Survival of population *S. aureus* at initial inoculum level of 10^4 cfu.mL^{-1} after 6 and 24 h at 37ºC in presence of different concentrations of nisin, lysozyme, lactic acid and their combination.

Mixture	*S. aureus* ($\log_{10}$ cfu.mL^{-1}) after	
	6 h	24 h
Control	7.9	8.3
N	1.0	2.0
L	4.4	9.1
LA	2.1	3.0
$N_{1/2}$&$L_{1/2}$	3.8	9.9
$N_{1/2}$&$LA_{1/2}$	4.9	3.0
$L_{1/2}$&$LA_{1/2}$	6.1	8.2
$N_{1/3}$&$L_{1/3}$&$LA_{1/3}$	5.7	8.5
$N_{4/6}L_{1/6}$&$LA_{1/6}$	4.4	3.0
$N_{1/6}$&$L_{4/6}$&$LA_{1/6}$	6.8	9.0
$N_{1/6}$&$L_{1/6}$&$LA_{4/6}$	6.3	8.0

Synergism was presented between nisin- lactic acid and nisin- lysozyme- lactic acid at initial inoculum level 10^4 cfu.mL^{-1}.

Impact of factors and interactions between nisin, lysozyme and lactic acid could be evaluated by polynomial equations (cf p. 83.) and visualised on iso-response curves (**Figure 22**).

The constants derived from the polynomial model are reported in Table 32.

Table 32. Coefficients calculated for inhibition of *S. aureus* in trypticase soy broth for an inoculum level of 10^7 cfu.mL^{-1} after 24 h of incubation and after 6 h and 24 h of incubation for an inoculum level of 10^4 cfu.mL^{-1}.

Initial population level	10^7	10^4	
Inhibition time	24 h	6 h	24 h
ß1	4.29	0.93	1.29
ß 2	8.39	4.57	8.91
ß 3	3.94	2.24	3.72
ß 12	8.98	4.60	15.59
ß 13	19.67	13.55	2.01
ß 23	3.47	12.02	9.65
ß123	4.77	13.45	7.31
R^2	0.922	0.945	0.864
R^{2A}	0.766	0.835	0.593

Coefficient of correlation R^2 gave indications about the correlation between the model and experimental design. RA^2 is an adjusted coefficient of correlation. The coefficients of correlation

were rather good of 0.945 and 0.864, or 0.922 thus the model was close to experimental values.

For an initial population of 10^7 cfu.mL^{-1}, the lowest iso-response curves were observed in presence of nisin and lactic acid (**Figure 22A**). Iso-response curves were at a level of 7 to 9 $\log_{10}$cfu.mL^{-1} in the center of domain, indicating no inhibition of *S. aureus* population in presence of the different combination of inhibitors.

For initial inoculum level of 10^4 cfu.mL^{-1} (**Figure 22 Ba**), the presence of different antimicrobial agents and their combination inhibited *S. aureus* population at 6 h of incubation. Nisin, totally inhibited *S. aureus*, when its proportion was increased and iso-response curve marked 1 $\log_{10}$ cfu.mL^{-1} in the presence of nisin alone.

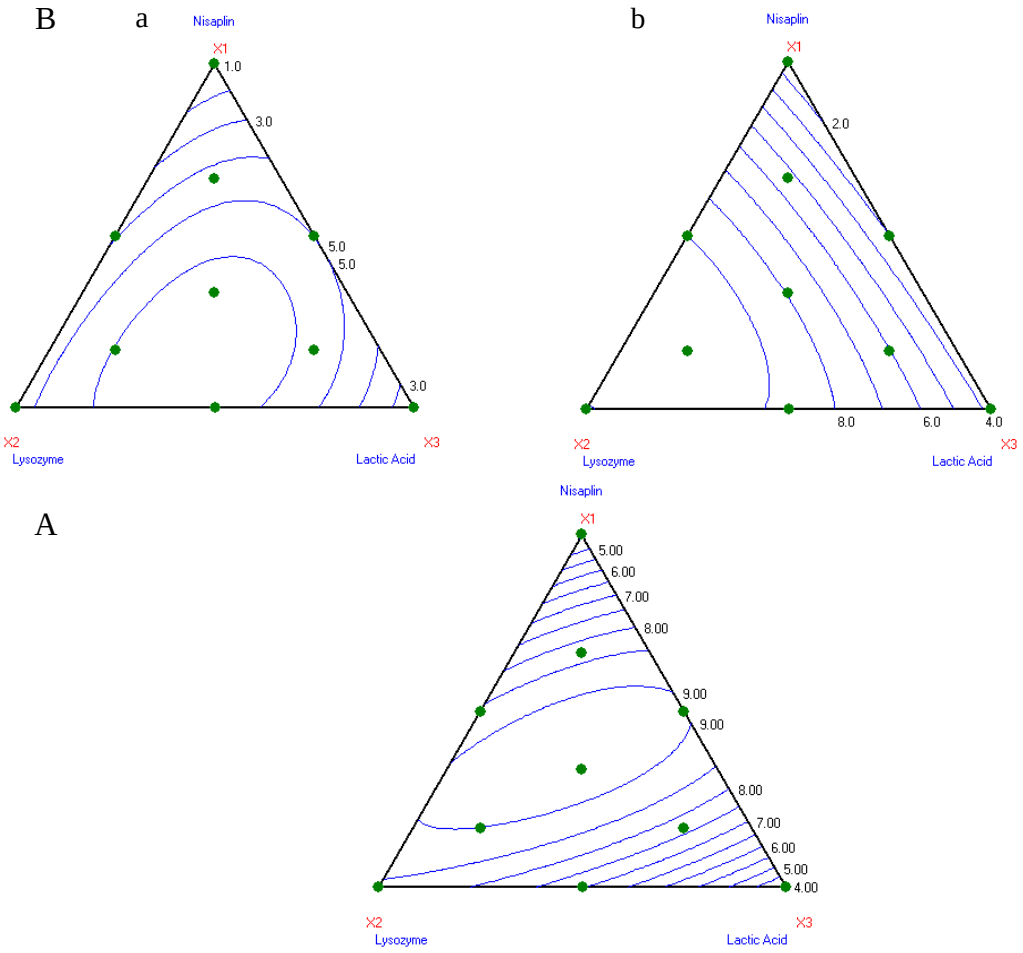


Figure 22. Response surface obtained with software analysis of the experimental design: Survival of *S. aureus* in trypticase soy broth, in the presence of combinations of nisin, lysozyme and lactic acid, after 6 (a) and 24 (b) h and inoculum level 10^7 (A) and 10^4 (B) at 37ºC.

The same effect with lower or higher amplitude was observed with lactic acid and iso-reponse curve was at 3 $\log_{10}$ cfu.mL^{-1}. After 24 h of incubation (**Figure 22 Bb**), synergetic interaction was observed between nisin-lactic acid, because a maximal decreased of *S. aureus* population was observed on axis nisin-lactic acid. Synergism was more visible at high nisin concentration. The presence of lysozyme decreased effectiveness of combination nisin, lysozyme and lactic acid. The synergistic effect between nisin, lysozyme and lactic acid, which was proved in previous analysis, was covered with statistical analysis and were not representative data.

Some research showed the synergy between nisin and other antimicrobials agent against *S. aureus* strain. When nisin was combined with endolisin synergistic effect was observed. The synergy occurred in vitro and was confirmed in challenge assays in pasteurized milk contaminated with *S. aureus* Sa9 (Garcia *et al* 2010a). Combination of endolisin and nisin was also successful at low pathogen concentration, opposite to the bacteriophages that require a minimum host threshold to be effective (Cairns *et al.* 2009). The presence of high pressure of 193 Mpa at subzero temperature (-20°C) did not cause synergy between nisin and lysozyme against *S. aureus* strains (Malinowska – Pańczyk and Kołodziejska 2009).

A synergistic activity of nisin and lactic acid was demonstrated against *S. aureus* for the concentration tested (0.125 g.L^{-1} nisin and 0.25% lactic acid). However, no synergy activity between nisin-lysozyme-lactic acid (0.165 g.L^{-1} nisin, 0.0025 g.L^{-1} lysozyme, 0.08% lactic acid) was demonstrated by Dohlert experiment design. The optimized combination was only efficacy against *L. monocytogenes* and can not be generalized to an other bacteria.

III 4. Effectiveness of selected combination nisin & lysozyme & lactic acid and nisin & lactic acid against *Listeria monocytogenes* CIP 82.110 and *Staphylococcus aureus* CIP 4.83

The objective of this study was to verify the antimicrobial activity of combinations nisin-lactic and nisin- lysozyme - lactic acid against *L. monocytogenes* and *S. aureus* in TSBYE medium at 37ºC. Only combination nisin (0.165 $g.L^{-1}$) – lysozyme (0.0025 $g.L^{-1}$) – lactic acid (0.08%) was retained, because on inhibitory effect against *S. aureus* was observed (**Table 33).** Each components was tested alone or in combination with one and two others inhibitors. Only one concentration for each antimicrobial agent was used (**Table 33)**.

Table 33. Serial mixture of nisin, lysozyme and lactic acid.

Nº experiment	Abbreviation	Nisin concentration ($g.L^{-1}$)	Lysozyme Concentration ($g.L^{-1}$)	Lactic Acid Concentration (%)
0	Control	0	0	0
1	N	0.165	0	0
2	L	0	0.025	0
3	LA	0	0	0.08
4	N&L	0.165	0.025	0
5	N&LA	0.165	0	0.08
6	L&LA	0	0.025	0.08
7	N&L&LA	0.165	0.0025	0.08

The antibacterial activity of these combinations was verified against *L. monocytogenes* (**Figure 23**) and *S. aureus (***Figure 24**).

At 3 h of incubation, nisin at the concentration of 0.165 $g.L^{-1}$ had an bactericid effect with reduction to 2 $\log_{10}$ $cfu.mL^{-1}$ population *L. monocytogenes*, but regrowth was observed at 24 h of incubation. It was confirmed by ours previous studies (Figure 13 and Table 19), because this concentration was lower than nisin MIC (0.25$g.L^{-1}$). Lysozyme, lactic acid and their mixture at these concentrations had no antimicrobial activity against tested strain, as it has already been observed in Figure 16 and Table 21. Combination nisin and lactic acid completely inhibited *L. monocytogenes* after 6 h. The regrowth was observed after 24 of incubation with maximal 8 $\log_{10}$ $cfu.mL^{-1}$ population. However combination nisin and lactic acid conserved its effect with a final level population of 6 $\log_{10}$ $cfu.mL^{-1}$, compared to 10^8 $\log_{10}$ $cfu.mL^{-1}$ for the control. Combination of three components could totally inhibit *L. monocytogenes* at 3 h with a final population 5 $\log_{10}$ $cfu.mL^{-1}$, compared to 9 $\log_{10}$ $cfu.mL^{-1}$ for the control at 24 h of incubation were obtained.

Synergy could be made evident between nisin, lysozyme and lactic acid. This synergistic interaction had been already proved in Table 25 and isoreponses curve (Figure 17).

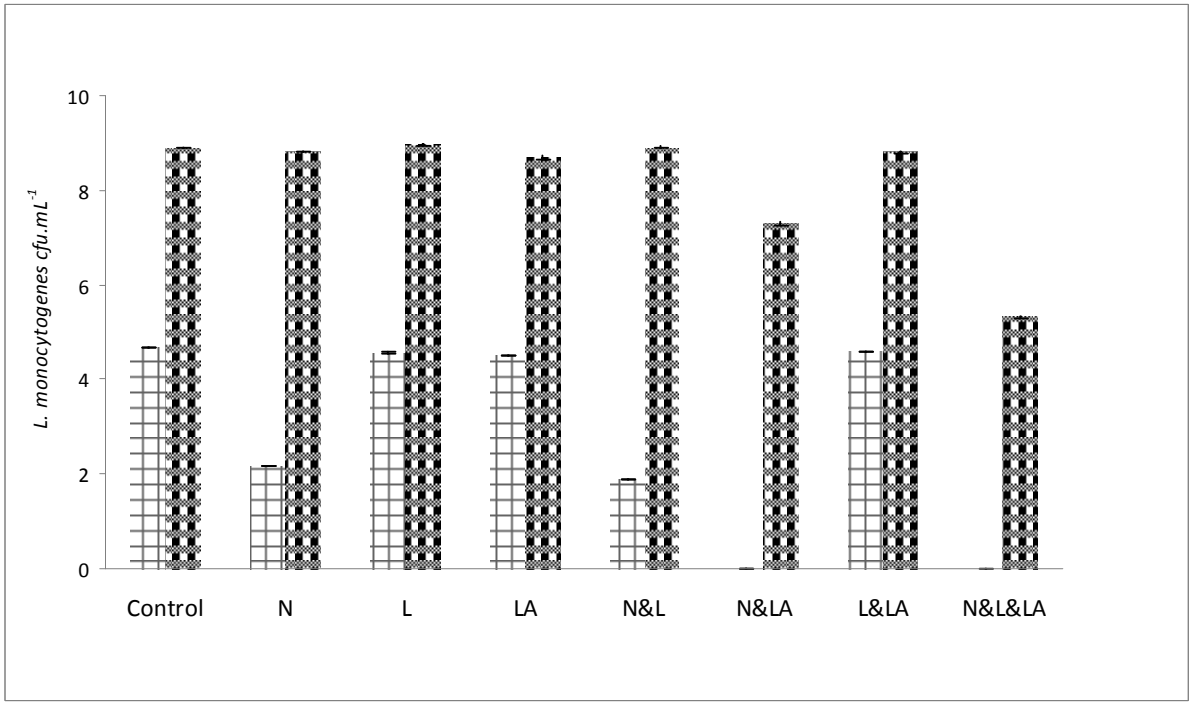


Figure 23: Survival of *L. monocytogenes* in the presence different combinations: (N: nisin 0.165 g.L^{-1}, L: lysozyme 0.0025g.L^{-1}, LA: lactic acid 0,08%, N&L: nisin 0.165 g.L^{-1}. lysozyme 0.0025g.L^{-1}, N&LA: nisin 0.165g.L^{-1} lactic acid 0.08%, L&LA: lysozyme 0.0025 g.L^{-1} lactic acid 0.08%, N&L&LA: nisin 0.165g.L^{-1}, lysozyme 0.0025g.L^{-1} et lactic acid 0.08%) in TSB-YE medium at 37°C after 3 h and 24 h of incubation.

Nevertheless this interaction did not totally inhibited the population, a regrowth was observed at 24 of incubation. Inoculum level 10^4 cfu.mL^{-1} was selected as initial contamination. It was supposed that this inoculum level was higher than the one observed in product accidental contaminated by *L. monocytogenes*. These combinations nisin lysozyme and lactic acid could be effective to control lower inoculum level and content lower concentration of each antimicrobial agent.

Lysozyme and lactic acid alone did not modify the growth of *S. aureus* (**Figure 24**), the population was identical to the control. Nisin had a bactericid effect and reduced to 1 log $_{10}$ cfu.m.L^{-1} *S. aureus* population at 6 h, but a regrowth was observed at 24 h of incubation. When inhibitors were associated simultaneously, a bactericide effect was observed in the presence of nisin and lactic acid, and with nisin and lysozyme with reduction to 2 $\log_{10}$ cfu.mL^{-1} population at 3 h. This effect is lower to the one induced by nisin alone. After 24 h only nisin- lactic acid had a population level lower the control (6 $\log_{10}$ cfu.mL^{-1} compared to 8 $\log_{10}$ cfu.mL^{-1}). The combination nisin lysozyme had no antibacterial effect. The combination nisin, lysozyme and

lactic acid inhibit *S. aureus* population after 3 h and 24 h with a reduction of 2 $\log_{10}$ compared to the control. These results were demonstrated in Table 32.

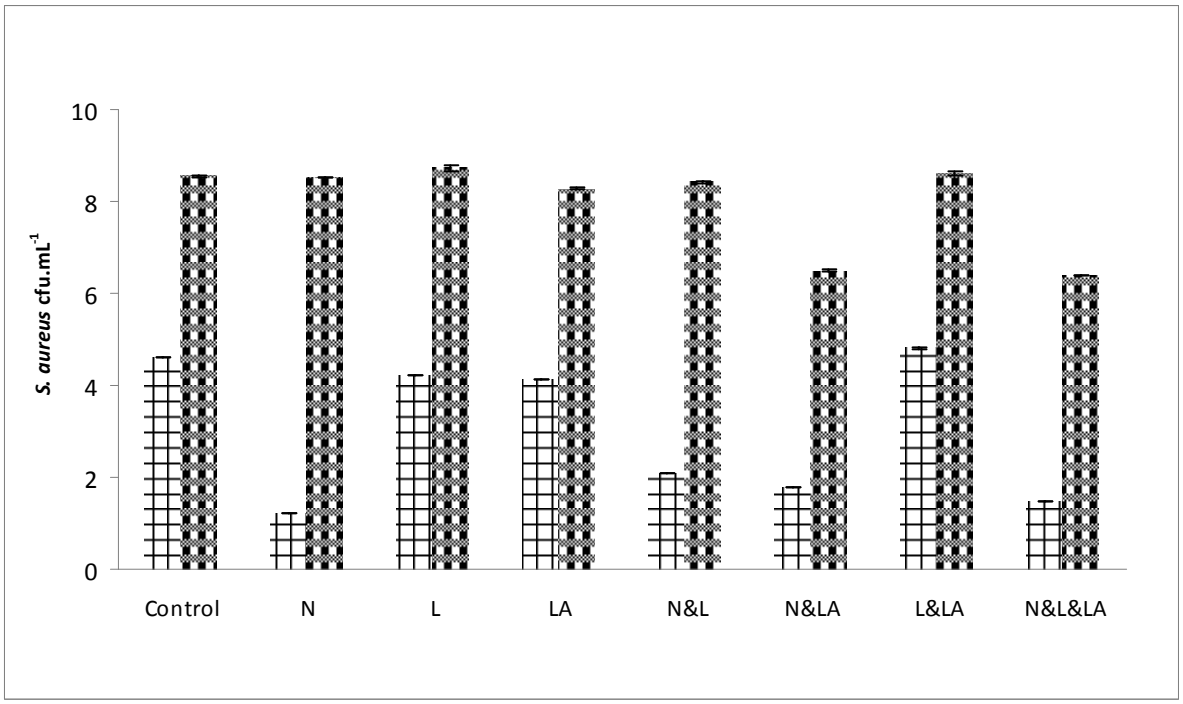


Figure 24. Survival of *S. aureus,* in the presence different combinations: (N: nisin 0.165 g.L^{-1}, L: lysozyme 0.0025g.L^{-1}, LA: lactic acid 0,08%, N&L: nisin 0.165 g.L^{-1}. lysozyme 0.0025g.L^{-1}, N&LA: nisin 0.165g.L^{-1} lactic acid 0.08%, L&LA: lysozyme 0.0025 g.L^{-1} lactic acid 0.08%, N&L&LA: nisin 0.165g.L^{-1}, lysozyme 0.0025g.L^{-1} et lactic acid 0.08%) in TSB-YE medium at 37°C after 3 h and 24 h of incubation.

The synergetic effect between nisin, lysozyme and lactic acid, which was observed against *L. monocytogenes,* was also demonstrated against *S. aureus.* Moreover this combination was more effective than combination of nisin-lactic acid.

Combination of nisin, lysozyme and lactic acid permitted inhibition of totally *L. monocytogenes* and 1 $\log_{10}$cfu.mL^{-1} *S. aureus* respectively at 6 h. Although the effectiveness of this mixture was decreased at 24 h of incubation, it was observed reduction of 4 $\log_{10}$cfu.mL^{-1} and 2 $\log_{10}$cfu.mL^{-1}, respectively.

III 5. Impact of nisin & lysozyme on cell membrane of *Listeria monocytogenes* CIP 82.110

Physiological characteristics of the membrane and/or the cell wall of bacteria are probably involved in insensitivity/resistance mechanisms limiting antibacterial agent diffusion through the cell wall, interaction with and penetration of the cell membrane (Jasniewski *et al.* 2008). To get more insight into the interaction of nisin (0.165 $g.L^{-1}$), lysozyme (0.0025 $g.L^{-1}$) and lactic acid (0.08%) with intact bacterial membrane, we studied the effect of nisin and lysozyme, lactic acid and their combination on membrane of *L. monocytogenes*. Action of the antimicrobial in the targeted viable cell membranes was studied by determination of potassium efflux and membrane potential ($\Delta\Psi$).

1. Impact of inhibitors on membrane potential ($\Delta\Psi$)

Nisin induced variation in membrane potential $\Delta\Psi$, in a range of 3 UA, in *L. monocytogenes* CIP 82.110 cells (**Table 34**). The amplitude was increased proportionaly to the concentration of nisin (data not shows). In contrast lysozyme and lactic acid didn't modify membrane potential $\Delta\Psi$. Besides, the presence of lysozyme or lactic acid in combination with nisin did not change the membrane potential $\Delta\Psi$.

Table 34. Impact of antibacterial agents alone and in combination on membrane potential of *L. monocytogenes* CIP 82.110 cells.

Mixtures	Variation in fluorescence emission of DiSC3-5 (UA)
N	3 +/- 0.5
L	0
LA	0
N&L	4 +/- 0.5
N&LA	3 +/- 0.5
N&L&LA	4 +/- 0.5

The synergitic effect of nisin, lysozyme and lactic acid at these concentration was not due to enhanced variation in membrane potential $\Delta\Psi$ of *Listeria monocytogenes* CIP 82.110. The presence of nisin induced modification in membrane potential $\Delta\Psi$.

2. Effect of inhibitors on potassium efflux in *Listeria monocytogenes* CIP 82.110

Nisin induced potassium efflux in *L. monocytogenes* (**Table 35**).

Table 35. Impact of valimocin and different concentration of nisin on potassium efflux in *L. monocytogenes.*

K^+ efflux ($mg.L^{-1}$)	H_20	Nisin concentration ($g.L^{-1}$)		
		1	2	4
	3.1	4.4	4.8	5.1

Increasing of nisin concentration significantly modified potassium efflux in cytoplasm of *L. monocytogenes.* Nisin at 1 $g.L^{-1}$ induced potassium efflux (4.4 $mg.L^{-1}$), comparative to control (3.1 $mg.L^{-1}$), respectively. Impact of combinations nisin, lysozyme and lactic acid on potassium efflux in *L. monocytogenes* was studied (**Table 36**). The presence of nisin was induced potassium efflux in *L. monocytogenes*.

Table 36. Impact of antimicrobial agent alone and in combination on potassium efflux in *L. monocytogenes* CIP 82.110.

K^+ efflux ($mg.L^{-1}$)	H_20	Nisin	Lysozyme	Lactic acid	Nisin Lysozyme	Nisin Lactic acid	Lysozyme Lactic acid	Nisin Lysozyme Lactic acid
	1.9	3.7	1.8	2.2	3.8	4	2.2	3.8

Lactic acid (2.2 $mg.L^{-1}$) and lysozyme (1.8 $mg.L^{-1}$) did not induced potassium efflux, because values were similar to control (1.9 $mg.L^{-1}$). Also presence of nisin in combination with lysozyme and/or lactic acid did not modify the amplitude of potassium efflux, because their values corresponded to nisin value alone (3.7 $mg.L^{-1}$).

We proved that the interactions and combinations between nisin, lysozyme and lactic acid against *Listeria monocytogenes* CIP 82.110 were significant. Antagonism interactions were observed between nisin- lysozyme, lysozyme- lactic acid during *L. monocytogenes* growth in TSBYE medium at 37ºC, under the condition tested. However synergetic interactions were proved between nisin – lactic acid and nisin-lysozyme - lactic acid, while nisin or lactic acud was used at high concentration.

The presence of lysozyme and lactic acid did no influence the variation in membrane potential $\Delta\Psi$ and potassium efflux in *Listeria monocytogenes* CIP 82.110 due to nisin. This might be due to the fact that lysozyme and lactic acid presented others mechanism of action against *Listeria* strain than nisin. It has been known that nisin acted on cytoplasmic membrane of sensitive cells *Listeria monocytogenes* CIP 82.110, where it formed pores, then lead to dissipation

of membrane potential and pH gradient (Budde and Jakobsen 2000). Results obtained from study of impact nisin on *Listeria monocytogenes* CIP 82.110 showed that nisin induced variation in membrane potential (ΔΨ), potassium efflux. These results were agreement on membrane potential (ΔΨ) with Budde and Jakobsen (2000) and Bruno *et al.* (1992).

The synergistic effect observed in presence of nisin, lysozyme and lactic acid was not due to enhanced modification of ΔΨ on potassium efflux.

III 6. Effectiveness of antibacterial activity the paper with nisin & lysozyme & lactic acid

The antimicrobial release systems have been used mainly in pharmaceutical applications and active packaging. The aim of controlled release systems intended for food packaging applications is to transfer the antimicrobial agent from the polymeric carrier to food to maintain a predetermined concentration of the active compound in the packed food for determine a period of time (Buonocore 2003).

The previous experiments were carried out to optimize the combination nisin lysozyme and lactic acid, which could be incorporated into Paper. The objective was to ensure antibacterial activity against *Bacillus licheniformis* CIP 52.71, *Listeria monocytogenes* CIP 82.110 and *Staphylococcus aureus* CIP 4.83.

1. Antibacterial effects of combination nisin & lysozyme incorporated onto paper against *Bacillus licheniformis* CIP 52.71

The combination nisin (0.625 $g.L^{-1}$) and lysozyme (1.25 $g.L^{-1}$) was determined by using combined concentrations of the two antimicrobials arranged in a checkerboard array (Davidson and Parish 1989). This formula had the highest antibacterial activity against *B. licheniformis*, but when 20 µl (v/v) of mixture was incorporated onto paper, no inhibition was observed. Too low concentration of combination or nisin and lysozyme diffusion problem through the paper matrix or in the agar gel could explained these results.

Forethought, nisin and lysozyme concentration were increased to 1 and 5 $g.L^{-1}$ and 10 and 50 µl of them were individually incorporated separately into paper (**Table 37**) to determine the inhibition area of *B. licheniformis.*

Table 37. Effect of nisin or lysozyme incorporated onto paper on inhibition area against *B. licheniformis* in TSAYE medium after 24 h at 37ºC.

Agent	Quantity deposed (µg)	Inhibition diameter (mm)
Nisin	10	10
	50	21
Lysozyme	10	16
	50	9

Paper, containing nisin and lysozyme proved antimicrobial activity against *B. licheniformis* in TSAYE medium. The results showed that inhibition diameter of lysozyme were more visible but smaller than that lysozyme. However the inhibition diameter did not change

proportionally to concentration of nisin and lysozyme. A problem of solubilisation nisin and lysozyme were observed. This analysis was done one time, so it was impossible to calculate standard deviations.

The concentration of nisin (0.3 $g.L^{-1}$) and lysozyme (1.25 $g.L^{-1}$) were multiplied by 1000 (MIC values in liquid TSBYE). 10 and 20 µl (v/v) of nisin (0.3 $kg.L^{-1}$) and lysozyme (1,25 $kg.L^{-1}$) were deposed incorporated onto paper (**Table 38**).

Table 38. Effect of combination of nisin and lysozyme incorporated onto paper on inhibition area against *B. licheniformis* in TSAYE medium after 24 hours at 37ºC

Quantity of mixture deposed (µg)	Inhibition diameter (mm)
10	8.5
20	8.5

The paper showed inhibitory effect against *B. licheniformis*. The inhibition diameter (8.5 mm) was the same for 10 and 20 µl of mixtures incorporated onto paper. No synergistic effect nisin and lysozyme was observed. This analysis was done one repetition, so it was impossible to calculate standard deviations.

2. Antibacterial effects of nisin & lysozyme & lactic acid incorporated onto paper against *Listeria monocytogenes* CIP 82.110 and *Staphylococcus aureus* CIP 4.83

The combination nisin 0.165 $g.L^{-1}$, lysozyme 0.0025 $g.L^{-1}$ and lactic acid 0.08% or nisin 0.125 $g.L^{-1}$ and lactic acid 0.25% were incorporated onto matrix paper. The inhibition diameters were zero, because quantities of nisin, lysozyme, and lactic acid, were too small for the antimicrobial effect against *L. monocytogenes* and *S. aureus*.

Due to the sorption of antimicrobial molecule, concentrations of nisin, lysozyme, lactic acid were strengthened 10 times, then put into paper matrix, and tested against *L. monocytogenes* and *S. aureus* with agar diffusion technique.

Analysis by spot confirmed the previous results. Synergy between nisin-lactic acid was proved *L. monocytogenes* and *S. aureus.* However synergetic effect of nisin- lysozyme- lactic acid was only observed against *L. monocytogenes* (**Table 39-40**).

The paper with the combination of nisin 0.125 $g.L^{-1}$ lactic acid 0.25% induced inhibition zone of (11 mm) against *L. monocytogenes* and (12 mm) against *S. aureus* (**Table 39**), whereas nisin or lactic alone were not inhibitory.

Table 39. Diameter of inhibition zone obtained against *L. monocytogenes* and *S. aureus* by the combination nisin 1.25 g.L^{-1} and lactic acid 2.5 %, with method agar diffusion technique at 37ºC after 24h.

Strains	*L. monocytogenes*		*S. aureus*	
Mixture	spot	paper	spot	paper
N	$4_{\pm 0.5}$	0	$4_{\pm 1,5}$	0
LA	0	0	0	0
N&LA	$6_{\pm 1}$	11_{+0}	$6_{\pm 1,1}$	$12_{\pm 0.3}$

The paper with the combination of nisin (1.65 g.L^{-1}) lysozyme (0.025 g.L^{-1}) lactic acid (0.8%) showed antimicrobial activity against *L. monocytogenes* (**Table 40**). Nisin indicated clear zone (14.5 mm) and lysozyme indicated fuzzy zone (14 mm). The combination nisin, lysozyme lactic acid led fuzzy zone (10 mm). Lysozyme had the most effective antimicrobial agent, but nisin and the mixture did not improve significantly the antibacterial activity. The same combination showed no antibacterial activity against *S. aureus* (**Table 40**). Whereas nisin incorporated onto paper led to an inhibition diameter of (11 mm).

Table 40. Diameter of inhibition zone obtained against *L. monocytogenes* and *S .aureus* by the combination nisin 1.65 g.L^{-1} & lysozyme 0.025 g.L^{-1} & lactic acid 0.8 %, with method agar diffusion technique at 37ºC after 24h.

Strains	*L. monocytogenes*		*S. aureus*	
Mixture	spot	paper	spot	paper
N	$8_{\pm 4.7}$	$14.5_{\pm 1.5}$	$10_{\pm 2}$	$11_{\pm 0}$
L	$13_{\pm 6.2}$	$14_{\pm 2.5}$	0	0
LA	0	0	0	0
N&L&LA	$16_{\pm 5.8}$	$10_{\pm 1.5}$	$4_{\pm 1.7}$	0

Paper, containing, mixture of nisin and lactic acid at higher concentrations than their MIC values had an antimicrobial activity against tested strains *Listeria monocytogenes* CIP 82.110 or *Staphylococcus aureus* CIP 4.93. The presence of lactic acid enhanced antimicrobial activity of nisin. This result confirmed the synergistic interaction between nisin and lactic acid, which was presented in previous, microbiological part. For the combination nisin, lysozyme, lactic acid, the inhibition areas obtained from spots were more visible than with paper. The retention of antimicrobial molecule in paper matrix could hypothesize why paper contained nisin, lysozyme and lactic acid had smaller inhibition areas. In condition that nisin or lysozyme were used at high concentration, we might suppose a problem of solubilization.

Nisin had been increasingly used as ‘bio-preservative’ for direct incorporation in food as well as in antimicrobial packaging. Imran *et. al.* (2010) demonstrated the antimicrobial activity of

film based on hydroxypropyl methylcellulose (HPMC) and nisin against *Listeria* species. The combination of nisin with lysozyme in package pasteurization of vacuum package RTE low fat turkey bologna caused the reduction of *Listeria monocytogenes* population (Matthews *et al.* 2008). Cao-Hang *et al.* (2010a,b) reported antimicrobial caseinate film containg 0.04 mg nisin/cm^2, which led to 1.1 log $cfu.g^{-1}$ reduction in *L. innocua* in surface inoculated semi- soft cheese sample and 1.1, 0.9 and 0.25 log $cfu.g^{-1}$ in depth inoculated. Mangalasary *et al.*(2010) presented no significant difference in reduction *L. monocytogenes* population by cellulose coating with nisin blended antimicrobial agent at level of 5.49 and 21.9 $mg.L^{-1}$.

III 7. Release of nisin & lysozyme, quantification from paper matrix to agarose gel.

The aim of this part was to quantify nisin and lysozyme into the cellulose matrix, and determine antimicrobial agents release from paper.

1. HPLC quantification of active compounds

Preliminary analyses showed that nisin and lysozyme could not be detected under the HPLC isocratic conditions. In order to improve the determination of nisin and lysozyme, reverse- phase conditions were applied.

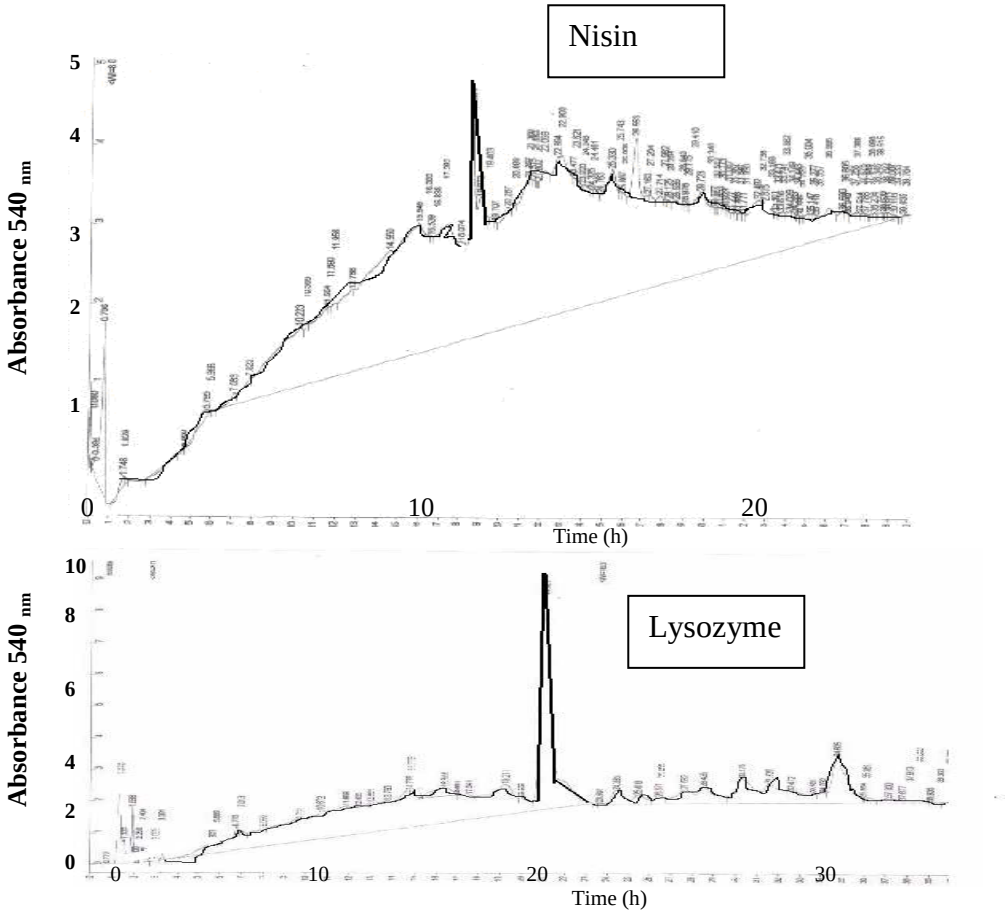


Figure 25. HPLC under gradient elution and by UV $_{540\ nm}$ detection of nisin 1.0 g.L^{-1} and lysozyme 1.0 g.L^{-1} (injected amount 50 µl).

Under the reverse phase conditions nisin and lysozyme might be eluted at about 20 min retention time (**Figure 25**). Many different peaks were observed. The presence of these peaks proved that commercial nisin and lysozyme were not 100% pure. Nisaplin, commercial product with nisin, contains 2,5% of nisin, 74.4% sodium chloride, 23.8% denatured milk solids and 1.7%

moisture (Deegan *et al*. 2006, Taylor *et al*.. 2007, Delves–Broughton *et al.* 1996). Lysozyme was a white, crystal powder.

Table 41. Area of peak nisin and lysozyme obtained by HPLC method.

Molecule	Injection (µg)	Concentration ($g.L^{-1}$)	Quantity ($\mu g.L^{-1}$)	Area UA
Nisin	50	0.5	25	109
Nisin	50	1.0	50	108
Lysozyme	50	0.5	25	214
Lysozyme	50	1.0	50	377
Lysozyme	400	1.0	400	515

In Table 41 the areas of nisin peak were identical at concentration 0.5 and 1.0 $g.L^{-1}$. In case of lysozyme, it was obtained positive relation between areas of lysozyme peak and lysozyme concentrations, because the areas of lysozyme peak proportionally increased with the volume of injection. HPLC method seemed to be only, suitable for lysozyme quantification. The elution times were the same for nisin and lysozyme and the sensitivity of method was not sufficient to measure nisin and lysozyme migration.

Following to these results and to improve the quality of nisin and lysozyme quantification, a purification process was applied to nisin and lysozyme. Extraction with ethanol and acetone was used before HPLC analysis.

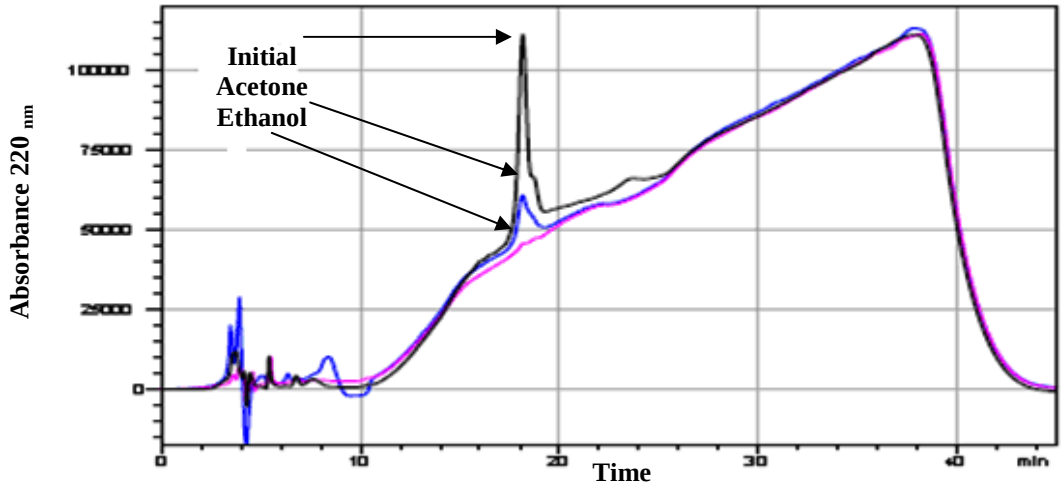


Figure 26. HPLC quantification under gradient elution and UV $_{220\ nm}$ detection of nisin 1 $g.L^{-1}$, nisin 1 $g.L^{-1}$ after purification by acetone, and nisin 1 $g.L^{-1}$ after purification by ethanol, injection 10µL.

Initial (untreated), acetone and ethanol nisin were analyzed at the wavelength of 220 nm and injection of 10 µl (**Figure 26** and **Table 42**). No nisin peaks were detected after ethanol extraction. The peaks areas with injection of volume 10 µl were superpose with the other ones with the injection. Moreover the peaks were identical for different nisin concentrations. In

consequence, there was not possibility to quantify nisin. Extraction with acetone caused ¾ loss of nisin.

Table 42. Area of peak nisin initial and after purification with acetone or ethanol obtained by HPLC method.

Molecule	Injection (µg)	Concentration ($g.L^{-1}$)	Quantity ($\mu g.L^{-1}$)	Area (UA)
Nisin initial	10	1.0	10	75
Nisin acetone	10	1.0	10	22
Nisin ethanol	10	1.0	10	0

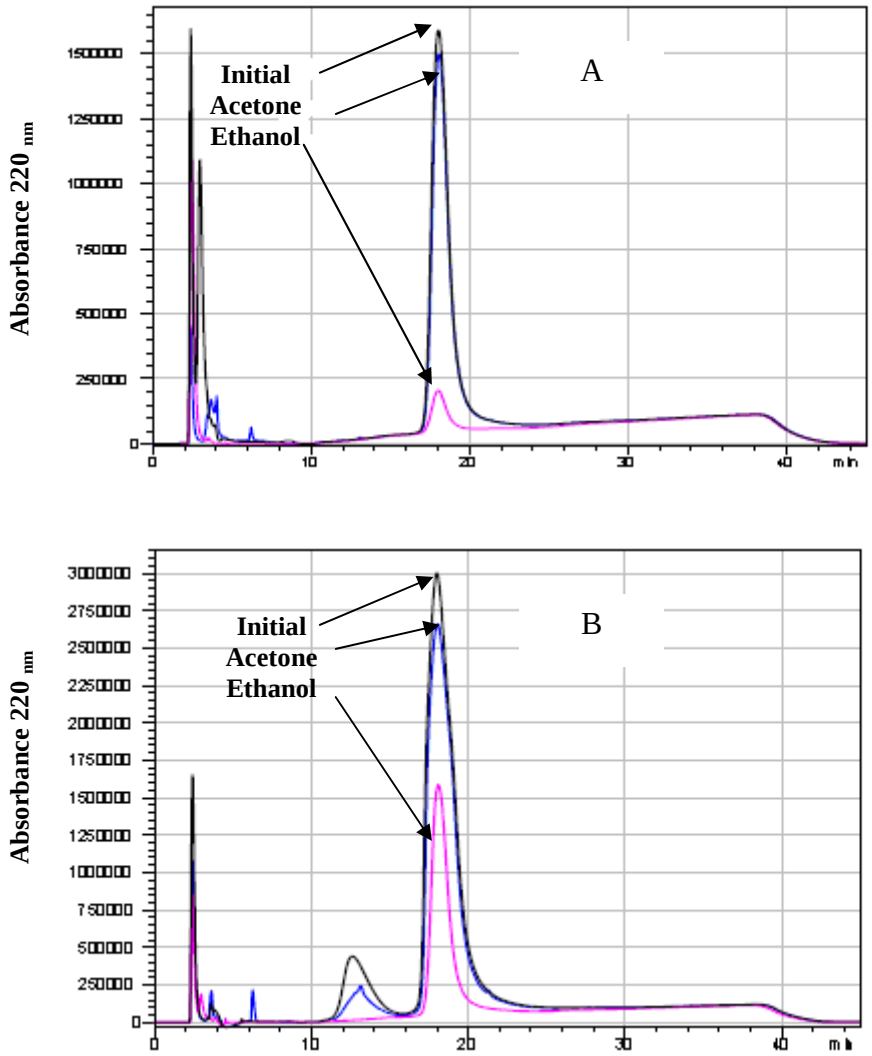


Figure 27. HPLC quantification under gradient elution and $UV_{254\ nm}$ detection of lysozyme standard 1 $g.L^{-1}$, lysozyme after purification by acetone, and lysozyme after purification by ethanol, injection 10µL (A) and 50µL (B).

Lysozyme initial (untreated), acetone and ethanol were eluted at about 17-18 min the retention time and at the wavelength of 220 nm (**Figure 27** and **Table 43**). There was no significant difference between the peak of initial (untreated) lysozyme and lysozyme after acetone extraction, whilst lysozyme peak after ethanol extraction was smaller than two other ones.

Table 43. Area of peak lysozyme initial and after purification with acetone or ethanol obtained by HPLC method.

Molecule	Injection (µg)	Concentration ($g.L^{-1}$)	Quantity ($\mu g.L^{-1}$)	Area (UA)
Lysozyme initial	10	1.0	10	15
Lysozyme acetone	10	1.0	10	14.6
Lysozyme ethanol	10	1.0	10	3
Lysozyme initial	50	1.0	50	33.5
Lysozyme acetone	50	1.0	50	29.6
Lysozyme ethanol	50	10	50	16.4

Most of the loss in nisin, lysozyme after extraction with ethanol might have been a consequence of degradation in the aqueous mixtures of ethanol. The HPLC assay showed that lysozyme could be determined without any purification. However the nisin was impossible to quantify with our HPLC method.

2. BCA method for active components determination

Due to no possibility of nisin quantification by HPLC method, we developed an assay based on bicinchoninic acid (BCA) method to measure nisin content in agarose gel. Agarose was chosen from various possible gel, because of its neutral structure. Agarose has a relatively uniform internal structure. Diffusion in agarose is regarded as diffusion in a porous solid matrix where, pores (from 0.1 to 1.0 µm) are connected with each other and filled with water (Ripoche *et al.* 2006).

The preliminary tests were necessary to choose the experimental conditions of nisi diffusion into agarose (**Figure 28**).

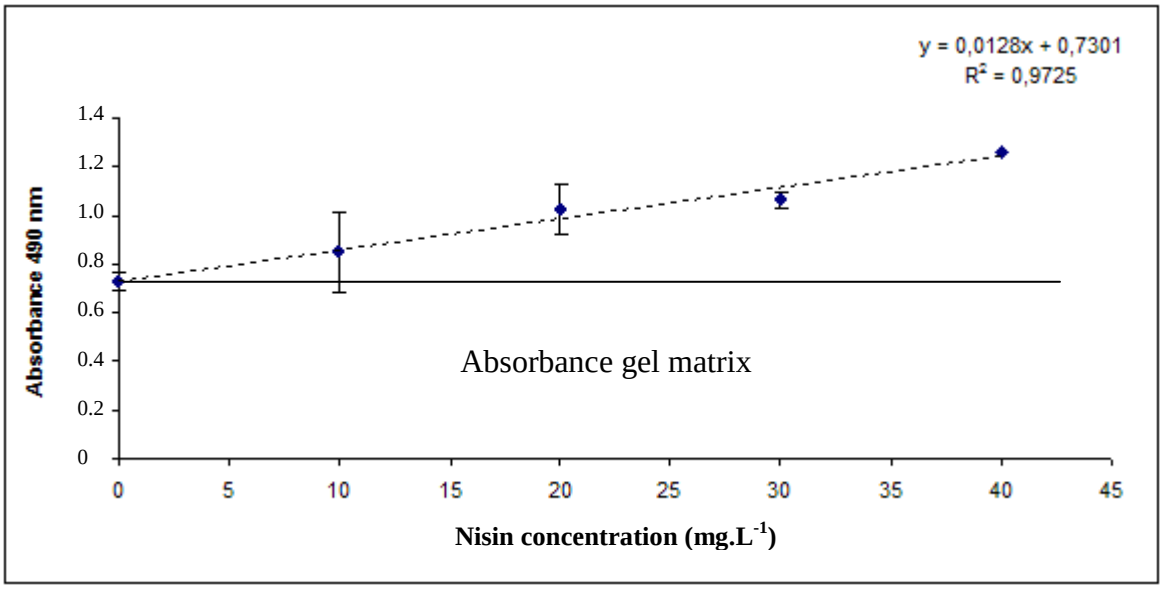


Figure 28. Quantification of nisin in agarose 2% .

Although gel matrix had important effect on results (Absorbance = 0.731 without nisin), the regression of absorbance versus nisin content presented good correlation coefficient (R^2 0.9725) with significantly different absorbance for the various nisin content (P>0.5). The absorbance values increased slowly with nisin concentration. Others factors had an influence on diffusion. It could be some extra reactions between agarose or nisin and reagents of BCA. The results indicated that BCA method is suitable for nisin quantification in agarose during diffusion experiments.

3. Nisin diffusion from cellulose support

Nisin at concentration of 20 $mg.L^{-1}$ were incorporated into cellulose support, the nisin diffusion into agarose 2% and 5% was studied for 5 days (**Figure 29**).

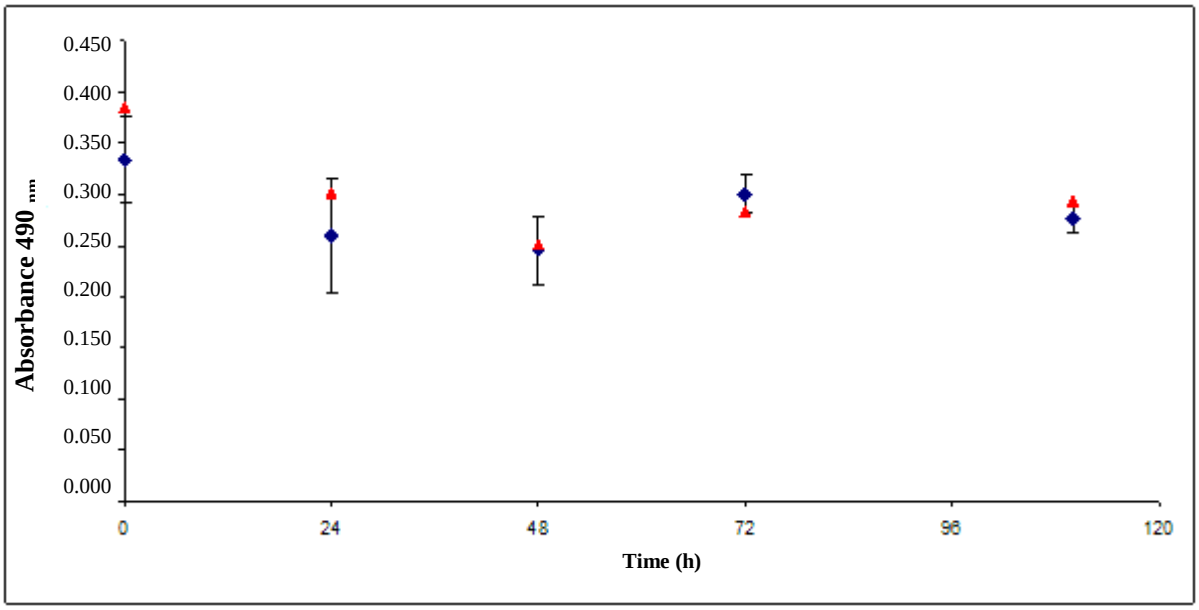


Figure 29. Diffusion kinetic diffusion of nisin from paper into agarose 2%▲ and 5 ♦%)

These results made evident that nisin diffusion occurred. The nisin diffusion was the most significant during the first 48 h, and after this time the diffusion was apparently stopped. Agarose concentration did not influence diffusion kinetic, because the results for agarose 2% and 5% presented no significant differences. To evaluate nisin diffusion from cellulose support to the gel (**Figure 29)**, the evolution of nisin quantity into cellulose during diffusion time (**Figure 30)** and nisin concentration in agarose during the migration was calculated (cf p53.) and presented in Figure 31.

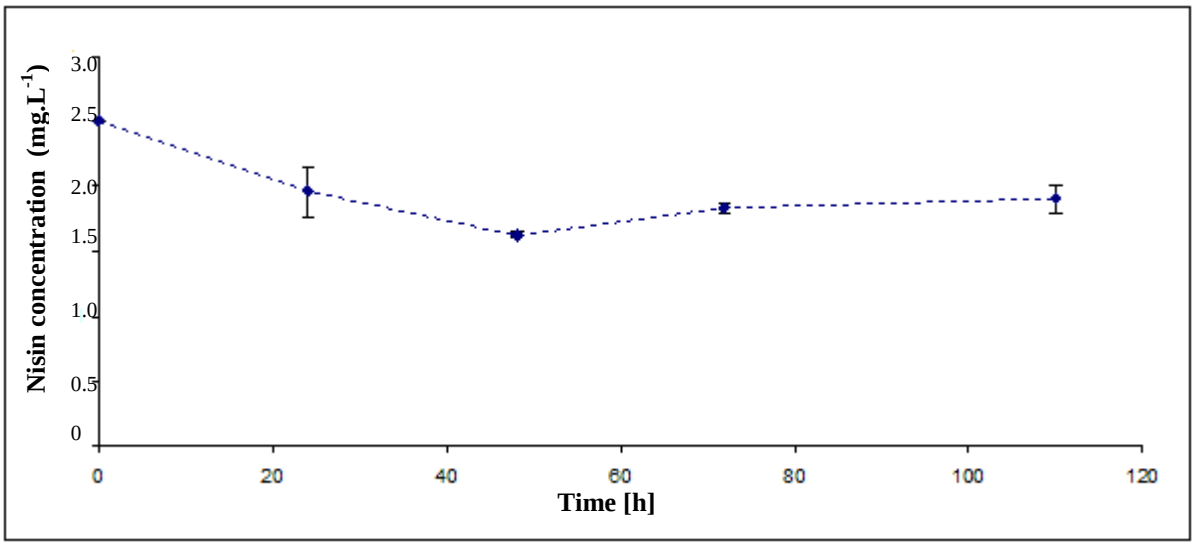


Figure 30. Evolution of nisin quantity incorporated into cellulose during diffusion.

Diffusion was confirmed by the decrease in nisin concentration from 2.5 mg/10 cm^2 of paper as initial concentration incorporated into paper to 1.63 mg/10 cm^2 after 48 h of contact with agarose. The amount of nisin into cellulose remained constant for the rest of the contact time. Nisin contents in agarose were calculated from Figure 30 and presented in Figure 31.

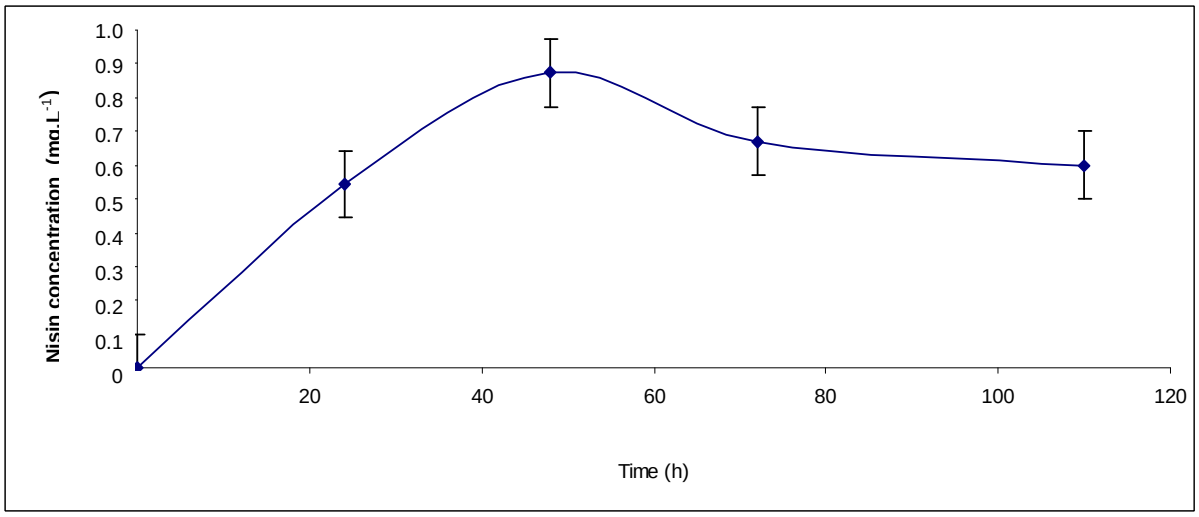


Figure 31. Evolution of nisin concentration in agarose during 5 days of migration from cellulose.

Nisin diffusion in agarose was observed during 48 h. Nisin maximal concentration (0.87 mg/g) was determined in agarose at 48 h. Then nisin concentration slowly decreased in two next days of diffusion.

Nisin diffusion was demonstrated and it was proved that nisin in cellulose was only able to diffuse for 48 h at a speed of 0.03 mg/h. Only 30% of nisin was diffused from paper to gel matrix after 5 days.

Some hypotheses such as nisin solubility, natural network of cellulose could explain the limited diffusion of nisin in agarose. The solubility of nisin did not influence diffusion, because nisin content in agarose 0.87 ($mg.L^{-1}$) was measured at 3.0 pH. Liu and Hansen (1990) measured the solubility of nisin in agarose as 57 $mg.mL^{-1}$ at pH 2, which was much higher than our concentration measurement.

It was possible that network of cellulose fibers formed a particular complex with nisin and blocked the diffusion (partition coefficient). However this aspect was positive to antimicrobiological activity of this cellulose support. Nisin is an antimicrobial agent which inhibits food pathogens and its contact with food is limited It might be supposed that nisin incorporated into cellulose and tested into natural food environment as cheese, fish or meat could remain at the surface of food and lead to higher release.

From microbiological point of view 0.25 $g.L^{-1}$ nisin was sufficient to inhibit *L. monocytogenes* at the laboratory conditions (cf. III a). It appeared to be interesting to determine if the amount of 1,78 mg nisin incorporated into 10 cm^2 cellulose support would be effective in inhibition of *L. monocytogenes* in agarose and food product.

Although there is a well-developed theory for diffusion processes. The present work indicated that nisin diffusion from cellulose support was limited by time and gel matrix, according to the bibliography. The shape, the density, the length and the diameter of the pores within and on the surface of agarose could affect the diffusivity (Bassi *et al.* 1987). Nisin concentration, temperature and agarose content could influence nisin diffusivity in agarose gel. Sebti *et al.* (2003) showed that nisin diffusion was independent of nisin concentration in agarose gel, which corroborated the hypothesis for the analytical solution of Fick's second law. Carnet –Ripoche *et al.* (2006) and Chollet *et al.* (2009) demonstrated that nisin diffusion could be favored by the presence of fat in agarose gel. This presence of fat in gel caused major microstructure changes, reducing fat level in a denser and less open microstructure with a smaller pore size of network (Pereira *et al.* 2006). About additional problems in the gel, Cao-Hang *et al.* (2010a,b) showed that 0.04 mg nisin/ cm^2 caseinate film hadn't migrate to inside semi-soft cheese, but its effect had been sufficient to stabilize on cheese surface contaminated by *L. innocua*. The commercial preparation " Nisaplin", contains 97,5% protein and salt which could have different affinities with gel matrix

and films polymers. These compounds could form conjugation by- products and interfere or compete with nisin diffusion in gel (Chollet *et al.* 2009). Further search be expected to study individually the effect of the salt and proteins present in the commercial form of nisin.

CONCLUSIONS
&
PERSPECITVES

The present thesis was focused on the improvement of the paper wrapping materials by adding nisin and lysozyme in combination and on the determination of the synergism, which could occur between these antimicrobial agents.

The results of experiments on *Bacillus* strains showed that the purpose might be difficult to achieve, and this fact has been confirmed by many different experiments and methods. So, this model with *Bacillus* strains has been replaced by *Listeria monocytogenes* CIP 82.110 and *Staphylococcus aureus* CIP 4.83 strains. Lactic acid as the third antimicrobial agent was chosen for further analysis with *L. monocytogenes* and *S. aureus.*

The mechanisms of synergy are complex and specific, and include the standardization and critical evaluation of testing and quantification methods and the characterization of the molecular mechanism of action.

Synergy between nisin, lysozyme and lactic acid, which was proved against *L. monocytogenes* and was not confirmed against *S. aureus* by statically analysis, but was demonstrated by classical methods. Combination of nisin 0.165 $g.L^{-1}$, lysozyme 0.0025 $g.L^{-1}$ and lactic acid 0.08% permitted a total inhibition *L. monocytogenes* and 1 $\log_{10}cfu.mL^{-1}$ increase for *S. aureus* respectively at 6 h. Although the effectiveness of this mixture decreased at the24 h of incubation, a reduction with 4 $\log_{10}cfu.mL^{-1}$ and 2 $\log_{10}cfu.mL^{-1}$ was netherless observed, respectively.

The effectiveness of antmicrobial combination is associated to the inoculum size in laboratory systems and might not be realized in cheese or meat, due to the interactions with, proteins, fats, natural microflore of food. Our results indicated that the inoculum size of 10^4 $cfu.mL^{-1}$ had permit to observe the synergy effect between nisin, lysozyme and lactic acid. The higher (10^7 $cfu.mL^{-1}$) inoculum size can lose the interaction between the antimicrobial agents and lower (10^3 $cfu.mL^{-1}$) inoculum size was not sufficient to observe the synergy, because nisin or lactic acid alone inhibited totally *L. monocytogenes* population.

Paper, contained, mixture of nisin and lysozyme at higher concentrations could assure the antimicrobial activity against tested strains, *Listeria monocytogenes* CIP 82.110 or *Staphylococcus aureus* CIP 4.93. The presence of lactic acid did not enhance antimicrobial activity of nisin. The inhibition areas obtained from spots were more visible than with paper. The retention of antimicrobial molecule in paper matrix could hypothesize why paper contained nisin or lysozyme had smaller inhibition areas. As nisin and lysozyme have great antibacterial activity,

but are expensive. Experiments in combination nisin, lysozyme or lactic acid were performed and showed a good effect.

It was proved that nisin in cellulose was only able to diffuse for 48 h at a speed of 0.03 mg/h. Only 30% of nisin diffused from paper to gel matrix after 5 days. Extra analysis in diffusion should be performed after the incorporation combination of nisin, lysozyme and lactic acid in cellulose.

From microbiological point of view 0.25 $g.L^{-1}$ nisin was sufficient to inhibit *L. monocytogenes* in the laboratory conditions. It appeared to be interesting to determine if the combination of nisin 0.165 $g.L^{-1}$, lysozyme 0.0025 $g.L^{-1}$ and lactic acid 0.08% incorporated into 10 cm^2 cellulose support would be effective in inhibition of *L. monocytogenes, S. aureus*, Bacillus *strains* in agarose, then possibly in cheese or meat model system and finally in cheese or meat product.

As a perspective, it could be interesting to determine the effectiveness of antimicrobial activity of cellulose, which contains nisin, lysozyme and lactic acid. The intrinsic factors, such as: nutrients, pH, water activity, in food might affect the growth of microorganism or antimicrobial activity and diffusion of our antimicrobial agent.

Modeling nisin diffusion is not easy to understand and is important for antimicrobial packaging, which could be protected and effective as food packaging. Many published results and our results show a quick desorption of nisin. Consequently to study the nisin release from films, some new systems will be investigated in the laboratory.

Encapsulation and liposome are widely used in controlled drug release for pharmaceutical formulations and have potential application in food industry. Liposome encapsulation of our antimicrobial agents before incorporation onto cellulose could improve the antimicrobial effectiveness of paper.

Nowadays the research and food industry market should be cooperate closely to respond to increasing consumers demand, and explores and/or improves food safety through using new packaging materials, implementing flexible modern and standard technology. The antimicrobial packaging is the best example of this cooperation. Food is protected from microbial spoilage by nisin, lysozyme and lactic acid. Cellulose packaging with antimicrobial agents prolongs and enhances of shelf life of food product. However the development of antimicrobial paper

packaging is a long, hard work with many problems, which are resolved by science and lots of experiments in the laboratory. We were able to optimize the mixture of nisin, lysozyme and lactic acid and try out only diffusion of nisin incorporated in a cellulose support. Next thesis could be focused on the determination diffusion of this mixture in cellulose then its antimicrobial effectiveness in model and real food.

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VU LES RAPPORTS ETABLIS PAR :
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à soutenir devant un jury de l'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE, une thèse intitulée :

"Microbiological growth control by nisin, lysozyme and lactic acid combination : Application to active packaging. "

en vue de l'obtention du titre de :

DOCTEUR DE L'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE

Spécialité : **« Procédés Biotechnologiques et Alimentaires »**

Fait à Vandoeuvre, le 09 février 2011
Le Président de l'I.N.P.L.,
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Abstract
Food and packaging are closely related. Many chemical and physical reactions exist between a food, its packages and the environment, which alter the composition, quality and physical properties of the food and/or the package. Thanks to active or antimicrobial packaging, food products can be distributed over a wide geographical area over a long period of time without unacceptable quality loss. Natural antimicrobials such as nisin, lysozyme or lactic acid improve shelf-life, eliminate undesirable pathogens and delay microbial spoilage.
The present PhD thesis was focused on the improvement of paper wrapping materials by adding nisin, lactic acid and lysozyme in combination and on the determination of the synergism, which could occur between these antimicrobial agents.
Synergy between nisin, lysozyme and lactic acid, which was proved against *Listeria monocytogenes* CIP 82.110 and was not confirmed against *Staphylococcus aureus* CIP 4.83 by statistical analysis. The paper, containing a mixture of nisin, lysozyme and lactic acid could ensure a antimicrobial activity against *Listeria monocytogenes* CIP 82.110 or *Staphylococcus aureus* CIP 4.93. Nisin diffusion from packaging to simulated food was demonstrated. It was proved that nisin in cellulose was only able to diffuse for 48 h at a speed of 0.03 mg/h. Only 30% of nisin was diffused from paper to gel matrix after 5 days. Extra diffusion analyses on combination of nisin, lysozyme and lactic acid should be performed to confirm the antimicrobial effectiveness of this packaging.
Keywords: listeria, staphylococcus, antibacterial effect, packaging

Résumé
Les aliments et les emballages sont interdépendants. Plusieurs reactions chimiques et physiques existent entre les aliments, l'emballage et l'enviromment, lesquelles peuvent changer la composition, la qualité et les propriétés physiques des aliments, voire de l'emballage. L'emballage actif ou antimicrobien permet la distribution d'un aliment dans le monde entier sans perte de qualité, pendant une longue période de transport. Les antimicrobiens naturels comme la nisine, le lysozyme ou l'acide lactique contrôlent la contamination microbienne d'un aliment, améliorent son stockage, éliminent les pathogènes indésirables et retardent la prolifération microbienne. L'amélioration d'un emballage en papier, contenant de la nisine, le lysozyme, l'acide lactique, ainsi que l'étude de la synergie d'action entre antimicrobiens constituent les objectifs de cette thèse.
La synergie entre nisine, lysozyme et l'acide lactique a etait déterminée avec *Listeria monocyotgenes* CIP 82.110 mais n'a pas observée pour *Staphylococcus aureus* CIP 4.83 par analyses statistiques. Le papier « activé » avec un mélange de nisine, lysozyme et acide lactique permet d'assurer une action antimicrobienne vis à vis de *Listeria monocytogenes* CIP 82.110 et de *Staphylococcus aureus* CIP 4.93. La diffusion de la nisine du papier vers un aliment simulé a était démontrée pendant 48h à la vitesse de 0.03 mg/h. En 5 jours, seulement 30% de la nisine a migré vers l'agarose. Des analyses complémentaires de diffusion vont permettre de mieux comprendre l'efficacité d'un emballage antimicrobien contenant un mélange de nisine, de lysozyme et d'acide lactique.
Mots clefs: listeria, staphylococcus, effet antibacterien, emballage