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TITOLO TESI

Use of essential oils and biocontrol cultures for the improvement of shelf-life of fresh cut products

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CHAPTER 1

GENERAL INTRODUCTION

1.1 MINIMALLY PROCESSED FRUITS AND VEGETABLES

FRESH-CUT PRODUCTS AND THEIR MARKET TREND

Fresh-cut fruits and vegetables can be defined as any fresh fruit or vegetable that has been physically modified from its original form (by peeling, trimming, washing, and cutting) to obtain 100% edible product that is subsequently bagged or prepackaged and kept in refrigerated storage (IFPA, 2005). Fresh-cut produce includes any kind of fresh commodities and their mixtures in different cuts and packaging. Items such as bagged salads, baby carrots, stir-fry vegetable mixes, and fresh-cut apples, pineapple, or melon are only some examples of this type of product (Rojas-Graù et al., 2011).

The market for chilled fresh-cut produce has witnessed dramatic growth in recent years, stimulated largely by consumer demand for fresh, healthy, convenient and additive-free foods which are safe and nutritious. In fact, organizations such as the World Health Organization (WHO), Food and Agriculture Organization (FAO), United States Department of Agriculture (USDA), and European Food Safety Authority (EFSA) recommended an increase of fruits and vegetables consumption to decrease the risk of cardiovascular diseases and cancer (Allende et al., 2006). The food industry has responded to this demand with creative product development, new production practices, innovative use of technology and skillful marketing initiative (James & Ngarmsak, 2011).

Consumers generally purchase fresh-cut products for convenience, freshness, nutrition, safety and the eating experience. In fact, the consumption of these products allows to save time on food preparation. Another reason for the success of fresh-cut product is the absence of waste material. Waste is generated in peeling and coring fruits. However, when utilizing fresh-cut produce, 100% is consumable, and there is a substantial decrease in labor required for home produce preparation and waste disposal (Garcia & Barrett, 2005).

Different kind of Fresh-cut fruits and vegetables have been introduced or expanded since the early 1980s. Some fresh-cut produce currently available in supermarkets is include in Table 1.1.

Table 1.1-*Some kind of prepared fruits, Leafy salads and Mixed-Tray salads, currently available in supermarkets (Garner E. 2008).*

Prepared Fruits	Leafy Salads	Mixed-Tray Salads
Classic salad	Sweet + crunchy salad	Potato + egg salad
Pineapple chunks	Watercress	Sweet + crunchy salad
Luxury fruit salad	Crispy salad	Lettuce + tomato + cucumber + celery
Melon medley	Iceberg lettuce	Mediterranean salad
Melon + grape	Italian salad	Fresh + crispy
Pineapple pieces	Rocket salad	Ribbon salad lettuce + cucumber
Sliced melon selection	Rocket	Mixed pepper salad
Tropical fruit salad	Baby leaf salad	Prawn + pasta salad
Fruit salad	Spinach + watercress + rocket salad	Tuna niçoise
Pineapple slices	Mixed salad	Pasta + cheese salad
Fresh fruit salad	Alfresco salad	Mixed salad white + red cabbage
Mango chunks	Bistro salad	Sweet pepper salad
Grape + kiwi + pineapple	Caesar salad	Oriental edamame soya bean
Apple + grape	Italian leaf salad	Greek salad
Pomegranates	Herb salad	Crunchy lettuce salad + cucumber
Fruit fingers	Ruby salad	Crisp mixed salad
Fruit selection	French style salad	King prawn + pasta salad
Rainbow fruit salad	Crisp mixed salad	Tuna + pasta
Mango + lime wedge	Fine cut salad	Egg salad
Mixed fruit salad	Crispy leaf salad	Poto + peas + bean salad
Fruit cocktail	Leaf salad	Pasta + pepper salad
Seasonal melon medley	Watercress salad	Salmon + potato
Mango pieces	Four leaf salad	Chicken + bacon Caesar salad
Grape + melon	Seasonal baby leaf salad	Avocado spinach + tomato
Apple segments	Mixed leaf salad	King prawn noodle salad
Melon selection	Crunchy mixed salad	Tomato + cheese pasta salad
Summer berry medley	Santa plum tomato salad	
Apple slices + grapes	Tender leaf salad	
Fruit medley	Watercress + spinach + rocket	
Red grape		

Whilst the manufacture of fresh-cut produce requires relatively little product transformation, it necessitates investment in technology, equipment, management systems and strict observance of food safety principles and practices to ensure product quality.

Nowadays, the consumption of fresh-cut fruits and vegetables is increased, and this because the consumer is looking healthy fruit and vegetable products. In the beginning of 1980s, fresh-cut productions were very popular just in fast food sector, but in the last years they became available at a retail level. The production and commercialization of fresh-cut vegetables are still higher than fresh-cut fruits. In particular, salads represent the dominant product among the minimally processed products (Rojas-Graù et al., 2011). The sector of fresh-cut fruits is rapidly increased in

the last years and will probably overshadow salad sales in the future, because fresh-cut fruits are more attractive to young consumers (Mayen & Marshall 2005).

Nowadays, fresh-cut produce is one of the fastest growing food categories in U.S. supermarkets, where packaged salads are the most important item sold (Figure 1.1). Fresh-cut fruit and vegetable sales have grown to approximately $15 billion per year in the North American food service and retail market and account for nearly 15% of all produce sales. According to the United Fresh Produce Association (2007), the largest portion of U.S. fresh-cut produce sales at retail is fresh-cut salad, with sales of $2.7 billion per year. However, the fast food sector is increasing the demand for packaged fresh-cut fruits by offering healthier choices on their menus. Scott (2008) reported that the U.S. sales of fresh-cut fruit items increased for every product, ranging from 7% to 54% growth.

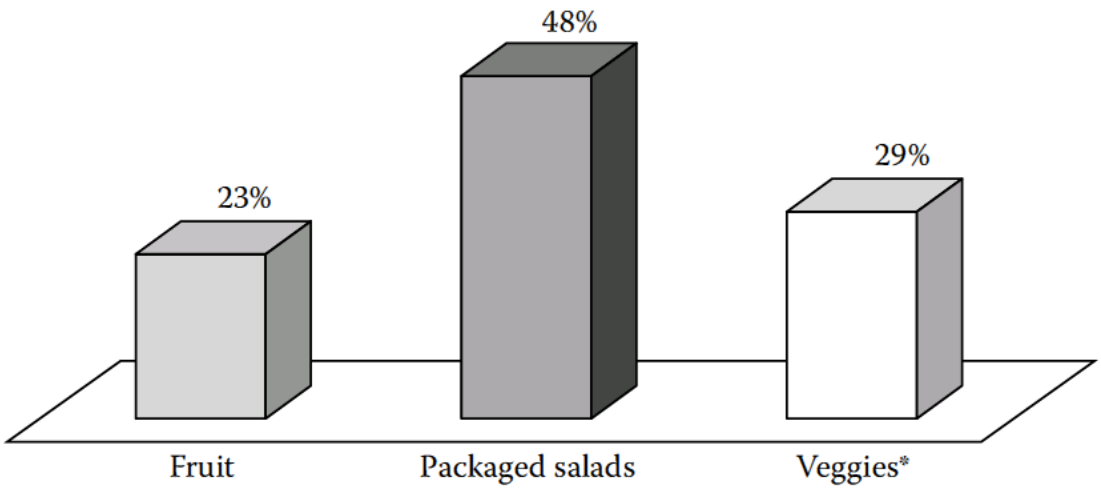


Figure 1.1-*Fresh-cut product sales via supermarket channels in U.S.; $6 Billion total. *Carrots = 45% of vegetables (Rojas-Graù et al., 2011adapted from Cook R., 2008).*

The fresh-cut vegetables and fruits European market trend is different among countries. The fresh-cut industry is rising in many European countries with the United Kingdom, France, and Italy as share leaders. In Italy, for example, the sales exceeded 42,000 tons of production, corresponding to €375 M ($450 M U.S.) in 2004 (Nicola et al., 2006). Over the last decade, ready-to-eat mixed salad packs have been one of the greatest successes of the UK food industry (Rojas-Graù et al., 2011). Currently, the countries with higher growth in the fresh-cut fruits and vegetables market are Germany, The Netherlands, Spain, and United Kingdom. The average European consumes up to 3 kilos of fresh-cut products a year, but the differences are quite

substantial within Europe. For instance, in the United Kingdom the rate is 12 kg per capita per year, France comes second consuming 6 kg per capita, and Italians consume around 4 kg. Other European countries, where the consumption is not far less than those already mentioned, are Belgium, The Netherlands and Germany. In the countries of eastern Europe, with increasingly healthier economies, they are beginning to see great growth in this sector (Rojas-Graù et al., 2011).

QUALITY OF FRESH-CUT FRUITS AND VEGETABLES

The quality of fresh-cut fruits and vegetables depends on several factors, which may be described by different attributes such as color, aroma, texture and nutritional value.

Appearance and color

The appearance of the product is an important factor affecting the consumer choice., It may comprise size, shape, color, gloss and absence of visual defects including morphological, physiological, physical or pathological ones. An important aspect, during the shelf-life of fresh-cut products, is the preservation of the tissue color avoiding the surface browning. For example, it is well documented that lettuce and carrots may be subject to changes in color due to biochemical processes, in particular, chlorophyll degradation and browning, in case of lettuce, or carotene degradation, whiteness and browning for carrots (Rico et al., 2007). This is probably the major defect of fresh cut fruits and vegetables, able to reduce their quality and shelf-life (Lopez-Galvez et al., 1996). The occurring of browning is different between fruits and vegetables, for example, in case of lettuce, browning appears very slowly because of the *de novo* biosynthesis of polyphenols (Murata et al., 2004). In case of apple, the high amount of polyphenols caused a rapid enzymatic browning. Generally, the vegetable browning is related to tissue wounding (cutting, breaking, etc.), which induces the biosynthesis of phenolic compounds and consequently of the browning (Rico et al., 2007). The main enzymes involved in the synthesis of brown pigments are peroxidase (POD) and polyphenol oxidase (PPO) (Nicoli et al., 1991). These enzymes, in the presence of oxygen, converts phenolic compounds into dark-colored pigments. Moreover, some vegetables and fruits are susceptible to dehydration and subsequent discoloration due to the damaged cells or to the removal of protective skin. In carrots for example, in stress conditions, lignin is synthesized with the role of defense from microorganisms and other stress

factors. The lignin formation and the reversible surface dehydration of the outer layers lead to a discoloration of the tissues, increasing the whiteness (Rico et al., 2007).

Texture

Textural quality factors include firmness, crispness, juiciness and toughness depending on the product. Products that maintain firmness, crunchiness and other texture parameters, are desirables for the consumers because are generally associated to freshness of the produce (Bourne, 2002). In minimally processed vegetables, changes in texture parameters are generally associated to enzymatic and non-enzymatic processes. Enzymes involved in the loss of firmness are pectine methylesterase (PME) and poligalacturonase (PG), that cause the degradation of pectins (Vu et al., 2004). The stimulation of PME activity with mild heating treatments has been correlated with texture maintenance, therefore only the combined action of PME and PG lead to a loss of firmness. Considering lettuce, it is very difficult maintaining the texture during storage. The cutting process makes start undesirable biochemical reactions that lead to a loss of crispness. In particular, the tissue softening and the associated leakage of juice are the major textural defects of fresh-cut fruits and vegetables.

Sensory quality

The flavor of the products comprises the taste and aroma of the products. Aroma compounds are detected by olfactory nerve endings in the nose. Taste is the detection of nonvolatile compounds by different receptors in the tongue. Flavor includes tastes like sweet, sour, astringent, bitter and off-flavors. The flavor quality of fresh-cut fruits and vegetables is affected by their content of sugar, organic acids, phenolic compounds and volatile active molecules. Hundreds of volatile compounds are responsible of the flavor of the products, and some of them are presents in very low concentrations (part per billion). For this reason, the analysis of the flavor actually requires the use of gas chromatography and gas chromatography/mass spectrometry techniques. A wide range of volatile molecules were detected and belonging to several chemical groups such as alcohols, aldehydes, ketones, esters, furanes, glucosinolate, lactones, nitrogen and sulfur-containing compounds, terpenes and other compounds (Rico et al., 2007). Esters are usually the major components affecting the aroma in fruits. Several studies demonstrated that the sensory quality may decline before to the textural and physiological quality. A hypothetical postharvest

quality evolution, comparing flavor, visual nutritional and texture quality, is reported in Figure 1.2. Basing on sensory evaluation, to satisfy the consumers it is necessary to get more information as possible about the optimum volatile compound concentration ranges.

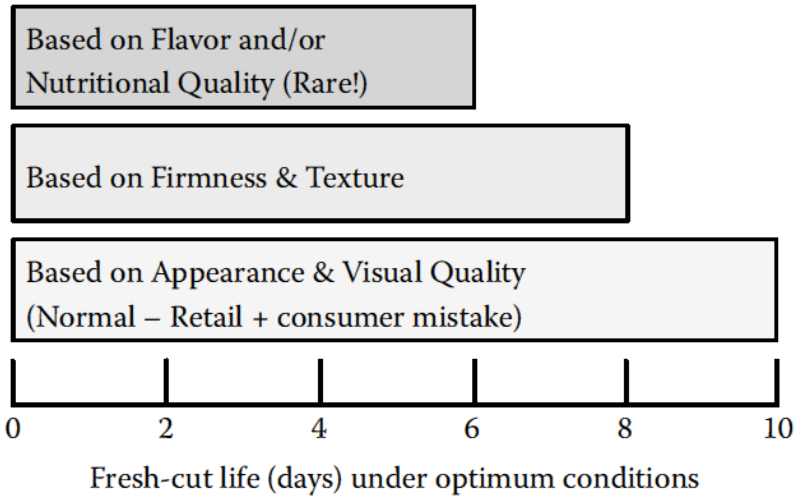


Figure 1.2-*Fresh-cut fruits life based on texture, nutritional and flavor quality attributes (adapted from Beaulieu J.C. 2011).*

Fresh-cut fruits and vegetables can be a source of vitamins, minerals and dietary fiber. Besides, they contain other minor constituents (flavonoids, carotenoids, polyphenols, and other phytonutrients), that may have a beneficial effect on human health and reduce the risk of cancer and heart diseases. Losses in nutritional quality are common during the storage, and they are enhanced by physical damage, high temperatures during storage, low relative humidity. Moreover, a decrease in the antioxidant activity during process has been reported for different kind of fruits and vegetables (Rico et al., 2007).

Intrinsic factors affecting quality of minimally processed vegetables and fruits

The main intrinsic factors affecting the quality of ready to eat fruits and vegetables are pH and respiration rate of minimally processed produce. Ready-to-eat fruits and vegetables are living tissue and if they are damaged in some way, the respiration rate increases (Laties, 1978). Tissues with a high respiration rate have a shorter postharvest life. The use of modified atmosphere during storage has the function to reduce the respiration rate of vegetable tissues. Also treatments

before and after wounding can affect the respiration rate and this can be evaluated by monitoring the concentration of oxygen and carbon dioxide in the headspace composition.

Another factor influencing the quality of minimally processed vegetables and fruits is the pH, which, for vegetables, should be between 5 to 6.5 to maintain the quality. However, some treatments, in particular the level of CO_2 in the modified atmosphere, can affect the pH of samples.

1.1.1 MICROBIOLOGICAL AND SAFETY ASPECTS OF FRESH-CUT FRUITS AND VEGETABLES

The spoilage of fresh-cut fruits and vegetables can be divided into physiological spoilage (due to enzymatic and metabolic activity of living plant tissue) and microbiological spoilage (due to microorganisms proliferation). During processing of fresh-cut fruits and vegetables, plant tissues suffer physical damage, with releasing of enzymes from substrates, and this makes them more perishables than the starting product (Artès et al., 2007). Moreover, the processing leads to an increase of respiration rate and ethylene production and in the end a faster metabolic rates (Ahvenainen, 1996; Surjadinata & Cisneros-Zevallos 2003; Artés et al. 2007; Soliva-Fortuny et al., 2003).

Besides, the release of nutrients on trimmed surfaces, enables the growth of microorganisms. The presence of microorganisms is correlated to the amount of sugar present on the surface of leaves. The presence of damaged areas on plant tissues represents a good substrate for microbial growth by providing nutrients (King et al., 1991; Zagory et al., 1999). Some of spoilage microorganisms produce pectinolytic enzymes degrading texture and providing more nutrients for microbial growth (Ragaert et al., 2011). The intrinsic properties of the product (e.g., pH of the tissue and nutrient availability) determine the growth rate and the type of microorganisms developing on the produce and, consequently, the type of spoilage pattern. Extrinsic properties (e.g., storage temperature and gas atmosphere) also influence the spoilage behavior of fresh-cut fruits and vegetables (Ragaert et al., 2011). The use of modified atmosphere can improve the shelf-life of fresh-cut products. The physiological state of the product, rather than the inhibition of spoilage bacteria, plays an important role in the beneficial effects of modified atmosphere storage of vegetables (Bennik et al., 1998).

MICROORGANISMS RELATED TO SPOILAGE OF FRESH CUT FRUITS AND VEGETABLES

Both fresh-cut fruits and vegetables are susceptible to many different contamination sources, such as seed, soil, irrigation water, animals, manure/sewage sludge use, harvesting, processing, and packaging. Total counts of microbiological populations on fresh-cut vegetables after processing range from 3 to 6 log CFU/g (Ragaert et al., 2011). In case of fresh-cut vegetables, the intrinsic properties favor growth of bacteria and yeasts, whilst molds are not so important. The predominant bacteria species are *Pseudomonadaceae*, *Enterobacteriaceae* and some species belonging to lactic acid bacteria (especially *Leuconostoc mesenteroides*) (Lund 1992; Nguygen & Carlin, 1994; Vankerschaver et al., 1996; Bennik et al., 1998). The yeasts species identified in fresh-cut vegetables are *Candida* sp., *Cryptococcus* sp., *Rhodotorula* sp., *Trichosporon* sp., *Pichia* sp., and *Torulaspora* sp. (Nguygen & Carlin 1994). In case of fresh-cut fruits, the growth of yeasts and molds is favor due to the lower pH values compared to vegetables (Beaulieu & Gorny, 2002). The most present yeasts are *Pichia* sp., *Rhodotorula* sp., *Candida pulcherrima*, *C. lambica*, *C. sake*, and *Debaryomyces polymorphus*. The principal molds reported on fruits are *Botrytis cinerea*, *Rhizopus stolonifer*, *Mucor piriformis*, *Rhizoctonia solani*, and *Phytophtora cactorum*.

Generally, the proliferation of microorganisms can have different impacts resulting in the production of enzymes and metabolites able to generate defects of texture and off-odors. The kind of spoilage depends from the composition of fruits and vegetables. For example, in sugar-rich vegetables and most of fresh-cut fruits the growth of yeasts and lactic acid bacteria is favored resulting in off-odors caused by microbial proliferation and the production of acids such as lactic acid, acetic acid, malic acid, succinic acid, and pyruvic one. In many vegetables and fresh cut fruits, the presence of off-odors normally happens when the bacterial count exceeds the threshold limit of 8 log cfu/g or 5 log cfu/g in the case of yeasts (Barry-Ryan & O'Beirne, 1998; Hao et al., 1999). Another problem related to the yeast growth is the production of ethanol and other volatile organic compounds such as 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl1-butanol, especially for fresh-cut fruits, which contain high concentrations of fermentable sugars (Ragaert et al., 2006; Ragaert et al., 2011). The fermentative processes, and so the ethanol production, can be also favored by too low O_2 concentrations or too high CO_2 concentrations regardless of the microbiological counts (López-Gálvez et al., 1997; Smyth et al., 1998). In some cases, for example in strawberries, the ethanol and other microbial metabolites such aldehydes

and ketones can be converted in acetate and butyrate esters by physiological processes (Hamilton-Kemp et al., 1996; Yu et al., 2000).

In produce with lower sugar content, as lettuce, the major spoilage microorganisms are *Pseudomonas* sp., which can produce metabolites resulting in off-odors. Moreover, many of these bacteria are able to produce pectinolytic enzymes resulting in changes of the texture of the product (Ragaert et al., 2011). The presence of soft and macerated tissues can happen when the *Pseudomonas* sp. count is above 7-8 log cfu/g, depending on the kind of vegetable. An overview of the mains spoilage mechanisms in different types of fresh cut fruits and vegetables are reported in Figure 1.3.

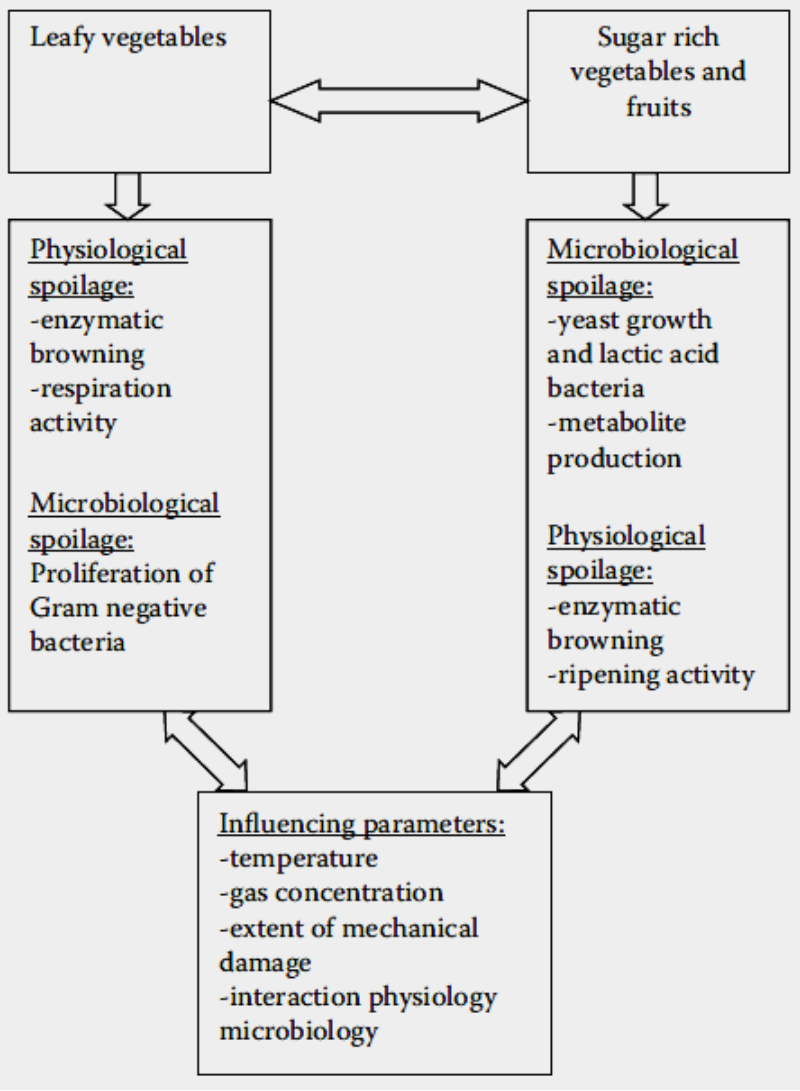


Figure 1.3- *Overview of dominating mechanisms of spoilage and influences on spoilage of leafy vegetables versus sugar-rich fruits and vegetables (adapted from Ragaert et al., 2011).*

PATHOGENIC MICROORGANISMS RELATED TO FRESH CUT FRUITS AND VEGETABLES

Fresh-cut fruits and vegetables are normally considered as safety products. However, in the last years some foodborne outbreaks were associated to this kind of products. In fact, fresh cut fruits and vegetables are considered as possible vehicles of foodborne pathogens. Pathogens may be present on the raw vegetables or due to cross-contamination during processing (Nguygen & Carlin, 1994; Beuchat, 1996; Seymour & Appleton, 2001). The incidence of foodborne outbreaks caused by contaminated fresh fruits and vegetables has increased in recent years (Mukherjee et al., 2006).

The pathogens most frequently linked to produce-related outbreaks include bacteria (*Salmonella*, *E. coli, L. monocytogenes*), viruses (Norwalk-like, hepatitis A), and parasites (*Cryptosporidium*, *Cyclospora)* (Tauxe et al., 1997), with *Salmonella* and *E. coli O157:H7* being the leading causes of produce-related outbreaks in the USA (Olsen et al., 2000). Fresh produce and sprouts have been implicated in a number of documented outbreaks of illness in countries such as Japan (Nat'l. Inst. Inf. Dis., 1997; Gutierrez, 1997), the USA (De Roever, 1998) and EU (Emberland et al., 2007; Pezzoli et al., 2007; Abadias et al, 2008b; Söderström et al., 2005).

Many pathogens were isolated from different kind of fresh-cut fruits and vegetables, although not all of them could be associated with foodborne outbreaks (Table 1.2).

Table 1.2- *Pathogenic organisms of concern or Potential concern in Fresh-cut Produce (adapted from Ragaert et al., chapter 3 Advances in fresh-cut fruits and vegetables processing, 2011).*

Pathogens of Concern	Pathogens of Possible Concern
Listeria monocytogenes	Nonproteolytic *Clostridium botulinum* types B, E, F
Escherichia coli (O157:H7)	*Aeromonas hydrophila/caviae*
Shigella spp.	*Bacillus cereus*
Salmonella spp.	*Yersinia enterocolitica*
Parasites	*Campylobacter* spp.
Viruses	

Among fresh-cut and vegetable categories, lettuce was most frequently associated with outbreaks of illness, followed by potatoes, tomatoes, melons, sprouts, berries, mushrooms, and peppers (Figure 1.4).

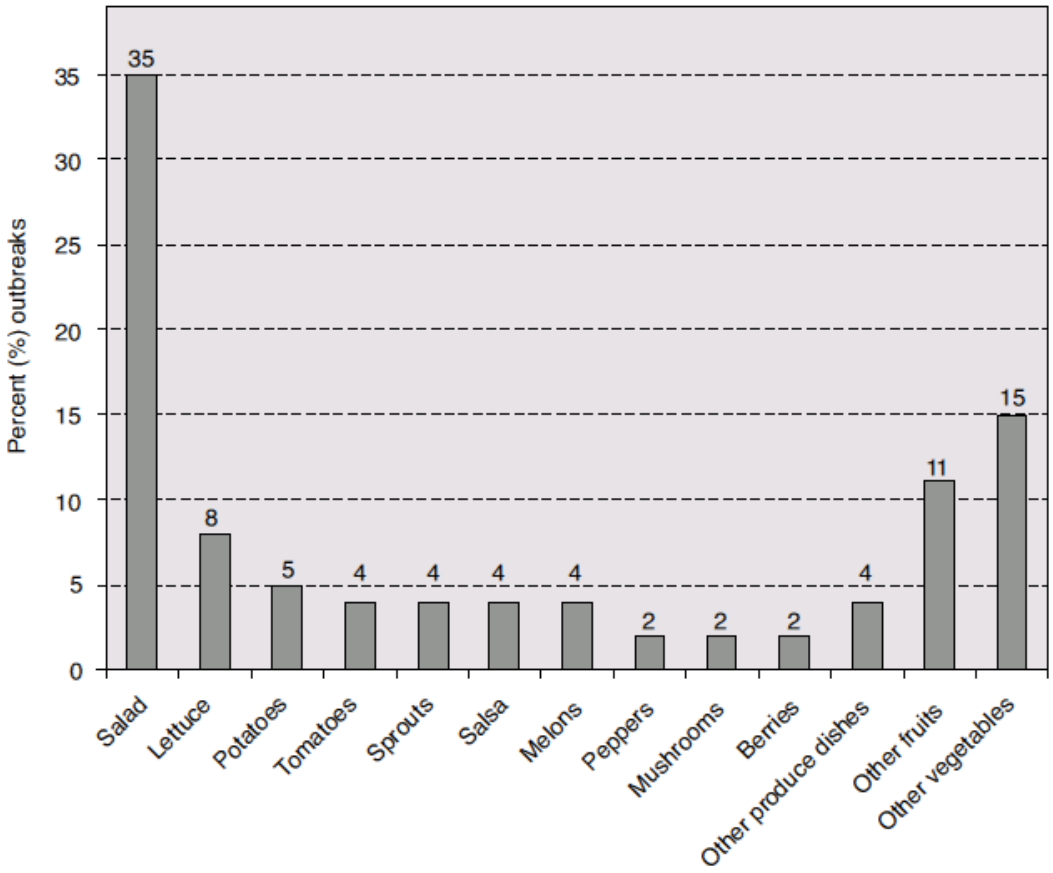


Figure 1.4- *Produce-linked outbreak vehicles between 1998 and 2006 (adapted from Buchholz et al., 2010).*

Salmonella and Shigella

Salmonella and *Shigella* spp. are Gram-negative, facultative anaerobic and nonspore forming bacteria. They are mesophilic fecal-associated pathogens. Their presence is mostly associated to fresh-cut vegetables and their growth on this kind of products is generally associated to temperature abuse (T>10 °C). On the contrary, in fresh-cut fruits, the low pH is a limit for the pathogen growth, with the only exception of melons because of their high pH.

These pathogens have a very low infectious dose of less than 100 cells. Shigellosis may also occur through consumption of contaminated water and foods, particularly salad vegetables. Imported food products from endemic regions where hygienic standards are insufficient have become a potential source of *Shigella* contaminated foods (Smith, 1987; ICMSF 1996). Laboratory studies revealed that *S. sonnei* can survive on shredded cabbage at 0 to 6°C for 3 days without decrease in number (Ragaert et al., 2011). Raafi et al.(1995) demonstrated that *Shigella*

spp. survived for several days both at 22°C and at refrigerator temperatures (5 and 10°C) when inoculated into commercial salads and vegetables (carrots, coleslaw, radishes, broccoli, cauliflower, lettuce, and celery). The experiment, conducted at 12°C on fresh-cut lettuce and shredded carrots, showed that *Shigella flexneri* and *S. sonnei* were able to proliferate (Ragaert et al., 2011).

Salmonella is frequently present on raw vegetables and fruits (Doyle, 1990, Beuchat, 1996, Abadias et al., 2008b). Normally its growth rate is reduced at less than 15°C and prevented at less than 7°C (ICMSF 1996). Zhuang et al. (1995) showed no changes in *S. montevideo* on tomatoes surfaces at 10°C after 22 days of storage, but at 20°C growth occurred after 7 days. *S. enteriditis*, *S. infantis*, and *S. typhimurium* were reported to be capable of growth in chopped cherry tomatoes. Abadias et al., (2008b), showed that the incidence of *Salmonella* in fresh cut vegetables was of 1.3%, but in other study conducted by Garcia-Villanova et al. (1987), was found that 7.5% and 3.3% of whole vegetables harbored *Salmonella*. Other studies of fresh, unprocessed produce conducted in Minnesota and Wisconsin (Mukherjee et al., 2006), the UK (Sagoo et al., 2003), in southern USA (Johnston et al., 2005), in USA (with imported fresh produce, FDA, 2001) and Malaysia showed widely varying incidences of *Salmonella*: 0, 0.2%, 3.3%, 3.5% and 35%, respectively. This microorganism was able to grow at low pH (3.99 to 4.37) under certain conditions (Asplund & Nurmi, 1991; Wei et al., 1995). However, refrigeration was the best preservation method to prevent an outgrowth of this mesophilic pathogen.

In the European regulation regarding criteria for foodstuffs (EU Regulation 2073/2005), is generally recommended for fresh-cut products the absence of *Salmonella* in 25 g of product.

Escherichia coli

E. coli is commonly found in the intestines of warm-blooded animals. Most types of *E. coli* are harmless, but some are pathogenic. The symptoms of *E. coli* O157:H7 infection include severe, sometimes bloody, diarrhea and abdominal cramps. *Escherichia coli* can be present in raw material and also in vegetables and fruits. The number of *E. coli* O157:H7 infections associated to fresh-cut vegetables and fruits have increased in the last years (Park et al., 1999). Survival and growth patterns of *E. coli* O157:H7 are dependent on vegetable type, package atmosphere, storage temperature, and bacterial strain (Francis & O'Beirne 2001). *E. coli* O157:H7 was able to grow on apples stored at 24°C for 6 days. (Dingmann, 2000). *E. coli* O157:H7 population in

shredded lettuce declined approximately 1 log throughout a 14-day storage at 4°C (Chang & Fang, 2007). At a higher temperature (22°C), populations of the same strain increased with about 3 log within 3 days. Similarly, populations of *E. coli* O157:H7 in lettuce stored at 5°C decreased with about 1 log in 18 days but it increased with about 3 log when lettuce was stored at 15°C (Li et al. 2001).

E. coli O157:H7 has the capability to grow at high temperatures and to survive at refrigerated temperatures. In addition, its low infectious dose (10 to 100 CFU/g) makes the presence of this pathogen in fresh-cut vegetables and fruits a risk for public health (Chang & Fang 2007).

Conflicting results were observed about the effect of modified atmosphere on the growth of *E. coli* O157:H7. Diaz and Hotchkiss (1996) showed that, although modified atmosphere packaging had beneficial effects on the shelf life of shredded lettuce, the extended shelf life allowed *E. coli* O157:H7 to grow to higher numbers within the shelf-life period compared to air-held shredded lettuce. Gunes and Hotchkiss (2002) observed that *E. coli* O157:H7 survived in fresh-cut apples but it was inhibited in modified atmospheres with high carbon dioxide concentrations at abusive temperatures.

E.coli is used as hygiene indicator for fresh cut fruits and vegetables because it is a true indicator of fecal origin and its presence is linked to the possible presence of other fecal pathogens (Ragaert et al., 2011).

Listeria monocytogenes

L. monocytogenes is widely distributed in natural environment including raw vegetables (Beuchat, 1996). *L. monocytogenes* is a bacterium that is able to grow in refrigerated produce such as lettuce (Carlin & Nguyen, 1994; Koseki & Isobe, 2005). The minimal temperatures for growth are between 0 and 4°C, it is not affected by the modified atmospheres applied for fresh-cut vegetables and fruits (Thomas et al., 1999; Castillejo-Rodriguez et al. 2000). Fresh-cut vegetables showed an incidence of *L. monocytogenes* varying from 0% to 19% in Europe (Carlin & Nguyen 1994). *L. monocytogenes* was also found in different kind of fresh-cut vegetables like green beens, tomato products and artichoke (Aguado et al., 2004). However, there were no *L. monocytogenes* outbreaks associated with fresh produce reported in the United States during 1999–2005 (DeWaal & Bhuiya, 2007), and in United Kingdom a screening on different vegetables revealed that, on the 151 samples analyzed, no *L. monocytogenes* was isolated.

The microbiological criteria about the presence of *L. monocytogenes* in fresh-cut fruits and vegetables are different and depending on the region. In Europe, the EU Regulation 2073/2005 considers fresh-cut fruits and vegetables as "ready-to eat foods able to support growth of *L. monocytogenes*," and it establishes as food safety criteria a maximum of 100 CFU/g at the end of the shelf life with the indication that during the entire shelf life, a maximum of 100 CFU/g must not be exceeded (Ragaert et al., 2011). However, depending on the shelf life of the fresh-cut vegetables (less than 5 days of shelf life) and the pH of the produce (pH below 4.4, as will be the case for the majority of fruits), they can also be considered as "ready-to-eat foods unable to support the growth of *L. monocytogenes*". The European classification of fresh-cut fruits and vegetables in possible risk of outgrowth of *Listeria monocytogenes* during shelf life is reported in Table 1.3.

Table 1.3- *Classification of Fresh-cut Fruits and Vegetables in Possible risk of outgrowth of Listeria monocytogenes during shelf life (adapted from Ragaert et al., chapter 3 Advances in fresh-cut fruits and vegetables processing, 2011).*

Group 1	Group 2	Group 3
• Fruit: melon parts • Sugar-rich vegetables with cut surfaces (e.g., cubes of zucchini, pumpkin, cucumber slices, eggplant cubes)	• Leafy fresh-cut vegetables (e.g., lettuce, celery, leek, chicory endive, spinach) • Red fruits with green crown part (e.g., strawberry, tomato)	• Fresh-cut produce with pH <4.4 (e.g., most fresh-cut fruit products) • Fresh-cut produce with shelf life <5 days • Mixed bell peppers due to low pH • Grated carrots due to antilisterial compound in carrots

It is the task of the food business operator to verify the capability of *L. monocytogenes* to grow in the concerned product, in the selected storage conditions within the prescribed shelf-life period. Generally, the absence of *Listeria* in 25 g of the product is recommended.

Outside Europe, there are often different criteria regarding *L. monocytogenes*. For example, the United States and Canada introduced a zero tolerance for some foods (absence of *L. monocytogenes* in 25 g), especially foods that are supportive of growth and have extended shelf lives. In these countries, decontamination techniques are often allowed in the production chain in order to reduce the bacterial load and avoid the presence of pathogens.

Spore forming pathogens

The growth of aerobic spoilage microflora rapidly decreases the redox potential of the food, improving conditions for the growth of *C. botulinum* (Francis et al., 1999). Normally *C. botulinum* is not able to grow below 10°C, but it is reported in literature that the types B, E and F are able to grow and produce toxin at temperature of 3°C (Francis et al., 1999). However, for becoming a real hazard for safety, the *C. botulinum* counts has to be more than 5-6 log CFU/g. Only in this condition the production of toxin occurs but in real condition, the natural microflora present on fresh-cut produce, inhibits the growth of *C. botulinum* at so high levels.

1.1.2 TREATMENTS AND FACTORS AFFECTING THE SHELF-LIFE AND SAFETY OF FRESH-CUT FRUITS AND VEGETABLES

Ensure the safety and the quality of fresh-cut products is an important target of the food industry. Every step from cultivation to the shelf is relevant for guarantee the safety and quality of fresh-cut produce. There are several guidelines for washing, sanitizing and packing the minimally processed fruits and vegetables. Some of these minimal processing procedures can cause to the products some negative effects, such as ethylene production, water loss, membrane deterioration, susceptibility to microbiological spoilage, loss of chlorophyll, decrease in acidity, tissue softening and lipid oxidation (Toivonen & De-Ell, 2002). Below, some of the traditional chemical and physical treatments are reported.

WHASHING OF FRESH-CUT PRODUCE

A correct washing process is probably one of the most important step affecting the subsequently shelf-life and safety of the minimally processed vegetables and fruits.

The main aim of the washing step is to remove the dirt, pesticides residue, and spoilage microorganisms. During the washing, the products are transported under water through pressurized water into a water tank. Water can be used alone or with a sanitizing agent. If it is used alone is enough for removing cell exudates released by cutting (Allende et al., 2008) but not sufficient to guarantee the safety of the produce, because pathogens, entrapped in plant residue or in biofilms along the process line, may survive and contaminate the clean products (López-Gálvez et al., 2009, Gil et al., 2011). Furthermore, at industrial level, the reuse of water is a

common practice and this may negatively affect the efficacy of the washing step (Lou, 2007; Allende et al., 2008). In conclusion, although washing can remove some of the surface microorganisms, it cannot remove all of them.

For these reasons, the disinfection of the water used for washing is necessary. Disinfection consists in the treatment of the process water, with the aim to inactivate pathogenic microorganisms, viruses and fungi and to prevent the microbial contamination of the produce (Gil et al., 2001). The use of commercial sanitizers can lead to a disinfection but not a sanitization of the produce. Normally, the used sanitizers do not have a microbiological benefit on produce, but they are used to extend the use of wash water or confer some improvement in quality during early to mid-storage (Gil et., 2011). Moreover, the reduction of microbial population can reduce the competition among microorganisms and could lead to a faster growth of potential pathogenic microorganisms (Gomez-López et al., 2007; Stringer et al., 2007; Allende et al., 2008b). It is well documented that the microbiology of fresh-cut vegetables and fruits affects also the quality of the produce, including sensory quality (Allende et al., 2008). Other authors have demonstrated that there are not relationship between the total microbial count and the shelf-life of the produce (Bennik et al., 1998; Gram et al., 2002). In this contest, the main aim of the washing is not to remove completely the microorganisms, but to ensure that the present microorganisms will not create a human health risk.

Concluding, sanitizing treatments are recommended to reduce the potential growth of microorganisms and possible contamination of fresh-cut vegetables and fruits. The Food and Drug Administration (FDA) elaborates guides with recommendations for a correct washing process. The last FDA guide suggests that washing raw agriculture commodities before processing the produce, may reduce the surface contamination. However, washing, even if a sanitizer is used, can only reduce but not eliminate the presence of microorganisms, including pathogens. Moreover, the importance of the water quality during the washing is well underlined. In fact, when antimicrobials are used with adequate quality water, the potential of produce contamination is reduced.

Different methods have been used for washing the produce and guarantee microbiological quality. Among them, several physical and chemical methods whose efficiency has been largely studied (Table 1.4).

Table 1.4- *Consideration concerning fresh-cut produce washing sanitizers (Gil et al. 2011, chapter 8 Advances in fresh-cut fruits and vegetables processing, 2011).*

Sanitizers	Considerations
Hypochlorite	The pH of the water should be kept between 6.0 and 7.5 to ensure the concentration of active chlorine (hypochlorous acid) is high enough. A rinse step of produce with potable water is necessary.
Chlorine dioxide	Produces fewer potentially carcinogenic chlorinated reaction products than chlorine, and it has greater activity at neutral pH.
Acidified sodium chlorite	More soluble than sodium hypochlorite (NaOCl) in water and has greater oxidizing capacity than hypochlorous acid.
Organic acid formulations	COD of water increases significantly after the addition of organic acid formulations such as Citrox and Purac. Tsunami is a good alternative as a sanitizing agent, but it is more expensive than chlorine.
Alkaline-based sanitizers	The high pH of alkaline washing solutions (11 to 12) and concerns about environmental discharge of phosphates may be limiting factors for use of certain alkaline compounds on produce.
Hydrogen peroxide	GRAS for some food applications but has not yet been approved as an antimicrobial wash-agent for produce.
Lactoperoxidase technology	The active molecule with disinfectant activity is hypothiocyanite, which does not remain in the finished product because it has very short life duration.
Ozone	A good option for washwater disinfection, reducing the need for water replacement, but it is not a substitute for the washing tank sanitizer.
UV-C illumination	Its efficacy as a washwater disinfectant is significantly impacted by turbidity.
Advanced oxidation processes	They are effective reducing microorganisms, chemical oxygen demand, and turbidity of water from the fresh-cut industry. Water could be reused for a longer time, but it is not a substitute for the washing tank sanitizer.
High pressure, pulsed electric field, oscillating magnetic fields	With the high capital expenditure together with the expensive process of optimization and water treatment, it is unlikely that the fresh produce industry would take up these technologies.

The efficacy depends on several factors such as the type of treatment and product, the target microorganisms, the time of the treatment, the concentration of the sanitizer, the operating pH and temperature (Sapers, 2001; Parish et al., 2003).

Chlorine based treatments

Chlorine based sanitizers are the most widely used for decontamination of fresh cut produce and in general in the food industry. Chlorine is normally used in a range of 50-200 ppm and with a contact times of 1-5 minutes (Rico et al., 2007). The antimicrobial activity of chlorine is

dependent on the pH of the washing solution. In fact, at acidic pH, the chlorine is more active because is in the form of active hypochlorous acid (HOCl). On the contrary, if the pH is above pH 7.5, only a little part of chlorine is in the active form while the other is inactive hypochlorite (OCl^-) without antimicrobial activity. For this reason the pH of the treatment solution should be ranged between 6 and 7.5. The use of washing solution with pH below 6 is not applicable because of the corrosion of processing devices and the ability to generate gas which is a health hazard for employees (Beuchat, 2000, Rico et al., 2007). Chlorine is widely used as sanitizer for washing vegetables and fruits, although several studies have demonstrated some concerns regarding its effectiveness when organic matter is present in the washing solution. In this condition, its efficacy is related to the time of contact with the product (Beuchat, 2000, Rico et al., 2007, Abadias et al., 2011, Gil et al., 2009).

Furthermore, chlorine may react with the organic matter and lead to the formation of undesirable product, such as chloroform and trihalomethanes, which are suspected of being potentially carcinogenic (Parish et al., 2003; Alegria et al., 2010; Abadias et al., 2011; Gomez-López et al., 2013). In some European countries including Germany, The Netherlands, Switzerland, and Belgium, the use of chlorine to wash minimally processed vegetables is prohibit (Artés & Allende, 2005), and in other countries there are some restrictions about its use.

Chlorine dioxide (ClO_2) has high oxidizing power, 2.5 times greater than free chlorine, and for this reason is more effective against microorganisms (Rico et al., 2007; Keskinen et al., 2009; Lee et al., 2004). Moreover, it does not react with organic matter and has a greater activity at neutral pH. Therefore, it does not form dangerous and carcinogenic compounds (Tsai et al., 1995). The use of chlorine dioxide in washing fruits and vegetables is accepted (FDA, 1998), and many studies have demonstrated its antimicrobial activity against spoilage and pathogenic microorganisms (*Listeria monocytogenes* and *E. coli*) in food models (Han et al., 2000; González et al., 2005; Allende et al., 2008). The main problems about the use of Chlorine dioxide are the stability, and the high costs related to the chlorine dioxide generation systems.

Organic acids

The use of acidifying agents may prevent the proliferation of spoilage microorganisms and the growth of pathogenic species. In fact, most of pathogens cannot grow at pH levels lower than 4.5 (Parish et al., 2003). Normally, fruits contain several organic acids such as acetic, benzoic, citric,

malic, sorbic, succinic and tartaric that may prevent the proliferation of bacteria. Otherwise, other fruits such as melon and pineapple, and the majority of vegetables, contain lower concentrations of organic acids and the pH is not below 5.0. This pH value cannot guarantee the microbial safety because of most of pathogens are able to grow at pH level above 5.0.

The antimicrobial activity of organic acids against psychrophilic and mesophilic microorganisms in fresh-cut fruits and vegetables is well documented (Cherrington et al., 1991; Uyttendaele et al., 2004; Bari et al., 2005). The activity of organic acids is due to the reduction of pH of environment, disruption of membrane transport and/or permeability, anion accumulation, or a reduction in internal cellular pH by the dissociation of hydrogen ions from the acid (Beuchat, 2000; Rico et al., 2007). Ascorbic acid and citric acid are probably the most used organic acids. They are generally recognized as safe (GRAS) and they are highly used in the dipping solution of ready to eat fruits to reduce microbial contamination and, because of their antioxidant properties, for the prevention of browning and other oxidative reactions. Ascorbic acid, in particular, also acts as an oxygen scavenger, removing molecular oxygen in polyphenol oxidase reactions. Polyphenol oxidase inhibition by ascorbic acid has been attributed to the reduction of enzymatically formed *o*-quinones to their precursor diphenols (Rico et al., 2007).

Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is considered as GRAS for some food application, but it has not been approved yet as an antimicrobial wash-agent for products. H_2O_2 is a powerful oxidant due to its capability to generate other cytotoxic oxidizing species such as hydroxyl radicals (Juven & Pierson, 1996). For this reason, H_2O_2 possess bactericidal, sporicidal and inhibitory activity. It is widely used as sterilizing agent for food contact surfaces decontamination. In normal condition, it produces no residue because it is broken down to water and oxygen by catalase (Sapers, 2003), but it depends on the presence or absence of peroxidase in the produce item (Parish et al., 2003). Sapers et al. (1999) have shown that solution of 5% hydrogen peroxide can achieve a higher log reduction for inoculated apples than 200 ppm of chlorine. Moreover, treatments by dipping in H_2O_2 reduce microbial population on fresh-cut produce as bell peppers, cucumber, zucchini, cantaloupe, and honeydew melon (Ukuku & Sapers, 2001; Ukuku et al., 2001; Beuchat & Ryu, 1997; Park & Beuchat, 1999). However, it is well documented that the use H_2O_2 in some kind of produce as shredded lettuce and mushrooms, can cause browning.

Lactoperoxidase technology, that is the use of H_2O_2 plus the sodium thiocyanate, is a relatively recent technology. In this case, the active molecule with disinfectant activity is OSCN–, this molecule does not remain in the finished product because it has very short life duration. For this reason this technology is suitable to be used in food processing, in particular for fresh cuts (Allende et al., 2008).

Ozone

Ozone (O_3) is a powerful antimicrobial and sporicidal agent (Singh et al., 2002; Guzel-Seydim et al., 2004). Ozone is one of the most known potent sanitizers, and it leads to a spontaneous decomposition to a non-toxic product (Grass et al., 2003). Ozone results from the rearrangement of atoms when oxygen molecules are subjected to high-voltage electric discharge. The product is a bluish gas with pungent odor and strong oxidizing properties (Horvath et al., 1985). The product is a bluish gas with pungent odor and strong oxidizing properties (Horvath et al., 1985). Solubility ratio for ozone increases as the temperature of water decreases (Bablon et al., 1991), but it is also affected by water pressure, pH an purity (Smilanick et al., 1999)

Molecular ozone reactions are selective and limited to unsaturated aromatic and aliphatic compounds. Ozone oxidizes these compounds through cycle-addition to double bonds (Bablon et al., 1991). Oxidation of sulfhydryl groups, which are abundant in microbial enzymes, may explain rapid inactivation of microorganisms and bacterial spores by ozone (Rico et al., 2007). Perez et al. (2002) showed that N-acetyl glucosamine, a compound present in the peptidoglycan of bacterial cell walls and in viral capsids, was resistant to the action of ozone in aqueous solution at pH 3 to 7. Glucosamine reacted relatively fast with ozone, but glucose was relatively resistant to degradation. This observation may explain the higher resistance of Gram-positive bacteria compared to Gram-negative ones; the former contains greater amounts of peptidoglycan in their cell walls with respect to Gram-negative bacteria. Moreover, ozone reacts with amino acids and peptides, saturated and unsaturated fatty acids and also with nucleobasis (Ishizaki et al., 1981).

Ozonized water is widely used as sanitizer in fresh-cut vegetables (Beltran et al., 2005; Selma et al., 2008). Several studies have shown the beneficial effects of O_3 in extending shelf-life of fresh-cut and not cut vegetables such as fresh-cut onion, escarole, carrot, spinach, broccoli, cucumber, apples, grapes, oranges, pears and strawberries (Beuchat et al.,1998; Kim et al., 1999; Skog &

Chu, 2001). Although the antimicrobial activity of ozone is well documented, a lack of knowledge is present about the effect of O_3 on pathogenic species (Rico et al., 2007).

Ozone has been declared as GRAS by FDA as antimicrobial agent for the treatment of raw and fresh-cut fruits and vegetables in gas and aqueous phases (Graham, 1997; Xu, 1999). The positive impact on water, decomposing many pesticides and reducing the oxygen demand is well documented (Rico et al., 2007). Moreover, the activity of ozone is not affected by the pH (Gil et al., 2011). When compared to chlorine, ozone has a greater effect against certain microorganisms and rapidly decomposes to oxygen, leaving no residues (White, 1992). The critical points about the use of ozone are related to the initial capital cost of the generator, the higher corrosiveness than chlorine and its high instability, making it difficult to predict how O_3 reacts in the presence of organic matter (Cho et al., 2003). Concluding, the use of ozone could be a good option for the wash-water disinfection for the fresh-cut industry, because it will reduce the need for water replacement and for high sanitizer concentration such as chlorine during vegetable washing (Gil et al., 2011) but its oxidant impact on the products needs to be considered.

Electrolyzed water

Electrolyzed water (EW), also known as electrolyzed oxidising water, is a novel antimicrobial agent which has been used in Japan for several years (Huang et al., 2008). It has been reported to possess antimicrobial activity against a wide range of microorganisms (Fabrizio & Cutter 2003; Kiura et al., 2002; Kimura et al., 2006). EW is generated by electrolysis of aqueous sodium chloride to produce an electrolyzed basic aqueous solution at the cathode and an electrolyzed acidic solution at the anode. By subjecting the electrodes to direct current voltages, negatively charged ions such as chloride and hydroxide in the diluted salt solution, move to the anode to give up electrons and become oxygen gas, chlorine gas, hypochlorite ion, hypochlorous acid and hydrochloric acid, while positively charged ions, such as hydrogen and sodium, move to the cathode to take up electrons and become hydrogen gas and sodium hydroxide (Hsu, 2005). The produced acidic EW (pH 2.1-4.5) has a strong bactericidal effect against pathogens and spoilage microorganisms, more effective than chlorine due to a high oxidation reduction potential (Bari et al., 2003; Rico et al., 2007). The main disadvantage of EW water is that the solution rapidly loses its antimicrobial activity if EW water is not continuously supplied with H^+, HOCl and Cl_2 by electrolysis (Kiura et al., 2002). Moreover, problems such as chlorine gas emission, metal

corrosion, and synthetic resin degradation, due to its strong acidity and free chlorine content, have been reported.

PHYSICAL TREATMENT

Several physical methods are used for extending the quality and guarantee the safety of minimally processed produce. Some of them are traditional methods as modified atmosphere packaging; but there are also alternatives to chemicals for disinfection of recycled or recirculating process water and fruit and vegetable sanitization, such as ultraviolet (UV) illumination, high pressure, pulsed electric field, pulsed light, oscillating magnetic fields, and ultrasound treatments, that they have been studied to reduce microbial contamination.

Modified atmosphere packaging

Modified atmosphere packaging (MAP) of fresh food is a growing sector, with an average annual growth rate of 13.6% over the last 5 years, and it will continue to grow due to research and development. MAP is a packaging system, consisting in different ratios of the components of the normal air (78% nitrogen, 21% oxygen, 0.03% carbon dioxide, in addition to trace amounts of noble gases) in order to improve the shelf-life and maintain the quality of the produce (Phillips, 1996). The packaging materials have to be selectively permeable to gases, and the modified atmosphere can be passive or active. In the first case, when the atmosphere is altered passively, the package is sealed under normal air conditions, and respiration of the product in the packaging causes an increasing of CO_2 or reduction of O_2 partial pressure until an atmospheric balance is reached. This condition is reached after a transient period, when gas partial pressure of packaging headspace reaches a steady state when diffusive exchanges through the film exactly compensate gas or vapor production or consumption. In case of active modified atmosphere, a gas mixture with defined concentrations of O_2 and CO_2 is injected into the package, so that the atmospheric equilibrium is reached quickly. For example, Charles et al. (2008), have shown that the equilibrium state in endives packaged in active MAP was reached faster (2 days) if compared to passive MAP (5 days). The changes of the package atmosphere composition is due to produce respiration and film gas permeability (Sivertsvik et al., 2002).

Low levels of O_2 and high levels of CO_2 are able to reduce the respiration rate; but too low O_2 concentrations can cause fermentative processes which cause the formation of off-flavors

compounds (Rico et al., 2007). Moreover, high level of CO_2 in sensitive product, can lead to the formation of brown spots on lettuce or yellowing of mushrooms, which are common visual degradations caused by high CO_2 content (Zagory & Kader 1988; Lopez Briones et al. 1992).

The recommended atmosphere composition depend on product species, variety and adopted processing. Normally fresh-cut produce are more tolerant to higher levels of CO_2 than intact products because the resistance to diffusion is smaller (Kader et al., 1989; Rico et al., 2007). The use of MAP to extend shelf-life for many foods has been well documented (Jayas & Jeyamkondan 2002; Brecht et al. 2003; Varoquaux & Ozdemir 2005; Rico et al. 2007; Guillaume et al., 2011). At this time, to support the development and achieve breakthroughs in the design of innovative MAP solutions in a rational way, there is still a need to formulate strategic options for reducing time-consuming step-by-step trials (Guillaume et al., 2011). There are several system that can be used in MAP of minimally processed fruits and vegetables and they are reported in Table 1.5

Table 1.5- *Some Available Systems Used or That Could Be Used in Commercial Applications for Modified Atmosphere Packaging of Fresh Fruits and Vegetables (adapted from Guillaume et al. chapter 10 Advances in fresh-cut fruits and vegetables processing, 2011).*

Systems	Formats	Active Agents	Trade Names	References
Gas-permeable and nonselective materials	Microperforated petrochemical material		All film manufacturers	Almenar et al. (2007), Schreiner et al. (2007)
Gas-permeable and permselective materials	Copolymer block or chemical modification of side chains in petrochemical material		Pebax® (Arkema, France), Saran Film® (Dow Chemical Co., USA), Intellipac™ (Landec Corp., USA)	Rogers (1975), Barron et al. (2002), Singh and Rao (2005)
Oxygen scavengers	Sachet, label	Iron: moisture-activated oxidation Glucose oxydase: self-activated enzyme-mediated oxidation	ATCO®LH (Laboratoire Standa, France), Ageless® (Mitsubishi Gas Chemical Company Inc., Japan) Bioka (Bioka LTD, Finland)	Tewari et al. (2002), Charles et al. (2003), Charles et al. (2008)
Carbon dioxide scavengers	Sachet, label	Calcium hydroxide: moisture-activated carbonation	ATCO®CO (Laboratoire Standa, France)	Charles et al. (2005), Charles et al. (2006)
Ethylene scavenger/ remover	Sachet, multilayered paper or cardboard Petrochemical films as low-density polyethylene	Silica gel to adsorb ethylene and potassium permanganate to oxidize it Mineral as zeolite	Ethylene Control Inc. (USA), Frisspack® (Dunapack Ltd., Hungary) Green bag (Evert-Fresh Corp., USA)	Brody et al. (2001), Correa et al. (2005)
Moisture controllers	Sachet	Silica gel	Condensationguard™ (Grace division, USA)	Paull (1999), Brody et al. (2001)
Antimicrobial	Multilayered paper or cardboard Sachet Label, coated petrochemical, or paper-based material	Sodium metabisulfite: moisture-activated sulfite emission Ethanol microcapsules: moisture-activated emission AIT microcapsules	Kontroll® (Kontek SRL, Italy) Ethicap® (Freund Industrial Co., Japan) Wasaouro® (Mitsubishi Kagaku Foods Co., Japan)	Valverde et al. (2005), Lurie et al. (2006), Martinez-Romero et al. (2007), Ayala-Zavala et al. (2008), Utto et al. (2008)

The use of an active system, with a package engineered to scavenge molecules as O_2, CO_2 and ethylene can find good applications in fresh-cut sector. The most important O_2 scavengers are based on the oxidation of ferrous ions, other active agents are unsaturated fatty acids, ascorbic acid, and enzymes (Brody et al., 2001). CO_2 scavengers already exist and they are composed of a physical absorbent such as zeolite or a chemical one such as calcium hydroxide, sodium carbonate, or magnesium hydroxide. The use of this CO_2 scavengers is not common in fresh-cut sector, because only on few products can give an advantage. Ethylene scavengers are already used to delay ripening of climacteric fruits and they are mainly based on ethylene oxidation by potassium permanganate.

Several studies have shown the good potential of the combination of MAP with antimicrobial packaging, that release volatile substances with antimicrobial activity to inhibit the growth of spoilage microorganisms on product surface.

The common packages used are made of plastic or polymer films. Plastic packaging may have different shapes (bags, pots, cups, and trays). Among the plastic materials, the most used are low density polyethylene, expanded polystyrene, polyvinyl chloride (PVC), polypropylene (PP) and polyethylene terephthalate (PET).

Different studies have shown the combined effect of MAP and low temperatures to reduce the respiration rate and ethylene synthesis in minimally processed lettuce, broccoli, melon, pineapple, apple, kiwi and papaya (O'Connor-Shaw et al., 1994; Barth et al., 1993; Nicoli et al., 1994).

The use of biodegradable films increased in the last years; the materials used in these edible coatings are lipids, polysaccharides and proteins. Those made of polysaccharides such as starch and alginate have shown the better results in extending the shelf-life of minimally processed fruits and vegetables (Rojas-Grau et al., 2008; Davis & Song, 2006).

Thermal treatments

Thermal treatment are extensively used in food industry. In case of ready to eat fruits and vegetables, there are some undesirable effects, such as loss of minerals and vitamins, formation of thermal reaction components and loss of fresh appearance, flavor and texture (Rico et al., 2007). To avoid or minimize these negative effects, it is important to reduce the extent of the heat treatment which has the greatest influence, with respect to the treatment temperature, on the undesirable quality changes.

Blanching is a widely used technique to decontaminate minimally processed vegetables; it consists in heating the produce in water or steam at 85-100°C for a short time. This treatment is very effective to reduce the initial microbial population of the product (Rico et al., 2007). However, it causes some negative changes in the product and particularly the loss of nutrients and the undesirable changes in color and texture indexes (Negi & Roy, 2000; Song et al., 2003).
An alternative to the blanching is the Heat-Shock method. In this case the heat treatment takes place during the washing step, at a temperature of 45-70°C for few minutes (less than 5) (Hisaminato et al., 2001). This method is able to repress the enzymatic browning of cut lettuce (Loaiza-Velarde et al., 1997). Moreover, in vegetable with a low level of phenolic compounds, where browning depends on the accumulation of phenolic compounds, heat-shock may redirect the protein synthesis away from the production of enzymes involved in phenolic compound accumulation. For this reason, in this kind of vegetables heat-shock can improve the organoleptic properties of the produce (Murata et al., 2004; Rico et al., 2007).

Irradiation and Ultraviolet light

The use of ultraviolet light (UV) light is well established for water treatment, air disinfection and surface decontamination. The UV region of the electromagnetic spectrum can be used for disinfection of liquid food products. The wavelength for UV processing ranges from 100 to 400nm (Guerrero-Beltràn & Barbosa-Cànovas, 2004). The wavelength between 250 and 270nm has the highest antimicrobial properties; normally a wavelength of 254nm is used for disinfection of water, food products, and surfaces (Bintsis et al., 2000).
The effects of UV light is dependent from the kind of microbial strain and type and composition of the food. The UV light causes a damage to the DNA (Liltved & Landfald, 2000), and moreover, it can increase the resistance of vegetable tissues to pathogen microorganisms (Nigro et al., 1998). This technique does not produce chemical residues, by-products or radiation (Guerrero-Beltràn & Barbosa-Cànovas, 2004). Also, it is a simple dry and cold process (Bachmann, 1975; Morgan, 1989) requiring very low maintenance and low cost, as it does not need energy as a treatment medium. Exposition to UV may induce the synthesis of healthy compounds such as anthocyanins and stilbenoids (Cantos et al., 2001). However, high UV can damage the vegetable tissue (Nigro et al. 1998). Some studies have demonstrated that the use of UV at 254 nm, on minimally processed lettuce, can reduce deterioration of the produce by

effectively reducing microbial populations. But negative effects were also found, and the application of UV increased the stress of the produce, respiration rate, and possibly induced a lignification-like process, which changed the appearance of the samples (Allende & Arles, 2003; Rico et al., 2007).

Another technique suitable to be used in food decontamination and that causes DNA damage in living cells in order to prevent the growth of microorganisms is the ionizing radiations. According to the Codex General Standard for Irradiated Foods, ionizing radiations foreseen for food processing are limited to high energy photons. Irradiation was approved by the FDA for use on fruits and vegetables at a maximum level of 1.0 kGy (IFT, 1983). Vegetative bacteria and molds are very sensible to irradiation; instead, more resistant are bacterial spores (Farkas, 2006). However, fungi with melanized hyphae have a radiation resistance comparable to that of bacterial spores (Saleh et al., 1988). Yeasts are as resistant as the more resistant bacteria. Viruses are highly radiation resistant (WHO, 1999). The suggested dose employed can be applied without unwanted changes, (e.g. off-flavours, in case of protein foods, and/or texture changes in fresh fruits and vegetables) (Farkas, 2006). Several studies have shown the potential application of irradiation in different kind of food produce. In particular, recently research was directed more on irradiation of minimally processed fresh produce. Good microbiological results without affecting color and texture parameters have been obtained in minimally processed carrots, lettuce and cantaloupes (Chervin & Boisseau, 1994; Foley et al., 2004; Goularte et al., 2004; Boynton et al., 2006).

High Pressure Processing

The use of high pressure processing has shown its great potential in food industry (Norton & Sun, 2008). The high pressure treatment microbial targets are the cell membranes, and in some cases, additional damaging events such as extensive solute loss during pressurization, protein denaturation and key enzyme inactivation are also required (Manas & Pagan, 2005). Moreover, high pressure treatments are very effective against a wide range of microorganisms. Normally, yeasts and molds, are more resistant to high pressure processing and they are inactivated by pressures between 200 and 300MPa (Smelt, 1998). Gram-positive bacteria are more resistant to pressure than Gram-negative bacteria.

High pressure processing can be applied to different range of foods, including juices and beverages, fruits and vegetables, meat-based products (cooked and dry ham, etc.), fish and pre-cooked dishes, with meat and vegetables being the most popular applications (Norton & Sun, 2008). The application of high pressures in a range 300-800 MPa can inactivate the microorganisms without affecting the color, texture and nutritional properties.

There are several limits about the use of high pressure processing in minimally process fruits and vegetables. In fact, pressure has shown to cause softening influence on texture of fruits and vegetables, and tissue firmness may be lost due to cell wall breakdown and loss of turgidity (De Belie, 2002). Trejo-Ayara et al. (2007) have found that textural changes in raw carrots are primarily caused by loss of turgidity induced by rapid compression and decompression. Moreover, the presence of air confined in the food matrix is subjected to compression and expansion during pressurization and decompression, disrupting food tissues, therefore making this unit operation unsuitable for fresh vegetables (Rico et al., 2007).

Hurdle Technologies

Hurdle technology is the combination of different preservation techniques as a conservation strategy (Rico et al., 2007). The control of temperature, water activity, acidity, redox potential and the use of preservatives, modified atmosphere and competitive microorganisms (e.g., lactic acid bacteria) represent the most important hurdles commonly used in food preservation (Leistner, 1999). By using hurdles, the intensity of the individual preservation techniques can be kept comparatively low, minimizing the loss of quality, while the overall impact on microbial growth may remain the same or better (Rico et al, 2007). The most important factor to consider is the selection of hurdles; this choice should be done carefully on the basis of the quality attributes of a product (Gorris & Tauscher, 1999). According to Leistner (1999), there are more than 60 potential hurdles for foods that improve the stability and/or quality of minimally processed products.

1.1.3 FUTURE TRENDS IN FRESH-CUT FRUITS AND VEGETABLES PROCESSING

In the last few years, a remarkable mutation occurred inside the social and family structure. The changes in lifestyles have led to a dramatic reduction in the times for meal preparation. An increasing number of people have at least one meal away from home. In this perspective,

industrial kitchens need to prepare and cook large numbers of meals in short periods of time (Oms-Oliu & Soliva-Fortuny, 2011). Simultaneously, consumers have become more appealed in food health and more interested in fresh and convenience products (Rocha & Morais, 2007). Moreover, nutrition experts agree in asserting that the consumption of sufficient amounts of fruits and vegetables is important in a healthy lifestyle while the presence of fiber, vitamins, minerals and phytonutrients can play an important role to prevent cardiovascular diseases, certain types of cancer, obesity, and diabetes.

A global trend is to encourage the consumption of fruits and vegetables, and in this regard different proposals have been carried out such as lower price of healthy foods to increase consumption. Although the knowledge about the health effects of fruits and vegetables has increased in the last time, the diets of a large part of consumers are still deficient in the recommended intake. In European countries, trends show that diets are moving away from the traditional "Mediterranean diet" (Rodrigues & de Almeida, 2001).

This situation is an opportunity for the introduction to the markets of new food products such as fresh-cut fruits and vegetables that may represent a strategy to increase the consumption of fruits and vegetables to the recommended levels for a healthy diet (Oms-Oliu & Soliva-Fortuny 2011).

In fact, minimally processed fruits and vegetables come across the consumer desire for convenience, quality, appearance, and healthy nutrition. A significant number of fresh-cut produces are already available in the markets of many developed countries. Ready to eat products are one of the major growing sectors in food retail establishments (Soliva-Fortuny & Martín-Belloso, 2003).

The market of fresh-cut produce is well consolidate in United States and some European country such as the Netherlands, United Kingdom, Germany and France whereas in countries such as Spain and Italy, its development is still limited. The reasons of the consolidation of the fresh-cut produce market in the cited countries appears to be due to the wide range of products and typology offered, the increase in exhibition space, and the increase in shelf-life up to 10−16 days for fresh salads. A different situation is present in countries such as Spain or Italy, where the shelf-life of fresh-cut salads is around a week. These shelf-life differences are related to the technology issues, but particularly to the logistic development that allows maintenance of the cold chain. The promotion of fresh-cut fruits and vegetables requires the appropriate combination of technologies for extending the shelf-life of the products, maintaining the sensory and organoleptic properties and guarantying the microbial safety of the product.

NEW APPROCHES TO CONTROL QUALITY AND SAFETY OF MINIMALLY PROCESSED FRUITS AND VEGETABLES

In order to obtain minimally processed vegetables endowed with high sensory quality, microbiological safety and high nutritional value, new approaches need to be developed. Actually, it is possible to get a shelf-life of at least 1 week for most refrigerated (5°C) products (Oms-Oliu & Soliva-Fortuny 2011). Nevertheless, some products would need a shelf-life of more than 2 weeks, so that success in their commercialization can be achieved. The maintenance of the correct product temperatures through the entire chill chain is the most important aspect to ensure quality and safety of fresh-cut fruits and vegetables. Even if these products are often packaged under modified atmospheres, the maintenance of a temperature close to 0ºC is necessary to keep the product safe for consumption.

The use of integrated approaches including the management of different aspects such as raw material, handling, processing, packaging, and distribution, can extend the shelf-life of the products. A good choice of different preservation techniques is expected to have significant prospects for the future of minimally processed fruits and vegetables (Oms-Oliu & Soliva-Fortuny 2011).

The disinfection step is important and necessary in order to minimize microbiological spoilage, and at the same time afford safe and high-quality fresh-cut fruits and vegetables. Even if chlorine is still the most commonly used sanitizer, future regulatory restrictions are expected and will require the development of substitutes. Therefore, innovative approaches have been studied for the sanitization of this kind of foods.

However, different studies have demonstrated that decontamination treatments such as hydrogen peroxide or acidic electrolyzed water can even enhance the microbial growth rate depending on the product and applied conditions (Gómez-López et al., 2008). Other alternatives are chlorine dioxide, low-dose gamma irradiation, ultraviolet light and pulsed light. All these technique have shown interesting prospects and advantages, but in all cases there are still some disadvantages and limits.

The use of a correct packaging has an important function in the preservation of fresh-cut products. Recently, new packaging has been developed due to the necessity to meet the demands of product safety, shelf-life extension, cost efficiency, environmental issues, and consumer convenience. Products are frequently packaged after flushing with different combinations of

gases (O_2, CO_2, and N_2). The use of low O_2 concentrations (1−5%) and high CO_2 concentrations (5−10%) in combination with storage at refrigeration temperatures (4ºC), is suggested as ideal storage situation for minimally processed vegetables to preserve sensory and microbial quality (Oms-Oliu & Soliva-Fortuny 2011).

The main issue is that the packaging films available do not have enough O_2 and CO_2 transmission rate to offset the high respiration rate of the products. This aspect, combined with the changes in temperature during storage (very common), lead to a rise in the respiration rate and consequently of the CO_2 level in the package headspace. Too low O_2 levels and excessive amounts of CO_2 in package headspace are often detrimental to fresh-cut fruits. To partially solve this kind of limitations, active packages are being developed. An active package is asked to respond to environmental changes such as temperature or atmosphere composition, or to physiological changes in the product (Oms-Oliu & Soliva-Fortuny 2011). Some films can change their permeability on the basis of the outer temperature. The incorporation of sachets in the packages can give advantages. Sachets may contain a different constituents that can absorb or release gases and provide another mechanism for regulating atmosphere composition and product quality (Ozdemir & Floros, 2004).

An alternative is the use of edible coatings that can be used as a complement or an alternative to MAP in order to improve the shelf-life of fresh-cut commodities. The use of edible coatings that can deliver active substances is an important advance. In particular, the incorporation of antimicrobial agents (chemical preservatives or antimicrobial compounds obtained from a natural source), antioxidants, and functional ingredients such as minerals and vitamins, can improve the functionality of edible coating. Micro- and nano-encapsulation can represent an useful method to incorporate functional ingredients and antimicrobials into edible coatings. This technology can be adapted for packaging solids, liquids, or gaseous substances in micro- and nanoscale forming capsules that can release their contents at controlled rates under specific conditions. The release can be solvent activated or signaled by changes in pH, temperature, irradiation, or osmotic shock (Vargas et al., 2008; Oms-Oliu & Soliva-Fortuny 2011).

Interesting results in prolonging the shelf-life and safety of minimally processed fruits and vegetables have been obtained through the use of biotechnological approaches. Fresh-cut productions are potentially contaminated by pathogenic microorganisms. Modern biotechnology provides molecular methods, fast and sensitive, for detecting human pathogens on fresh-cut produce. An example is the use of real-time polymerase chain reaction (RT-PCR) for detecting

pathogens in food matrices. RT-PCR systems have been verified for identification and quantification of *Listeria monocytogenes* (Liming et al., 2004) and *Salmonella* spp. (Cheung et al., 2004) on minimally processed products such as fresh-cut cantaloupe, mixed salads, and cilantro leaves. Other biotechnological techniques have been tested for detecting pathogens such as enzyme-linked fluorescence immunoassay and immune strip test (Huang et al., 2005), pulsed-field gel electrophoresis (Francis & O'Beirne, 2006), random amplified polymorphic DNA (RAPD), and restriction endonuclease analyses (REA) (Aguado et al., 2004). Finally, genetic engineering may generate fruits and vegetables best suitable for fresh-cut processing. The most desirable characters for such genotypes would include inhibited enzymatic browning, firm texture, slow tissue degradation, inhibited senescence, and protection against microbial proliferation. However, prospects of concrete employment of these genotypes depend on their acceptance by consumers (Oms-Oliu & Soliva-Fortuny 2011).

1.2 USE OF COMPETITIVE MICROFLORA IN FOOD BIOPRESERVATION

HYSTORICAL USE OF MICROORGANISMS IN FOOD PROCESSES

A wide range of microorganisms are commonly present on food; some of them have positive effects, for example those used in fermentation processes. On the other hand, some microorganisms can be dangerous for human healthy, such as pathogens.

Fermentation is a process dependent on the biological activity of microorganisms for production of a range of metabolites which can suppress the growth and survival of undesirable microflora in foodstuffs (Ross et al., 2002). Many microorganisms are used for the production of different kind of fermented products, and the properties of the final product are dependent on the used fermenting microorganisms. In many cases, the use of fermentation leads to a shelf-life extension and an improvement of the organoleptic properties of the final product. Species used for food fermentations belong to the genera *Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Lactobacillus,* and the newly recognized *Carnobacterium* (Table 1.6). These organisms have been isolated from grains, green plants, dairy and meat products, fermenting vegetables, and the mucosal surfaces of animals. Once used to retard spoilage and preserve foods through natural fermentations, they have found commercial applications as starter cultures in the dairy, baking, meat, vegetable, and alcoholic beverages industries.

Even if fermentation as preservative technique has well known for 8000 years; it has been only in the more recent past that microorganisms were recognized as responsible for the fermentation process. Moreover, the consumer demand for fermented produce is increased in the last years. For this reason also the availability of fermentative strains has increased. Nowadays, the production of fermented foods and beverages is dependent almost exclusively on the use of starter strains which have replaced the undefined strain mixtures traditionally used for the manufacture of these products (Ross et al., 2002). The use of starter strains allows to improve the performance and product quality of fermented processes, even if the intensive use of starters lead to some problems such as the bacteriophage proliferation that can affect cheese starter performance (Ross et al., 2002; Klaenhammer & Fitzgerald, 1994).

Table 1.6- *Biopreservation by lactic acid bacteria (Ross et al., 2002).*

Fermented products	Lactic acid bacteria[a]
Dairy product	
- Hard cheeses without eyes	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*
- Cheeses with small eyes	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis var. diacetylactis*, *L. lactis* subsp. *cremoris*, *Leuc. mesenteroides* subsp. *cremoris*
- Swiss-and Italian-type cheeses	*Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, *Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
- Butter and buttermilk	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis var. diacetylactis*, *L. lactis* subsp. *cremoris*, *Leuc. mesenteroides* subsp. *cremoris*
- Yoghurt	*Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
- Fermented, probiotic milk	*Lb. casei*, *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. johnsonii*, *B. lactis*, *B. bifidum*, *B. breve*
- Kefir	*Lb. kefir*, *Lb. kefiranofacies*, *Lb. brevis*
Fermented meats	
- Fermented sausage (Europe)	*Lb. sakei*, *Lb. curvatus*
- Fermented sausage (USA)	*P. acidilactici*, *P. pentosaceus*
Fermented vegetables	
- Sauerkraut	*Leuc. mesenteroides*, *Lb. plantarum*, *P. acidilactici*
- Pickles	*Leuc. mesenteroides*, *P. cerevisiae*, *Lb. brevis*, *Lb. plantarum*, *Leuc. mesenteroides*, *Lb. pentosus*, *Lb. plantarum*
- Fermented olives	*P. acidilactici*, *P. pentosaceus*, *Lb. plantarum*,
- Fermented vegetables	*Lb. fermentum*
Fermented cereals	
- Sourdough	*Lb. sanfransiscensis*, *Lb. farciminis*, *Lb. fermentum*, *Lb. brevis*, *Lb. plantarum*, *Lb. amylovorus*, *Lb. reuteri*, *Lb. pontis*, *Lb. panis*, *Lb. alimentarius*, *W. cibaria*
Fermented fish products	*Lb. alimentarius*, *C. piscicola*

[a] *B.=Bifidobacterium, C.=Carnobacterium, L.=Lactococcus, Lb.=Lactobacillus, Leuc.=Leuconostoc, O.=Oenococcus, P.=Pediococcus, S.=Streptococcus, T.=Tetragenococcus, W.=Weissella*

There is a relationship between fermentation and preservation, and the aim is the biopreservation of food by extending shelf-life and food safety. The use of starter microorganisms can inhibit the growth of spoilage and pathogenic microorganisms through antagonistic activity and the production of antimicrobial compounds and proteinaceous substances which can inhibit or reduce undesirable flora in food products (Ross et al., 2002). An example are Lactic Acid Bacteria (LAB) that are able to inhibit spoilage and pathogenic microorganisms; their activity is mainly due to the acids production and the production of a wide range of antimicrobial peptides and proteins that are called bacteriocins. Recently, the use of functional starter cultures in the food fermentation industry has been explored (De Vuyst, 2000; Leroy et al., 2006; Leroy & De Vuyst, 2004). Functional starter cultures are starters having functional properties. For example, LAB that

are able to produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, useful enzymes, or nutraceuticals, or probiotic strains (Leroy & De Vuyst, 2004).

LACTIC ACID BACTERIA (LAB)

Lactic acid bacteria (LAB) are the most used microbial group in fermentative processes. LAB improve the taste and texture of fermented products and inhibit food spoilage microorganisms by producing growth-inhibitory substances (bacteriocins) and large amounts of lactic acid, and other organic acids. Moreover, LAB can have a benefit effect on human health.

In the last two decades, major advances on fermented product technology have included improved culture selection procedures. Molecular technology has been applied to map the genetic constructs of starter culture organisms and, by using plasmid/gene transfer mechanisms, to improve starter culture performance (Doyle et al., 2013).

LAB are the most important microbial group associated to food, and especially to fermented products. LAB associated with food are generally restricted to the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, and *Weissella*. In particular, lactococcocci are involved in cheese manufacture, *Streptococcus salivarius* subsp. *thermophilus* for cheese and yoghurt manufacture and various members of the *Lactobacillus* genus for a variety of dairy, meat and vegetable fermentations (Table 1.6). LAB are Gram-positive, nonsporeforming cocci, coccobacilli, or rods. They are strictly fermentative, catalase-negative and lack a terminal electron transport chain.

On the basis of carbohydrate metabolism, LAB can be subdivided into homofermentative and heterofermentative. Homofermentative group includes the genera *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus* and some member of the *Lactobacillus* genus, which use the Embden–Meyerhof– Parnas pathway to convert glucose into lactate (Figure 1.5), where sugars can only be fermented by glycolysis. Heterofermentative group includes the genera *Leuconostoc*, *Oenococcus*, *Weissella* and some Lactobacilli. In this case, bacteria produce equimolar amounts of lactate, CO_2 and ethanol from glucose using the hexose monophosphate or pentose phosphate pathway alternatively referred to as the pentose phosphoketolase pathway (Figure 1.5), generating only half the energy of the homofermentative group.

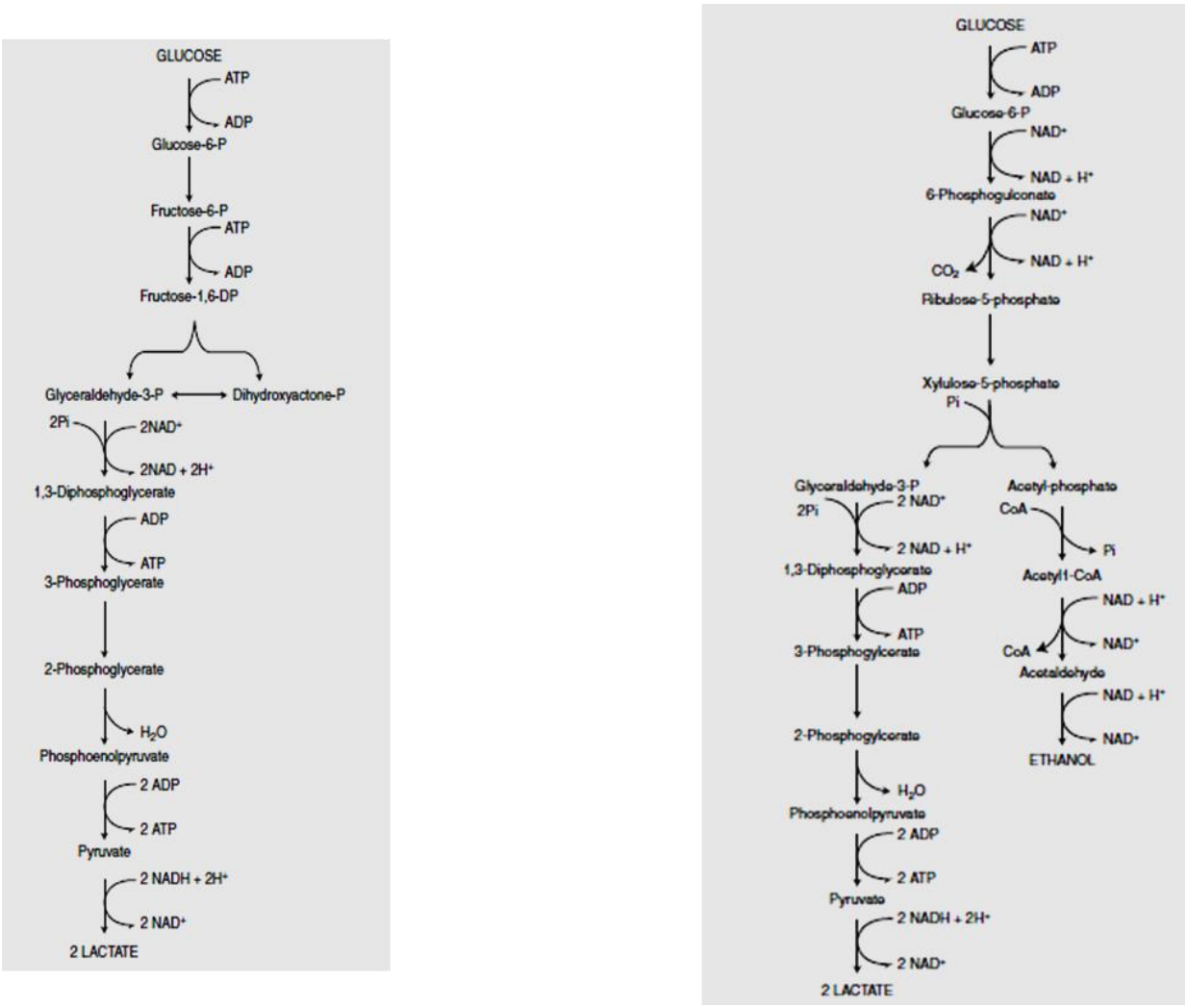


Figure 1.5- Homolactic fermentation pathway of glucose glycolysis (on the left). Heterolactic fermentation pathway of glucose (on the right).

LAB can be also classified on the basis of the rate of growth at different temperatures, pH of media and sodium chloride tolerance. A characteristic of lactic acid bacteria is their high acid-resistance, which allows them to grow until the pH reaches values lower than 5.0. This physiological characteristic is of great ecological importance, because it allows them to win the competition of other bacteria in environments rich in organic matter. LAB are able to develop in a very broad range of temperature (from 2 to 55 ° C), with an optimum between 20 ° and 45 ° C depending on the species (Stiles & Holzapfel, 1997). Some can develop only with high levels of water activity, while others have little demands on the aw (up to 0.85).

Antimicrobial activity of LAB

LAB have shown a wide range of antimicrobial activity. The antimicrobial properties of LAB in food are mainly due to the production of a wide range of antimicrobial metabolites (Ross et al., 2002). Various organic acids can be produced, including lactic, acetic and propionic acids which provide an acidic environment which inhibiting a broad range of microorganisms, including Gram-positive and Gram-negative bacteria, yeasts and molds.

Other antimicrobial compounds produced by LAB are ethanol, from the heterofermentative process; H_2O_2 produced during aerobic growth and diacetyl which is generated from excess pyruvate coming from citrate (Ray & Daeschel, 1992). There are also secondary metabolites produced by LAB with antimicrobial activity, for example the molecule reuterin (Chung et al., 1989) and the antibiotic reuterocyclin (Holtzel et al., 2000).

Many LAB are also able to produce bacteriocins and bacteriocin-like molecules. Bacteriocins are antimicrobial peptides produced by bacteria to compete against bacteria of the same species or other genera (Cotter et al., 2005). Despite bacteriocins can be produced by Gram-positive and Gram-negative bacteria, those produced by LAB have received particular attention in recent years due to their potential application in the food industry as natural preservatives (De Vuyst & Leroy 2007) and because LAB are designed as GRAS (generally recognized as safe) by the U.S. Food and Drug Administration (FDA). Bacteriocins are ribosomally synthesized peptides, proteinaceous inhibitor that act through depolarization of the target cell membrane or through inhibition of cell wall synthesis (Abee et al., 1995). They can have a wide or narrow spectrum of action. For example, lactococcins can inhibit only lactococci, instead the lantibiotic nisin has a broad range of antimicrobial activity (Ross et al., 2002).

Bacteriocins can be divided into three groups according to Klaenhammer (1993) (Table 1.7).

Table 1.7- *Classification of bacteriocins according to Klaenhammer (1993).*

Class I.	Lantibiotics
	I A: nisin-like, elongated, screw-shaped, cationic molecules
	I B: duramycin-like, globular molecules with low net negative charge
Class II.	Non-lantibiotics
	II A: pediocin-like antilisterial bacteriocins
	II B: two-peptide bacteriocins
Class III.	Large heat-labile proteins

The class I includes lantibiotics family and these bacteriocines are generally small, composed of one or two peptides of approximately 3 kDa. Type A includes the elongated flexible molecules that have a positive charge and act via membrane depolarization, such as nisin. Type B lantibiotics are globular in structure and interfere with cellular enzymatic reactions and examples include mersacidin and actagardine (Ross et al., 2002). The group II includes peptide bacteriocins or small, heat-stable, non-lanthionine-containing bacteriocins. This group can be divided into two classes, class IIa includes pediocin-like or *Listeria*- active bacteriocins, while class IIb comprise bacteriocins that are composed of two separate peptides.

The class III includes bacteriocins not very characterized and it consists of large heat-labile lytic proteins, often murein hydrolases proteins which are generally > 30 kDa.

The continual discovery of new extra members of these groups of peptides has meant that their classification has to be periodically updated. An update was proposed by Cotter et al. in 2005 (Figure 1.6).

Classification	Remarks	Examples
Class I (Lantibiotics)	Lanthionine-containing bacteriocins	
Class I lantibiotics	Unusual amino acids introduced by LanB and LanC	Nisin A, Subtilin, Epidermin, Pep5
Class II lantibiotics	Unusual amino acids introduced by LanM	Lacticin 481, Nukacin ISK-1, Mersacidin, Lacticin 3147 (two-peptide bacteriocin)
Class II	Non-lanthionine-containing bacteriocins	
Class IIa	Pediocin-like bacteriocins Specific against *Listeria monocytogenes*	Pediocin PA-1/AcH, Leucocin A
Class IIb	Two-peptide bacteriocins	Lactococcin G, Lactococcin Q
Class IIc	Cyclic bacteriocins	Enterocin AS-48, Gassericin A, lactocyclicin Q
Class IId	Single-peptide non-pediocin-like linear bacteriocins	Lactococcin A, Lacticin Q

Figure 1.6- *Cotter et al (2005) classification of LAB bacteriocins adapted by Nishie et al. (2012).*

In this case, class I includes lantibiotics which stands for lanthionine-containing antibiotics. Class II includes non lanthionine-containing bacteriocins. In this classification, large, heat-labile proteins and murein hydrolases were excluded from previously classified bacteriocins and named bacteriolysins. Subsequently, a new update was proposed by Heng and Tagg in 2006 (Figure 1.7).

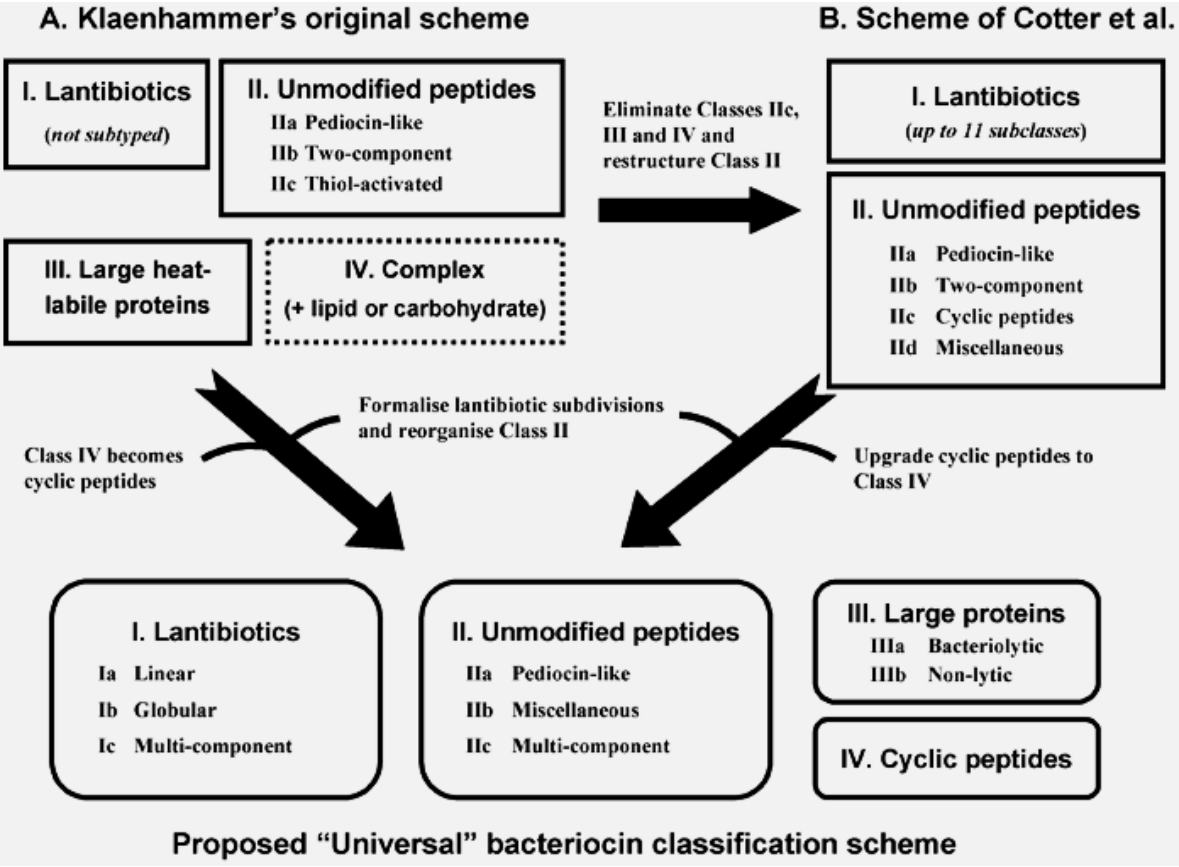


Figure 1.7- Bacteriocins classification scheme proposed by Heng and Tagg (2006).

Recently, bacteriocins produced by LAB have been characterized and they exhibit a wide range of antimicrobial activity, including activities against Gram-negative bacteria (Svetoch & Stern 2010; Lee et al. 2011). It seems that the mechanisms of action of bacteriocins are related to the permeabilization of the cell membrane. Moreover, they are cationic and amphiphilic or hydrophobic (Nissen-Meyer et al., 2009). However, it is demonstrated that each bacteriocin possesses more than one modes of action on the target microorganism (Hasper et al., 2006). The main target of lantibiotics are Lipid II, a membrane-bound cell wall precursor, as a berthing (Nishie et al., 2012). After that, lantibiotics, as nisin, kill the target microorganism by permeabilizing the plasma membrane, leading to the leakage of intracellular molecules.

Two-peptides lantibiotics, as lacticin 3147, interact with Lipid II and then cause an inhibition of the cell wall biosynthesis and form pores (Wiedemann et al., 2006). Bacteriocins of class IIa such as Pediocin PA1, are able to use components of the mannose phosphotransferase system (man-

PTS) of target cells as receptor, and then they act through the dissipation of the proton motive force via membrane pore formation (Nishie et al., 2012).

Two-peptides bacteriocins of class IIb, consist of two different unmodified peptides. Examples are lactococcin G and lactococcin Q, whose mode of action is the permeabilization of the cell membrane that induce the release of intracellular substances such as monovalent cations, phosphate and ATP (Oppegard et al., 2007). A similar behavior is exercised by class IIc of cyclic bacteriocins such as enterocin AS-48. This class is able to permeabilize the cell membrane of target cells, resulting in release of ions, dissipation of the membrane potential and finally cell death (van Belkum et al., 2011).

The non-categorized bacteriocins of class IId are diverse in their structure and so in the mode of action (Nishie et al., 2012). In fact, lactococcin A and lactococcin B are suggested to utilized the man-PTS as receptor, permeabilize the cytoplasmic membrane and cause leakage of solutes across the membrane. Instead, lacticin Q does not require a receptore for its membrane-permeabilizing activity, and acts via a toroidal-pore mechanism (Yoneyama et al., 2009).

Although the number of known bacteriocins is very large, nisin is absolutely the most characterized bacteriocin and the only one to have realized widespread commercial use (Ross et al., 2002).

In recent years, bacteriocins produced by LAB with an expanded range of antimicrobial activity, in particular against Gram-negative bacteria have been identified (Svetock & Stern 2010; Lee et al., 2011).

USE OF LAB AND BACTERIOCINS IN FOOD BIOPRESERVATION

The capability of LAB to produce bacteriocins and other antimicrobial molecules such as organic acids, diacetyl, acetoin, reuterin, reutericyclin, peroxidase, etc., and their general acceptability in foods, make them interesting to be used as alternatives to chemicals in food preservation (Doyle et al., 2013). LAB as "antimicrobials" can be used in a food system in different ways. The used of purified bacteriocins such as nisin are already used in food industry to prevent the growth of *Listeria* and spoilage microorganisms (Leroy & De Vuyst 2010). An alternative is the use of living cultures of LAB as protective cultures, to obtain the desired antimicrobial effect to food (Jones et al., 2011).

Several possible strategies for the application of bacteriocins in the preservation of foods may be considered: inoculation of the food with LAB (starter or protective cultures) able to produce the bacteriocin in the product (production in situ); addition of the purified or semipurified bacteriocin as a food preservative; use of a product previously fermented with a bacteriocin- producing strain as an ingredient in food processing (Schillinger et al., 1996). The success of the first application depends on the ability of the bacteriocin-producing LAB to grow and to produce the bacteriocin, while in the case of a semi-purified preparation the dosage can be most accurate and its effect most predictable. Finally, a crude bacteriocin preparation, obtained by growing a bacteriocin-producer LAB in a complex or natural substrate, is now employed for the industrial-scale production of nisin (Randazzo et al., 2009).

Antimicrobial properties of LAB can be delivered to food produce by living cells in the form of protective cultures. These capabilities are due to production of several metabolites such as bacteriocins, bacteriocin-like inhibitory substances (Jones et al. 2011), organic acids, and may also be a result of other microbial interactions (competitive exclusion, quorum sensing). Protective cultures can be developed to inhibit specific target microorganisms such as yeast and mold or pathogens such as *Listeria monocytogenes*. As these cultures are viable, it is important that they do not affect the products from a sensory point of view. For this reason, their use in food has generally been limited to cultured products, where they are typically added as adjunct cultures and have little effect on the finished product with regard to organoleptic properties. Protective cultures for specific food safety and quality applications are available commercially, and they are gaining in popularity as biopreservative agents in food (Doyle et al., 2013).

The desirable criteria of biopreservatives agents should be nontoxic, regulatory approved (GRAS, Generally Recognized as Safe), low cost, no negative organoleptic effects, effective in low concentrations, stable at storage conditions, and no medical application (Jones et al., 2011).

LAB and in particular bacteriocin-producing LAB have been tested on several food products. For example, several studies have shown the efficacy of nisin and/or nisin producing strains against pathogens such as *Clostridium butulinum* and against *L. monocytogenes* in cheeses such as Camembert, Ricotta, and Manchego (Annanou et al., 2007; Davies al., 1997; Nunez et al., 1997). Other bacteriocins have been tried in dairy products, such as pediocin AcH against *L. monocytogenes*, *S. aureus*, and *E. coli* O157:H7 (Buyong et al., 1998; Alpas & Bozoglu, 2000), lacticin 3147 against undesirable LAB, *L. monocytogenes* and *B. cereus* in Cheddar, Cottage cheese and yogurt (Ananou et al., 2007; Ross et al., 2002), and enterocin AS-48 against *B.*

cereus, *S. aureus* and *L. monocytogenes* in milk, Manchego cheese and meat (Ananou et al., 2007; Rodriguez et al., 1997; Munoz et al., 2004).

Several applications of LAB and bacteriocins in meat products are reported in literature. In particular, the tested bacteriocins in meat products include nisin, enterocin AS-48, enterocins A - B, sakacin, leucocin A, and particularly pediocin PA-l/AcH, also in combination with physicochemical treatments, modified atmosphere packaging, high hydrostatic pressure, (HHP), heat, and chemical preservatives, as an additional hurdle to control the proliferation of *L. monocytogenes* and other pathogens (Ananou et al., 2007). Moreover, some bacteriocin-producing LAB have been tested as bioprotective agents in meat products, in order to control these pathogens (Ananou et al., 2010, 2007, 2005a,b;). The data obtained on meat products have shown a lower efficacy of the tested strains and bacteriocin if compared to dairy products, probably due to their low solubility, irregular distribution, and lack of stability.

Some applications of bacteriocins and biocontrol agents have been reported also in vegetable products, in particular, bacteriocins such as nisin, pediocin PA-1/AcH and enterocin AS-48 have been tested in tinned vegetables, fruit juices, and salad, against pathogens such as *E. coli* O157:H7, *S. aureus*, and the spoilage bacterium *Alicyclobacillus acidoterrestris* (Grande et al., 2006; Ananou et al., 2005b; Cobo-Molinos et al., 2005; Cleveland et al., 2001; Alpas & Bozoglu, 2000).

The application of bacteriocin and bacteriocin-producing LAB have been reported to delay the fresh-fish deterioration. The use of nisin reduced the total aerobic bacteria populations of fresh chilled salmon, as well as the growth of inoculated *L. monocytogenes* in fresh salmon (Zuckerman & Ben Avraham, 2002). Also the use of bacteriocin producing cultures such as *Carnobacterium divergens* showed the inhibition of *L. monocytogenes* in fish (Duffesa et al., 1999).

However, the best effects of bacteriocins and bacteriocin-producing LAB on food products, have been obtained when the use of bacteriocins was combined with other preservation methods, in order to make a series of hurdles during the food processes to reduce food spoilage (Ananou et al., 2007). In fact, the use of chemical additives, physical treatments, or new physical methods such as HHP, pulsed electric field, vacuum, or modified atmosphere packaging, can increase the permeability of cell membranes, increasing the effects of many bacteriocins (Garriga et al., 2002; Ananou et al., 2010). In fact, the use of physical or chemical treatments increase the permeability of the outer-membrane increasing the effectiveness of some LAB bacteriocins against Gram-

negative cells, which are generally resistant. Examples of the application of bacteriocins in combination with other physical or chemical preservation methods are reported in Table 1.8.

Table 1.8- *Example of bacteriocins used in combination with other preservative methos in order to prolong the shelf-life and guarantee the safety of food products (adapted from Ananou et al., 2007)*

Bacteriocin	Other hurdles	Results
Nisin	HHP	Combination of HHP and nisin was effective to inactivate cheese indigenous microbiota. This combination was also effective against *S. carnosus* and *B. subtilis* spores, although a part of population survived the treatment
	pH and low temperature	A significant reduction in *L. innocua* was observed with a combination of low pH 5.5 and nisin at 20 °C. However, nisin-resistant cells regrew. Additional hurdles, such as refrigeration temperature, caused a dramatic reduction in population and allowed an increase of storage time to 10 days in liquid cheese whey.
	Pulsed electric fields (PEF)	The addition of nisin prior to PEF treatment increased the susceptibility of *L. innocua* to PEF treatment in whey.
	Sodium citrate and sodium lactate	The combination of low temperature, sodium lactate and/or sodium citrate with nisin controls *Arcobacter butzleri* on chicken.
Pediocin AcH	HHP and high temperature	The combination of HHP , higher temperature, and pediocin acts synergistically, causing reduction of viability of *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7, *Lb. sakei*, *Le. mesenteroides*
	Sodium diacetate	Combination of pediocin and sodium diacetate works synergistically against *L. monocytogenes* at room and low temperature
Enterocins A and B	HHP	Enterocins A and B were used in combination with HHP to the enhancement of safety in cooked ham against *L. monocytogenes*. Pathogen counts were below detection limits at the end of storage.
Enterocin AS-48	Heat treatment	The efficacy of AS-48 against *S. aureus* was greatly enhanced by combination with a moderate heat treatment in milk.
	STPP, lactic, acetic and citric acids	The combination of AS-48 and STPP or lactate acts synergistically against *S. aureus*. The activity of AS-48 increases in the presence of organic acids at pH 4.5. The combination with lactate reduces *S. aureus* population by 6 log units under neutral pH.
	Mild heat treatment, OM-permeabilizing agents or acidic/alkaline pH	The antimicrobial activity of AS-48 against *E. coli* O157:H7 enhanced by combination with mild heat treatment, OM-permeabilizing agents (EDTA and STPP), or under acidic or alkaline conditions in buffer and in apple juice.
	NaCl and low temperature	Highest effectiveness of AS-48 against *S. aureus* was obtained at 4 °C in combination with high concentrations of NaCl (6 and 7%).

1.3 ESSENTIAL OILS

Essential oils are complex mixture of volatile compounds, characterized by a strong sensorial impact and they are produced by many plants as secondary metabolites. Also called volatile oils, they may be obtained from all the organs of the plant, i.e. flowers, buds, seeds, leaves, roots, wood, stems, twigs, fruits or bark, and they are stored in secretory cells, cavities, canals, epidermis cells or glandular trichomes (Bakkali et al., 2008). Essential oils are extracted from various aromatic plants generally located in warm temperate countries such as the Mediterranean and tropical countries where they represent an important part of the traditional medicine. The main function in nature of essential oils may be different. In fact, they can act as internal messengers, as defensive substances against herbivores or as volatiles directing not only natural enemies to these herbivores but also attracting pollinating insects to their host (Harrewijn et al., 2001).

Essential oils are usually extracted from plants through several different methods, including steam, hydro-distillation or also, in the lasted years, supercritical carbon dioxide. The method of extraction depends on the use of the oil. For pharmaceutical and food purposes, the extraction by steam distillation is preferred, whereas for other uses extraction with lipophilic solvents or supercritical carbon dioxide is favored. The historical use of essential oils is in perfumes, cosmetics, soaps and other products; because of their bactericidal, fungicidal, virucidal, and medicinal properties and their fragrance, they are used also in embalmment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthetics remedies (Bakkali et al., 2009). Right now, an estimate number of about 3000 EOs are known, of which about 300 are commercially important, especially for pharmaceutical, agricultural, food, health, cosmetics and perfumes. Hundreds of new natural substances are being isolated and identified every year, but data concerning their biological activities are known only for some of them (Cowan, 1999).

Most of these substances have been recognized as safe (GRAS) (Newberne et al. 2000). Until now, these features have not changed much, while some of their mechanisms of action, in particular the antimicrobial action, is now more clear. In fact, originally essential oils have been used to enhance the aroma of foods, but they can also lead to a prolongation of the shelf-life due to their antimicrobial activity. Therefore, it is important to develop a better understanding of their mechanisms of action for the new applications in human health, agriculture, food and

environment. Due to the increasing interest on the part of the consumer towards natural products, some of essential oils constitute effective alternatives or complements to synthetic compounds of the chemical industry, without showing the same secondary effects (Carson & Riley, 2003).

EXTRACTION AND COMPOSITION

Different methods are known for the extraction of essential oils and the choice of the method depends on the nature of the material, the stability of the chemical components and the specification of the targeted product. The main extraction techniques used are distillation processes, in particular steam and hydro distillation. Other methods include the supercritical carbon dioxide, solvent, cold pressed and microwave extraction. The hydro distillation and steam distillation are still the most economical methods of extracting essential oil from spices and aromatic plant material. The main advantage of distillation is that it can generally be carried out with some very simple equipment, close to the location of plant production. Even in relatively remote locations, large quantities of material can be processed in a relatively short time. Moreover, distillation is less labor intensive and has a lower labor skill requirement than solvent extraction (Douglas et al., 2005). However, also the solvent extraction process cost is not expensive. The mainly disadvantages of distillation are related to the induction of thermal degradation, hydrolysis and water solubilization of some fragrance constituents. Extracts obtained by solvents contain residues that pollute the foods and fragrances to which they are added (Guan et al., 2007).

Supercritical carbon dioxide extraction is an environmental friendly technique, very suitable to obtain different plants extracts, due to the simple manipulations of some process parameters such as temperature and pressure able to easily change the solvent power of the supercritical fluid. However, the most serious drawback of supercritical carbon dioxide extraction, when compared with traditional atmospheric pressure extraction techniques, is the higher initial investment cost of the equipment (Coelho et al., 2012).

Cold press extraction is used exclusively for the extraction of citrus oil from the fruit peel, because the chemical components of the oil are easily damaged by heat.

The microwaves extraction fits with the new “green” technique in essential oil extraction, which typically use less solvent and energy. The main benefits of this technique are the reduction of extraction time and the absence of toxic solvent residue in the extract (Lucchesi et al., 2004). On

the other hand, the main disadvantage of microwave extraction are its high capital cost and possible need to filter the sample if fine particles are used for the extraction of compounds (Wang, 2010).

The different extraction method goes to influence the composition of the oils that will be different depending on the method used, affecting also their flavor profile and their antimicrobial properties. Moreover, independently on the extraction methods, essential oils may be subjected to numerous degradative processes (primarily oxidative) and for this reason, following the extraction, they need to be stored in the dark in order to prevent compositional changes.

There are several studies on the composition of essential oils obtained from herbs and fruits. For each essential oil, "characteristic" molecules can be identified, but their qualitative and quantitative composition is extremely changeable. This variability depends on several factors such as climate, soil composition, plant organ, age and vegetative cycle stage (Masotti et al., 2003; Angioni et al., 2006). Thus, in order to obtain essential oils at constant composition, these must be extracted in the same conditions, from the same organ of the plant that has been grown on the same soil, under the same climatic conditions and was collected in the same season.

Most of the essential oils have been characterized by gas chromatographic and mass spectrometry analysis. Precisely, through these techniques, it is possible to obtain a detailed analysis of the composition of the essential oils or their head space (Salzer, 1977; Daferera et al., 2000; Juliano et al., 2000; Jerkovic et al., 2001; Delaquis et al., 2002). Normally, essential oils contain about 20–60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts (Bakkali et al. 2008). The major components and the relative percentage of some essential oils with high antimicrobial properties are reported in Table 1.9.

Table 1.9-*Major components of some essential oils well known for their antimicrobial properties (Burt, 2004)*

Common name of EO	Latin name of plant source	Major components	Approximate % composition[b]	References
Cilantro	*Coriandrum sativum* (immature leaves)	Linalool E-2-decanal	26% 20%	(Delaquis et al., 2002)
Coriander	*Coriandrum sativum* (seeds)	Linalool E-2-decanal	70% –	(Delaquis et al., 2002)
Cinnamon	*Cinnamomum zeylandicum*	Trans-cinnamaldehyde	65%	(Lens-Lisbonne et al., 1987)
Oregano	*Origanum vulgare*	Carvacrol Thymol γ-Terpinene p-Cymene	Trace-80% Trace-64% 2–52% Trace-52%	(Lawrence, 1984; Prudent et al., 1995; Charai et al., 1996; Sivropoulou et al., 1996; Kokkini et al., 1997; Russo et al., 1998; Daferera et al., 2000; Demetzos and Perdetzoglou, 2001; Marino et al., 2001)
Rosemary	*Rosmarinus officinalis*	α-pinene Bornyl acetate Camphor 1,8-cineole	2–25% 0–17% 2–14% 3–89%	(Daferera et al., 2000, 2003; Pintore et al., 2002)
Sage	*Salvia officinalis* L.	Camphor α-Pinene β-pinene 1,8-cineole α-tujone	6–15% 4–5% 2–10% 6–14% 20–42%	(Marino et al., 2001)
Clove (bud)	*Syzygium aromaticum*	Eugenol Eugenyl acetate	75–85% 8–15%	(Bauer et al., 2001)
Thyme	*Thymus vulgaris*	Thymol Carvacrol γ-Terpinene p-Cymene	10–64% 2–11% 2–31% 10–56%	(Lens-Lisbonne et al., 1987; McGimpsey et al., 1994; Cosentino et al., 1999; Marino et al., 1999; Daferera et al., 2000; Juliano et al., 2000)

Among the various components, it seems that the phenolic compounds are those most responsible for the antibacterial properties of the essential oils (Cosentino et al., 1999). However, also the minor components can play a critical role in antibacterial activity, probably due to a synergistic effect with other components. For example this synergistic effect has been demonstrated for some species of *Thymus*, Oregano, Sage, *Eucalyptus* and *Malaleuca alternifolia* essential oils (Paster et. al., 1995; Marino et al., 1999; Marino et al., 2001; Lattaoui & Tantaoui-Elaraki, 1994; Mulyaningsih et al., 2010; Carson et al., 2006). The components can be divided into two different classes on the basis of biosynthetic origin (Croteau et al., 2000; Betts, 2001; Pichersky et al., 2006). The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight (Bakkali et al., 2008) (Figure 1.8).

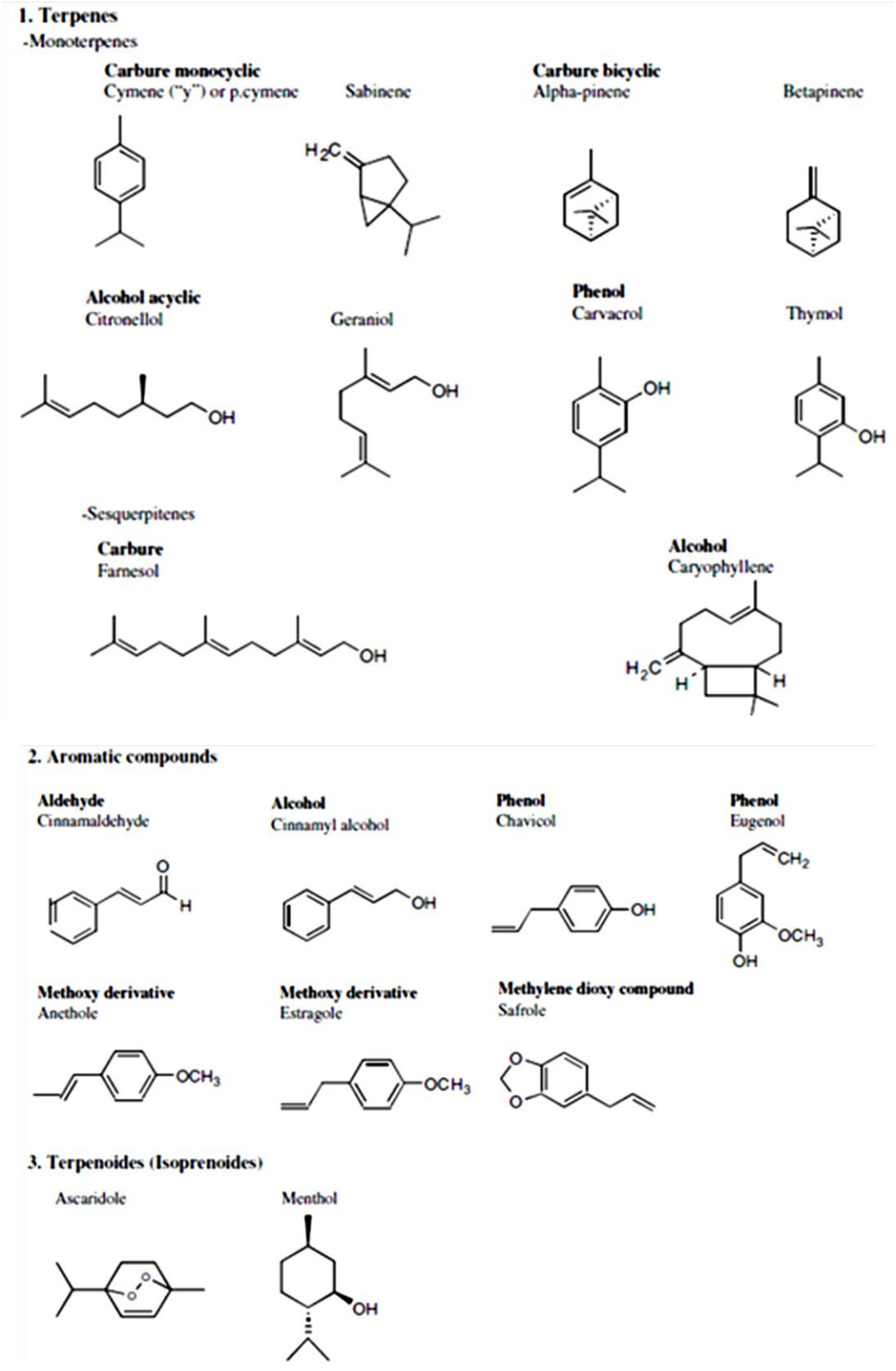


Figure 1.8-*Chemical structure of selected aromatic components of essential oils (Bakkali et al., 2008)*

The terpenes have different classes from a structural and functional point of view. They are substances composed of isoprene (2-methylbutadiene) units. The biosynthesis of terpenes consists on coupling of isoprene units is almost always in one direction, the so-called head to-tail coupling. This is shown in Figure 1.9.

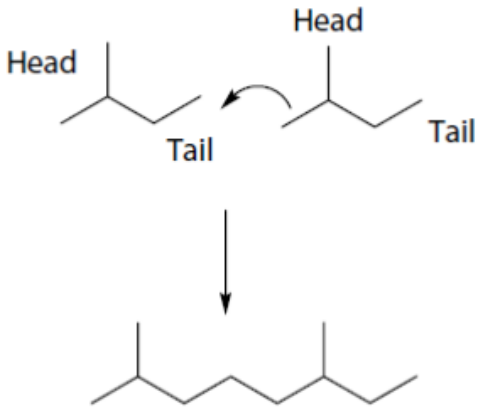


Figure 1.9-*Head-to-tale coupling of two isoprene units (Sell, 2010)*

The branched end of the chain is referred to as the head of the molecule and the other as the tail, and finally, secondary enzymatic modification (redox reaction) of the skeleton to attribute functional properties to the different terpenes. Terpenoids are terpenes that undergo biochemical modifications via enzymes that add oxygen molecules and move or remove methyl groups (Caballero et al., 2003). They can be hydrocarbons, alcohols, aldehydes, ketones, acids, acetals, esters, lactones, epoxides, ethers or phenols; they can also contain sulfur and nitrogen groups, can be saturated or unsaturated, with a linear, branched, cyclic or heterocyclic structure, and with a number of carbon atoms greater or lesser degree. The first terpenoids to be studied contained 10 carbon atoms per molecule and were called monoterpenoids. This nomenclature has remained and so those with five carbon atoms are known as hemiterpenoids, those with 15, sesquiterpenoids, and those with 20, diterpenoids, and so on. In general, only the hemiterpenoids, monoterpenoids, and sesquiterpenoids are sufficiently volatile to be components of essential oils. Monoterpenoids are the most representative molecules constituting 90% of the essential oils and allow a great variety of structures, for example carbures, alcohols, aldehydes, ketone, esters, ethers, peroxides, phenols (Bakkali et al., 2008).

When the molecule is optically active, the two enantiomers are very often present in different plants: (+)-α-pinene from Pinus palustris, (-)-β-pinene from Pinus caribaea and from Pinus pinaster, (-)-linalol from coriander, (+)-linalol from some camphor trees, etc. In some cases, it is the racemic form which is the most frequently encountered, for example (±)-citronellol is widespread, the form (+) is characteristic of *Eucalyptus citriodora*, the form (-) is common to the rose and geranium essential oils (Bakkali et al 2008) (Figure 1.10).

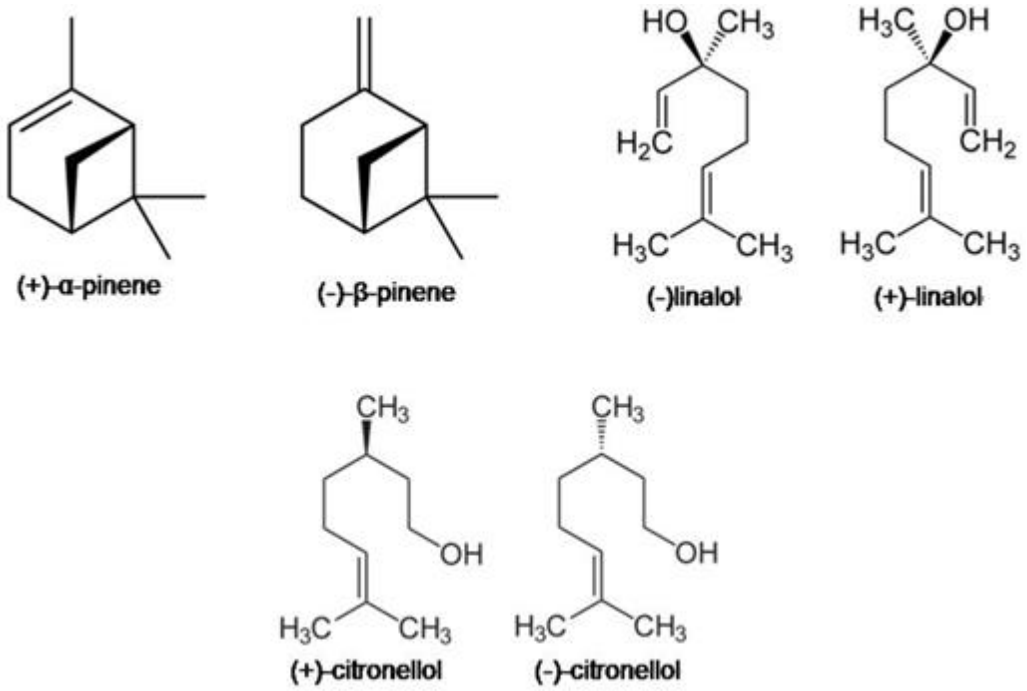


Figure 1.10-*Structural formula of (+)-α-pinene, (-)-β-pinene, (-)linalol, (+)-linalol, (+)-citronellol and (-)-citronellol; generally for pinene and linalol the two enantiomers are synthesized in different plants.*

By definition, sesquiterpenoids contain 15 carbon atoms. This results in their having lower volatilities and hence higher boiling points than monoterpenoids. Therefore, fewer of them (in percentage terms) contribute to the odor of essential oils but those that do often have low odor thresholds and contribute significantly as end notes. They are also important as fixatives for more volatile components (Sell, 2010). The structure and functions of the sesquiterpenes are similar to those of monoterpenes, and some examples are reported in Figure 1.11.

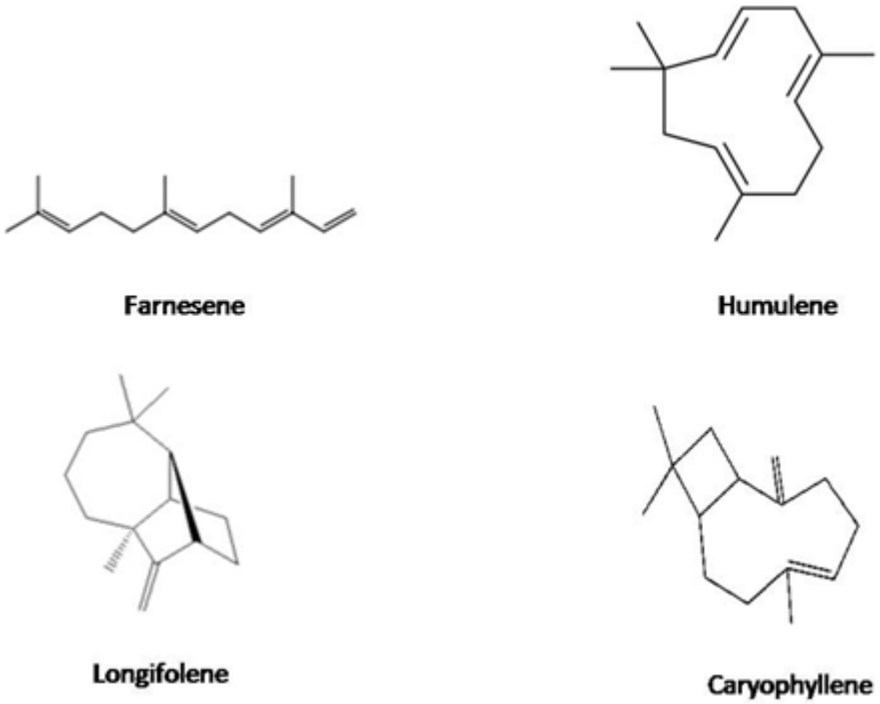


Figure 1.11- *Examples of acyclic (farnesene), monocyclic (humulene), bicyclic (caryophyllene) and tricyclic (longifolene) sesquiterpenes.*

Examples of plants containing terpenes in their essential oils are: angelica (*Angelica archangelica*), bergamot (*Citrus bergamia*), caraway (*Cuminum cyminum*), celery (*Apium graveolens*), citronella (*Cymbopogon nardus*), coriander (*Coriandrum sativum*), eucalyptus (*Eucalyptus globulus*), geranium (*Pelargonium graveolens*), juniper (*Juniperus communis*), lavender (*Lavandula officinalis*), lemon (*Citrus limonum*), lemongrass (*Cymbopogon citratus*), mandarin (*Citrus nobilis*), mint (*Mentha piperita*), orange (*Citrus sinesis*), peppermint (*Mentha piperita*), pine (*Pinus sylvestris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*). In Figure 1.12 some of these plants are reported

Figure 1.12- *Main plants which produce essential oils containing mainly terpenes and terpenoids.*

The aromatic compounds, compared to terpenes and terpenoids, are derivatives of phenylpropane, which are less frequently than the terpenes in essential oils. The biosynthetic pathways concerning terpenes and phenylpropanic derivatives are generally separated in plants but they may coexist in some, with one major pathway taking over (see cinnamon oil with

cinnamaldehyde as major and eugenol as minor constituent) (Bakkali et al., 2008). The structure of some of these aromatic compounds are reported in Figure 1.13.

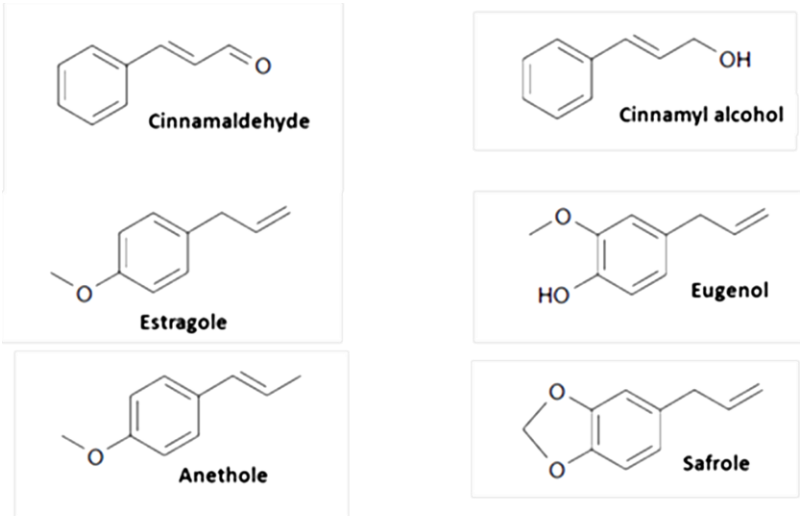


Figure 1.13-*Structures of some aromatic components of essential oils.*

Examples of plants containing these molecules are anise (*Pimpinella anisum*), cinnamon (*Cinnamomum zeylanicum*), clove (*Eugenia caryophyllata*), fennel (*Foeniculum vulgare*), nutmeg (*Myristica fragrans*), parsley (*Petroselinum sativum*), sassafras (*Sassafras albidum*), star anise (*Illicium verum*), tarragon (*Artemisia dracunculus*), and some botanical families (Bakkali et al., 2008). In Figure 1.14 some of these plants are reported.

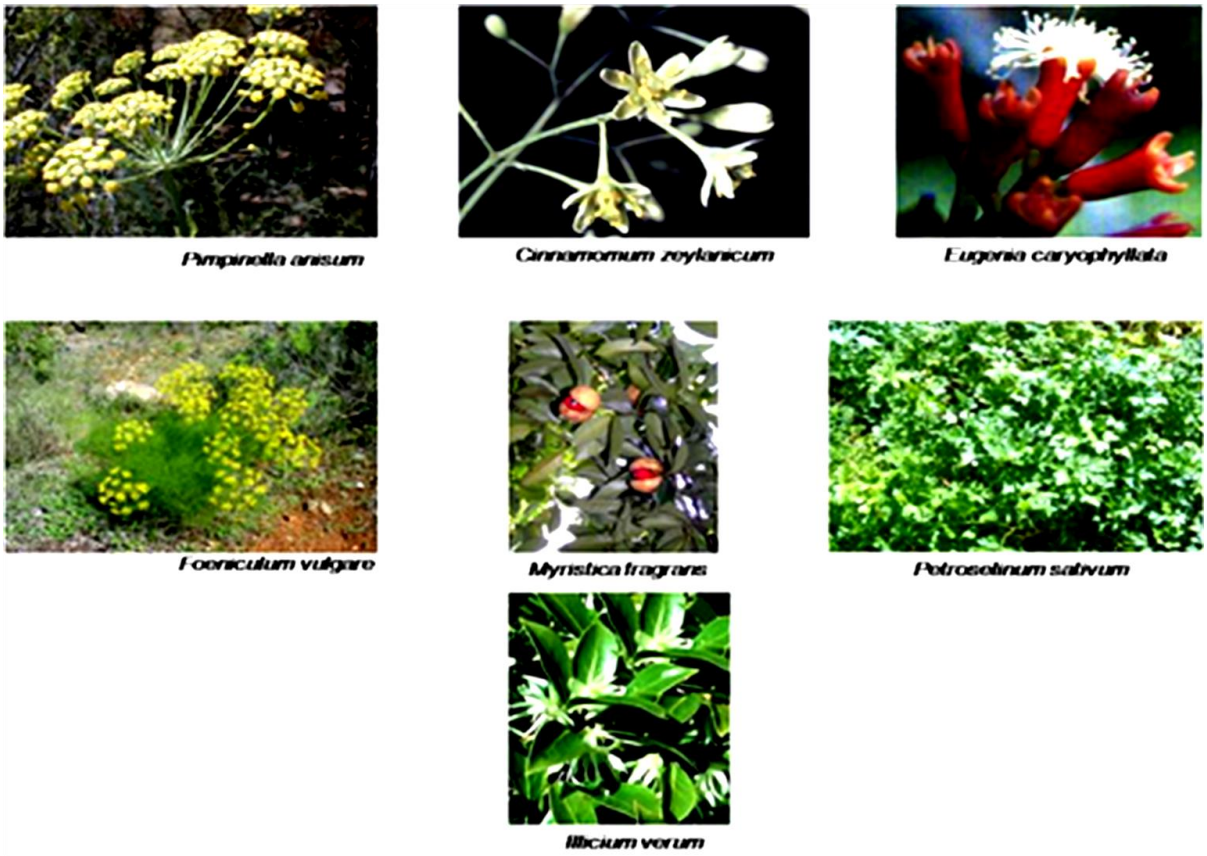


Figure 1.14- Main plants which produce essential oils containing mainly aromatic compounds.

PHYSICAL AND CHEMICAL PROPERTIES

The study of the chemical-physical characteristics of the essential oils obtained from certain plants, consisting mainly in terpenoids. Nowadays the comprehension of the oil composition is of great importance to understand their antimicrobial action, especially against pathogenic microorganisms (Delaquis et al., 2002; Dorman & Deans, 2000).

In particular, the activity and the physico-chemical properties of oils extracted from plants belonging to the *Labiatae* family, such as rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*) etc., and to citrus family (*Citrus bergamia*, *Citrus nobilis*, *Citrus sinensis*) has been studied by several authors (Basilico & Basilico, 1999; Juven et al. 1994, Lambert et al., 2001; Tasoou et al., 2000; Ben-Yehoshua et al. 1998).

The aromatic compounds possess similar physical properties while they are structurally heterogeneous between them. It can be said that with an increase of the number of carbon atoms of the structure as well of the molecular weight the water solubility of the molecule decreases,

while the solubility in less polar solvents increases. The water solubility of linear aldehydes increases with decreasing of the length of the carbon chain. The same can be applied for alcohols from 1 to 9 carbon atoms, for ketones and for the methyl esters. The characteristics of hydrophilicity and lipophilicity are very important for the biological activity of volatile organic compounds, the solubility in fats enables, to volatile molecules, to permeate in cytoplasmic membranes and the waxy cuticle. Among the features of these substances, the volatility, or rather the tendency of the molecules to pass from the liquid phase to the vapor phase, is one of the most important . Also the knowledge of pure compound solubility is essential, but more important is the knowledge of the solubility of their aqueous solutions. The volatility is described by the partition coefficient expressed as the ratio at equilibrium and constant temperature between the amount of volatile compound dissolved in one mL of air and the amount dissolved in one mL of water. For example, at the same concentration of compound in water, nonanal is present in the vapor phase in an amount 100 times greater than ethanol having an higher boiling point. The partition coefficients are valid only for pure aqueous solutions and well below the saturation point.

Generally alcohols have a low partition coefficient in water and are followed, with increasing volatility from ketones, esters and aldehydes. The addition of other solutes such as salts or sugars can change considerably the volatility of the present aromatic compounds .

Under the same conditions of temperature and concentration, the vapor pressure of a solute depends on the Aw of the system and on the concentration and nature of the other solutes. In fact, various solutes present in an aqueous solution interact with water molecules by changing the structure and characteristics (Guerzoni et al. 1994).

The chemical-physical characteristics of essential oils, such as their volatility and particularly their hydrophobicity, affect the bioactivity of these aromatic substances which generally is expressed as inhibition or stimulation of metabolic processes. The compounds in the gaseous phase may accumulate more rapidly in the cytoplasmic membranes of cells acceptor than they do if solubilized in the carrier. Once the compound has entered the liquid film that surrounds the cells and then in the cytoplasm, there are not physiological differences between volatile and non-volatile compounds (Guerzoni et al., 1994). In this regard, the evaluation of the effects of the bioactivity of molecules and factors influencing by the vapor pressure may be an important key to optimize the use of these molecules in food.

1.3.1 ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS

Different methods are known for testing the antimicrobial activity of essential oils and their components. The most used screening are the dilution and diffusion methods (Rios et al., 1988). In particular, the disk diffusion method (Farag et al., 1989; Elgayyar et al., 2001; Skandamis et al., 2001; Cimanga et al., 2002; Faleiro et al., 2002; Packiyasothy & Kyle, 2002; Burt & Reinders, 2003; Wilkinson et al., 2003), the agar wells method (Dorman & Deans, 2000), the agar dilution method (Rios et al., 1988, Hammer et al., 1999) and the broth dilution method (Delaquis et al., 2002, Lambert et al., 2001, Ultee et al., 2002, Smith-Palmer et al., 1998, Gill et al., 2002, Tassou et al., 2000, Pol & Smid 1999) are the most used. In broth dilution studies, a number of different techniques exists for determining the end-point and the most used methods are that of optical density (OD) (turbidity) measurement and the enumeration of colonies by viable count (Burt et al., 2004). Other methods used for the determination of the antimicrobial properties of essential oils are the time-kill analysis (Tassou et al., 1995; Ultee et al., 1998; Ultee et al., 2002) that is used for the determination of rapidity and duration of antimicrobial activity, and the scanning electron microscopy (SEM) that is used for study the damage of the cell wall and loss of cell contents (Lambert et al., 2001; Skandamis et al., 2001; Burt & Reinders, 2003).

Normally there is not a standardization of these methods, and researchers adapt their experimental methods to better represent possible future applications in their proper field (Burt 2004). This aspect makes difficult to compare published data. In fact, the antimicrobial activity is affected by some factors such as the method used to extract the EO from plant material, the volume of inoculum, growth phase, culture medium used, pH of the media and incubation time and temperature (Burt, 2004). Moreover, a solvent has to be used to vehicular the essential oils and dissolve them in a water solution and for this purpose several solvents have been used: ethanol, methanol, Tween-20, Tween-80, acetone in combination with Tween-80, polyethylene glycol, propylene glycol, n-hexane, dimethyl sulfoxide and agar (Burt, 2004). The solvent used as carrier for essential oils strongly affects the antimicrobial activity (Burt, 2004).

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) are the most used parameter for expressing the antimicrobial activity of essential oils or their components. Carson et al. (1995), defined MIC as the lowest concentration resulting in maintenance or reduction of inoculum viability. Instead, the MBC is defined as the concentration where 99.9% or more of the initial inoculum is killed (Carson et al., 1995). In table 1.10, are

reported the MICs values of some essential oils and their principal components in relation to the test method and the bacterial strain used.

Table 1.10-*Selected MICs of essential oils and their most bioactive components against food borne pathogens (Burt 2004)*

Plant from which EO is derived	Species of bacteria	MIC, approximate range (μl ml^{-1})[b]
Rosemary	*Escherichia coli*	4.5–>10
	Salmonella typhimurium	>20
	Bacillus cereus	0.2
	Staphylococcus aureus	0.4–10
	Listeria monocytogenes	0.2
Oregano	*E. coli*	0.5–1.2
	S. typhimurium	1.2
	Staph. aureus	0.5–1.2
Lemongrass	*E. coli*	0.6
	S. typhimurium	2.5
	Staph. aureus	0.6
Sage	*E. coli*	3.5–5
	S. typhimurium	10–20
	Staph. aureus	0.75–10
	L. monocytogenes	0.2
Clove	*E. coli*	0.4–2.5
	S. typhimurium	>20
	Staph. aureus	0.4–2.5
	L. monocytogenes	0.3
Thyme	*E. coli*	0.45–1.25
	S. typhimurium	0.450–>20
	Staph. aureus	0.2–2.5
	L. monocytogenes	0.156–0.45
Turmeric	*E. coli*	>0.2
	B. cereus	0.2
Tea bush (*Lippia* spp.)	*E. coli*	2.5–>80
	Shigella dysenteria	5–>80
	Staph. aureus	0.6–40
	B. cereus	5–10
α-Terpineol	*Escherichia coli*	0.450–>0.9
	Salmonella typhimurium	0.225
	Staphylococcus aureus	0.9
	Listeria monocytogenes	>0.9
	Bacillus cereus	0.9
Carvacrol	*E. coli*	0.225–5
	S. typhimurium	0.225–0.25
	Staph. aureus	0.175–0.450
	L. monocytogenes	0.375–5
	B. cereus	0.1875–0.9
Citral	*E. coli*	0.5
	S. typhimurium	0.5
	Staph. aureus	0.5
	L. monocytogenes	0.5
Eugenol	*E. coli*	1.0
	S. typhimurium	0.5
	L. monocytogenes	>1.0
Geraniol	*E. coli*	0.5
	S. typhimurium	0.5
	L. monocytogenes	1.0
Perillaldehyde	*E. coli*	0.5
	S. typhimurium	0.5
	L. monocytogenes	1.0
Thymol	*E. coli*	0.225–0.45
	S. typhimurium	0.056
	Staph. aureus	0.140–0.225
	L. monocytogenes	0.450
	B. cereus	0.450

MODE OF ACTION OF ESSENTIAL OILS AND THEIR PRINCIPAL COMPONENTS

Although the antimicrobial properties of essential oils and their components have been tested in the past (Nychas, 1995; Dorman & Deans 2000; Cosentino et al., 2003; Smith-Palmer et al., 2001; Prabuseenivasan et al., 2006, Holley & Patel 2005; Kalemba & Kunicka 2003), their mechanisms of action has not been studied in detail (Lambert et al. 2001, Hyldgaard et al., 2012). Considering the large number of different groups of chemical compounds present in the essential oils, it is likely that their antibacterial activity is not attributable to a specific mechanism but there are more targets in the cell (Skandamis et al., 2001; Carson et al., 2002, Faleiro, 2011). The locations or mechanisms inside the bacterial cells that seem to be the major sites of action of the components of the essential oils are shown in Figure 1.15.

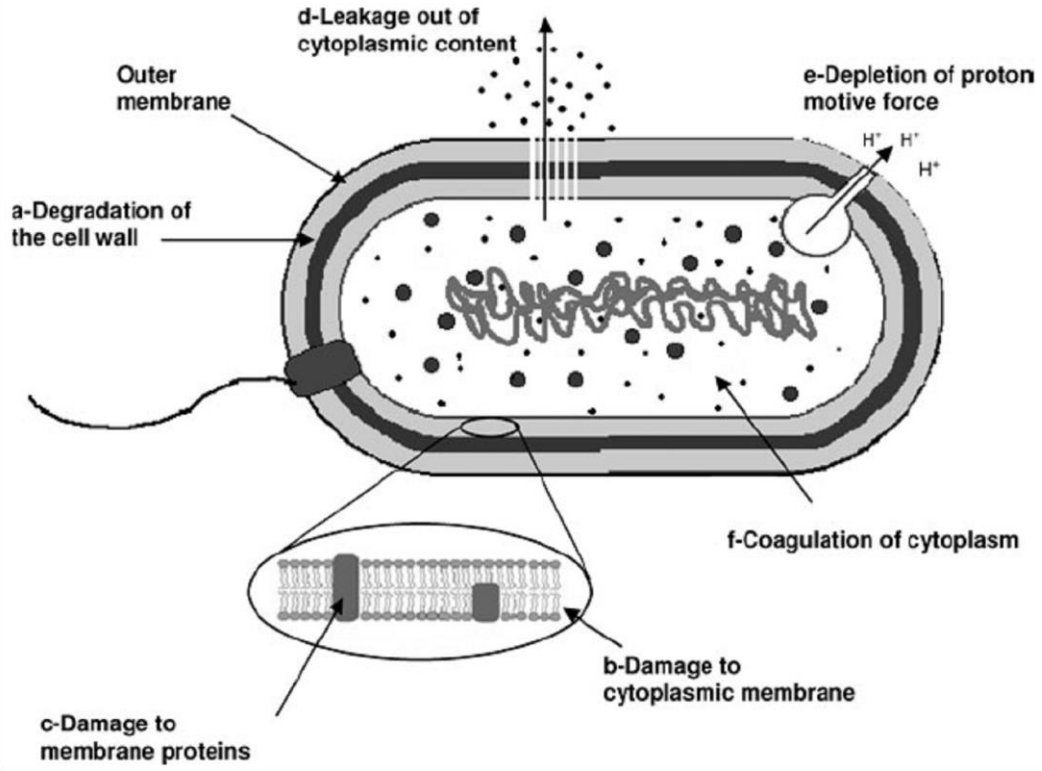


Figure 1.15- *Possible sites of action of essential oils or their compounds at the cellular level: cell wall degradation; damage of the cytoplasmic membrane; damage of membrane proteins; loss of cell contents; coagulation of cytoplasm and depletion of the proton motive force (Raybaudi-Massilia, 2009, adapted from Burt, 2004).*

Essential oils can have pronounced antimicrobial effects, although often their complexity and variability make difficult the correlation between antimicrobial activity and individual specific components, also in relation to possible synergistic and antagonistic effects.

The role and the mechanisms of action of the minor components of EOs is not well documented, but some antagonistic effects due to these minority fractions were observed. In *Malaleuca alternifolia* EO, not oxygenated hydrocarbon monoterpenes (p-cymene, γ-terpinene) increase the resistance of the more tolerant microorganisms probably due to the reduction of solubility of aqueous terpenes terpen-4-ol, 1,8-cineole and for this reason, also the availability of the active components against microorganisms.

As previously mentioned, the aromatic molecules among the various physical properties, are characterized by a poor solubility in water and a high hydrophobicity. For this reason, many studies indicate their antimicrobial effects as dependent on this characteristic and on their ability to act on the cell membrane. The bioactivity of many aromatic compounds may depend, in addition, by the vapor pressure, which can be considered an indirect measure of hydrophobicity. The factors responsible for the increase of the vapor pressure of the aromatic molecules lead to a rise the antimicrobial activity, since it increases their solubility in cell membranes (Caccioni et al., 1997; Gardini et al., 2001).

Precisely, it is their hydrophobicity to permit them to share in the lipids of cell membranes and mitochondria, altering the structures and making them more permeable (Knobloch et al., 1986; Sikkema et al., 1994), and leading to the loss of ions and other cell contents (Helander et al., 1998; Cox et al., 2000; Lambert et al., 2001; Skandamis et al., 2001; Carson et al., 2002; Ultee et al., 2002).

The bacterial cell can tolerate, up to a certain limit, the loss of some cell contents, but their excessive leakage or the loss of critical molecules and ions lead to the cell death (Denyer & Hugo, 1991). Gilbert et al. (1977) have found that when the concentration of antimicrobial agents is bacteriostatic, the loss of low molecular weight cytoplasmic constituents, could be due to a reversible disorganization of the cytoplasmic membrane. At bactericidal concentrations (defined by Gill and Holley (2004) as the concentration that can prevent the reproduction of the treated cells when transferred to a medium without antimicrobial agent), relations between the loss of cellular components and the death of the cell, due to antimicrobial agents, were detected.

Cellular targets

Many studies indicate the cell membrane as the primary target of bioactive aromatic compounds. Membranes disrupted by the action of terpenes can be observed both on bacteria and fungi (Lanciotti et al., 2004). The antimicrobial action of many essential oils (EOs) appears to be connected with the presence of phenolic compounds. The inhibitory effect of phenols can be explicate through interaction with the cell membrane of microorganisms, and it is often correlated with the hydrophobicity of the compounds (Sikkema et al., 1995; Weber & de Bond 1996). Indeed, the hydrophobicity of these molecules is responsible for their solubility in the cell plasma membranes and their bioactivity depends essentially on their partition coefficient (Caccioni et al., 1997; Lambert et al., 2001).

The lipophilic structure of cyclic monoterpenes promotes their partition from the aqueous phase to cell membranes resulting in expansion and increase in fluidity and permeability of the membrane, which leads ultimately to an inhibition of membrane enzymes (Cox et al., 2000). In some microorganisms, mild heat treatments increase the inhibitory effect of carvone, altering the membrane composition, the fluidity and favoring the partition of these molecules in the membrane phospholipids.

In bacteria, the permeabilization of membranes is associated with the loss of ions and the reduction of the membrane potential, the collapse of the proton pump and the depletion of the ATP pool (Knobloch et al., 1989; Sikkema et al., 1994; Helander et al., 1998; Ultee et al., 2000, 2002; Turina et al., 2006). EOs can coagulate the cytoplasm (Gustafson et al., 1998) and cause damage to lipids and proteins (Ultee et al., 2002; Burt, 2004). The damages to the cell wall and in the membranes, may lead to loss of macromolecules up to cell lysis (Juven et al., 1994, Gustafson et al., 1998; Cox et al., 2000; Lambert et al., 2001; Oussalah et al ., 2006).

In particular, the loss of specific ions, due to the action of the aromatic molecules on the cell membrane, has dramatic effects on the proton motive force, by decreasing the content of intracellular ATP. In this manner, the total activity of the cells is greatly compromised, as well as the cellular turgor (osmotic pressure), the transport of solutes and the regulation of metabolism (Lanciotti et al., 2004).

The oregano EO, for example, creates an alteration of membrane permeability with a consequent loss of protons, phosphorus and potassium (Lambert et al., 2001). Carvacrol leads to a dissipation of intracellular ATP in *B. cereus* due to the reduction of the synthesis or hydrolysis, accompanied

by the increase of permeability of the membrane to ATP (Ultee et al., 1999). On the other hand, a loss of ATP was already observed by Helander (1998) in Gram-negative bacteria.

The "Tea Tree" EO stimulates autolysis, coagulates the cytoplasm of the cell, resulting in loss of intracellular material in *E. coli* (Gustafson et al., 1998). Cox et al. (1998) showed that concentrations of tea tree oil inhibiting the growth or kill *E. coli*, also inhibit the glucose dependent respiration and stimulate the loss of intracellular K^+.

Juven et al. (1994) hypothesized that the inhibition against *S. typhimurium* and *S. aureus* by the thyme EO, was dependent on the hydrophobicity and the nature of the present phenolic constituents, which determined alteration of the functionality of membrane proteins after partitioning in the phospholipid bilayer.

In general, the EOs with the most remarkable antibacterial properties against pathogenic microorganisms, contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol (Farag et al., 1989; Cosentino et al., 1999; Dorman & Deans, 2000; Juliano et al., 2000; Lambert et al., 2001). It seems reasonable to assume that their mechanism of action is similar to that of other phenolic compounds, i.e., the alteration of the cytoplasmic membrane, the interruption of the proton motive force, the electrons flow, the active transport and coagulation of the cellular contents (Denyer & Hugo, 1991; Sikkema et al., 1995; Davidson, 1997).

The antimicrobial activity of the oils seems to be related to their composition, to the structural configuration of the constituents and to their functional groups, as well as to the possible synergistic interactions among the components. Consequently, the chemical structure of the individual compounds present in the EOs affects their precise mode of action and their antibacterial activity (Dorman & Deans, 2000).

The compounds with a phenolic structure, such as carvacrol, eugenol, thymol, have shown high activity against the tested microorganisms. Some members of this class of substances are known both as bacteriostatic or bactericidal agents, depending on the concentrations used. These compounds are highly actives despite their relatively low solubility in water. This is in agreement with data reported in the literature (Lattaoui & Tantaoui, 1994; Charai et al., 1996).

The relative position of the hydroxy group in the phenolic ring, does not appear to strongly affect the degree of antibacterial activity. For example, the action of thymol against *B. cereus*, *S. aureus* and *Pseudomonas aeruginosa* appears to be comparable with that of carvacrol (Lambert et al., 2001; Ultee et al., 2002). However, Dorman and Deans (2000) have shown that carvacrol and thymol have behaved differently against Gram-positive and Gram-negative species. The high

antimicrobial activity of phenolic components can also be explained in terms of alkyl substitution of the phenolic nucleus which, as well known, promotes the antimicrobial activity of these substances.

The importance of the phenolic ring (destabilized electrons) is demonstrated by the lack of activity of menthol when compared to carvacrol (Ultee et al., 2002). The addition of an acetate molecule seemed to increase the antibacterial activity: the geranyl acetate was more active compared to geraniol against various species of Gram-positive and Gram-negative (Dorman & Deans, 2000), even alcohols are known for their bactericidal activity, especially against vegetative cells. The terpene alcohols have exhibited a strong bioactivity against microorganisms proving potentially active, both as denaturing agents against proteins, and as solvent and dehydrating agents.

Numerous components of EOs belong to the group of ketones. The presence of oxygen in the structure increases the antimicrobial effects of terpenoids. Regarding the non-phenolic compounds, although are less interesting, the type of alkyl group affects its activity (alkenyl> alkyl). For example, limonene is more active than *p*-cymene (Dorman & Deans, 2000).

Moreover, also the stereochemistry has an influence on the bioactivity. It was noted that the α-isomers are less active than β-isomers. *Cis*-isomers are less effective than *trans*-isomers; cyclic compounds such as methyl-isopropyl cyclohexane are the most active; unsaturations in the ring of cyclohexane further enhances the antibacterial activity, such as terpinolene, terpineol and terpineolene.

The components of the EOs appear to act on membrane proteins embedded in the cytoplasmic membrane (Knobloch et al., 1989). Enzymes such as ATPase are localized in the cytoplasmic membrane and they are surrounded by lipid molecules. Two possible mechanisms through which the cyclic hydrocarbons may act have been proposed. The lipophilic hydrocarbon molecules may accumulate in the lipid bilayer and distort the protein-fat interactions, or alternatively direct interactions of lipophilic compounds with hydrophobic parts of proteins are possible (Juven et al., 1994; Sikkema et al., 1995).

Cytotoxic effects (for cytotoxicity is meant the effects of a chemical, physical or biological agent, able to induce a damage to the cell) were observed *in vitro*, through the method of agar diffusion, using the filter paper disk or through the method of dilutions using agar or broth liquid culture, on the majority of Gram-positive and Gram-negative bacteria (Williams et al., 1998; Kalemba &

Kunicka, 2003; Schnebelen Arnal et al., 2004 , Burt, 2004; Hong et al., 2004; Rota et al., 2004; Si et al., 2006; Sonboli et al., 2005) and in viruses (Logu et al., 2000; Jassim & Naji, 2003; Reichling et al., 2005) and fungi (Manohar et al., 2001; Pitarokili et al., 2002; Hammer et al., 2004; Kosalec et al. , 2005) also included yeasts (Harris, 2002; Hammer et al., 2004; Duarte et al., 2005; Pauli, 2006; Carson et al., 2006).

Recent works on *Saccharomyces cerevisiae*, have shown that the cytotoxicity of some EOs, on the basis of the ability to form colonies, was considerably different depending on their chemical composition. Treatments with EOs on cells in stationary growth phase showed 50% mortality with 0.45 µL / mL of EO of *Origanum compactum*, 1.6 µL / mL of EO of *Coriandrum sativum*, > 8 µL / mL of EO of *Cinnamomum camphora*, *Artemisia herba-alba* and *Helichrysum italicum* (Bakkali et al. 2005).

A number of characteristics of Gram-negative bacteria including the virulence and pathogenicity are regulated through the quorum sensing (mechanism by which the bacterial population measure its cell density, is a communication from cell to cell, based on the synthesis, the exchange and the perception of small signal molecules between bacteria, and that regulate the expression of certain sets of genes). Its interruption is an example of anti-pathogenic effect. Several EOs including cinnamon (*Cinnamomum zeylanicum*), mint (*Mentha piperita*) and lavender (*Lavandula officinalis*) have shown a potential anti-quorum sensing activity on pigment production by *C. violaceum* (Khan et al., 2009). It is not clear if are the larger or the smaller constituents of the EOs that act on the system of quorum sensing. The common mechanism of interference with quorum sensing includes:

a) the inhibition of the biosynthesis of signals or the inhibition of the activity of enzymes that produce N-acyl-homoserine lactones (AHLs) (small molecules that act as signals for the quorum sensing);
b) the degradation of enzymatic signals;
c) the inhibition of molecules of signal reception. It is also possible that the final effect on the inhibition of particular traits related to quorum sensing may be the result of an action of the various multi-target components of EOs on bacterial quorum sensing system.

1.3.2 USE OF ESSENTIAL OILS IN FOOD MICROBIOLOGY: CURRENT USES AND FUTURE PROSPECTS

The combination of the antimicrobial properties and the flavor of essential oils that are suitable for use in food products, has led to increase research for the uses of EOs as potential food preservatives. For a safe use of EOs as antimicrobial agents in food products, they must not only be safe for consumption but also reduce the initial microbial load during production and extend the shelf-life of food products (Moreira et al., 2005).

Taking into consideration the consumer demand for alternatives to chemical-based antimicrobials for food applications, EOs are potentially an ideal alternative, given the new attraction towards natural products. It is important to develop a better understanding of their biological mode of action for the new applications on human health, agriculture and the environment. Some of them are effective alternatives or complements of synthetic compounds of the chemical industry, but without showing the same negative secondary effects (Carson & Riley, 2003).

Further studies on foods are additionally required to assess changes in the organoleptic properties of food products after application of EOs. Also their economic sustainability needs to be revised, as well as their mechanisms actions avoiding the possibility to originate resistant pathogens (Hili et al., 1997). However, it seems that EOs applied to foods may be able to inhibit a wide range of microrganisms but, on the other hand, they may also cause an imbalance in the intestinal microflora (Dorman & Deans, 2000).

The large amount of papers on the study of the antimicrobial activity of EOs and their constituents (Burt, 2004; Fisher & Phillips, 2008; Lanciotti et al., 2004; Holley & Patel, 2005) is a sign of an increased interest on their potential use in order to control the growth of pathogen and spoilage microorganisms in food products. Large part of this literature is referred to EOs *in vitro* antimicrobial activity, to the research of more bioactive molecules, to the understanding of the mechanisms of action and to the factors affecting their bioactivity. Nevertheless, the application of EOs for antimicrobial purpose in foods is still limited and sporadic and the reasons are numerous. Among them, the most important are i) the variability of the composition of EOs (due to the geographic origin, agricultural techniques, season, methods of extraction, etc..) able to influence their effective overall antimicrobial activity (Burt, 2004); ii) the interaction of bioactive molecules with the food matrix (in particular with proteins, lipids, starch, etc..) that limits the contact of these molecules with the microbial cells, thereby reducing the effects on cell viability

(Gutierrez 2008b); iii) the lack of knowledge of the mechanism by which these molecules exert their antimicrobial activity and the influence that technological and composition parameters may have on their activity. Another important factor that limits the use of these substances is the amount of EOs or their constituents needed to exercise a tangible and satisfactory antimicrobial activity. In fact, the EO must be compatible with the organoleptic characteristics of the food in which it will be used both in qualitative and quantitative terms.

In this perspective, the use of EOs in combination with other inhibiting factors in the context of "hurdle technologies" (Alzamora et al., 2003) could represent an interesting strategy to obtain minimally processed food products which can preserve the characteristics of freshness typical of untreated fresh product, and at the same time to safeguard the hygienic and microbiological aspect. Several recent researches have studied the stabilization of minimally processed foods combining the presence of EOs and their constituents with bacteriocins, organic acids, low aw, low pH and modified atmosphere (Alzamora et al. 2003; Chouliara et al. 2007; Lopez-Malo et al., 2005; Lopez et al, 2006; Serrano et al. 2008).

A great advantage of EOs is the fact that they are usually without genotoxic risks in the long term, where for genotoxicity means the ability of a substance to induce changes in the nucleotide sequence or of the double helix structure of the DNA of a living organism. Also, some of them show a very clear anti-mutagenic capacity that could be connected to an antitumor activity.

Active components and toxicity should be clearly documented before the oil use in the food industry or in the clinical area (Rios & Recio, 2005). As demonstrated recently by Dusan et al. (2006), high doses of some essential oils can have adverse effects on the intestinal cells and therefore the effect for the entire intestinal tract must be evaluated to achieve a safe use. The use of oils that are suitable to be used as a flavoring in the food industry and that are GRAS, therefore, can be considered a good starting point for the use of essential oils as antimicrobials in food.

CHAPTER 2

OBJECTIVES

Fruits and vegetables are strongly recommended in the human diet due to their content in vitamins, antioxidants, minerals and dietary fibers. However, outbreaks of food-borne diseases associated to the consumption of fresh and minimally processed fruits and vegetables, have augmented dramatically since the 1970s. In fact, a wide literature shows the presence on fresh produce and related minimally processed products of pathogenic species (Harris et al., 2003; CDC, 2007; Powell & Luedtke, 2000; Abadias et al., 2011; Olaimat & Holley, 2012; Van Boxstael et al. 2013). The application of decontamination methods is the most important tool to guarantee the safety and shelf-life of minimally processed products. Several chemical sanitizers have been employed to decontaminate raw material, however, a wide literature showed, in addition to their potential toxicity, their inability to completely eradicate or kill microorganisms on fresh produce (Beuchat, 1998; Brackett, 1999; Abadias et al., 2008a,b). These reasons have stimulated the research of alternative methods to decrease minimally processed fruits and vegetables decay and increase the product safety and shelf-life. In fact, the intrinsic characteristics of minimally processed fruits may favor the growth of pathogens and spoilage microbiota.

Currently, several investigations have been focused on the search for natural antimicrobials able to increase the quality and safety of the minimally processed fruits and vegetables (Beuchat, 1998; Allende et al., 2008; López-Gálvez et al., 2009; Vandekinderen et al., 2009; De Azeredo et al., 2011). A wide literature shows the great potential as antimicrobials in model and food systems of essential oils (EOs) and their components (Nychas, 1995; Dorman & Deans 2000; Cosentino et al., 1999; Smith-Palmer et al., 2002; Prabuseenivasan et al., 2006, Holley & Patel 2005., Kalemba & Kunicka, 2003). Moreover, the action of single constituents of these oils has been studied to identify their cell targets and the most active molecules, and to balance their intrinsic variability (Karatzas et al., 2000; Vazquez et al., 2001; Sado Kamdem et al., 2011; Zheng et al., 2013; Kurekci et al., 2013; Picone et al., 2013). However the action mechanisms of EO and their main components are not fully understood limiting the industrial exploitation of these natural antimicrobials.

Also the use of protective cultures has been proposed for their potential application in minimally processed fruits and vegetables (Schillinger et al., 1996; Bennik et al., 1999; Rodgers, 2001). Protective cultures of lactic acid bacteria (LAB) to increase safety and shelf-life of minimally processed fruits and vegetable have been developed in last decades (Vescovo et al. 1996; Bennik et al. 1999; Leroy et al. 2003; Palmai & Buchanan, 2002). Several authors showed the potential of LAB strains to increase the safety of minimally processed fruits and vegetables due to the

inhibition pathogenic microorganisms. Selected strains of *Pseudomonas syringae*, *Pseudomonas graminis*, *Gluconobacter asaii*, *Candida* spp., *Dicosphaerina fagi* and *Metschnikowia pulcherrima* showed great potential as biocontrol agents in minimally processed fruits to their ability to antagonize under laboratory conditions several foodborne pathogens (Leverentz et al., 2006; Abadias et al., 2009; Trias et al., 2008a, 2008b; Alegre et al., 2012, 2013). However, the application of bioprotective cultures at industrial level for commercial products is scarce because satisfactory conditions under laboratory settings are unable to guarantee the success under real processing and distribution conditions (Trias et al., 2008a; Abadias et al., 2009).

In this context, the main aims of this thesis were:

- to evaluate the potential of some essential oils and their components to improve the safety and the shelf life of Lamb's lettuce (*Valerianella locusta*) and apples (*Golden delicious*);
- to isolate, identify, characterize for their technological features LAB isolated from apples and fresh-cut lettuce in order to select potential bio-control agents
- to evaluate the effect of the addition of the selected LABs, alone or in combination with essential oils or their components, on the shelf-life and safety (through execution of challenge-test) as well as organoleptic properties of minimally processed Lamb's lettuce (*Valerianella locusta*) and apples (*Golden delicious*).

Since the lack of knowledge of cell targets of essential oils represent one of the most important limit to the use of this molecules at industrial level, another aim of this thesis was the study of the action mechanisms of essential oils and their components against pathogens frequently associated to minimally processed vegetables (*Listeria monocytogenes*, *Escherichia coli* and *Salmonella enteritidis*). In particular, the study of the modifications in the fatty acids of the cytoplasmic membrane and the volatilome of the microorganisms mentioned above, grown in the presence of sublethal concentrations of antimicrobial molecules, was performed. In addition, the evaluation of the effects of some of the natural antimicrobials considered on gene expression of the entire genome of a pathogenic microorganism (*Escherichia coli*) and a potential bio-control agent (*Lactococcus lactis*), was performed.

CHAPTER 3

Natural antimicrobials to prolong the shelf-life of minimally processed apples

Abstract

Minimally processed fruits are susceptible to microbial proliferation and to a fast loss of sensory quality. In this experimental work, to increase shelf-life and quality parameters (texture and colour) maintenance of sliced apples (*Malus communis*, var. *Golden delicious*), the use of natural antimicrobials was proposed as alternative to the traditional sanitization methods. Citron EO, hexanal, 2-(*E*)-hexenal, citral and carvacrol, alone or in combination, were added to the traditional dipping solution (0.5% of ascorbic and 1% of citric acid) of sliced apples. As controls, apples dipped in the traditional solution were used. The products packaged in ordinary atmosphere were stored at 6°C and, immediately after washing and during storage, the yeast cell loads were monitored until the spoilage threshold (6 log cfu/g). In addition, the volatile profiles, electronic nose analyses, colour and texture analyses were monitored during the storage. Cell load data showed that the use of natural antimicrobials changed the naturally occurring yeast growth parameters with respect to the control. The combination of citron/carvacrol prolonged the yeast lag phase of about 6 days in comparison with control sample, while the use of citral and the mixture hexanal/2-(E)-hexenal decreased the maximum reached yeast cell load and growth rate respectively. After 8 days of storage, samples treated with hexanal/2-(E)-hexenal and citral, although characterized by an initial browning, showed equivalent or even better colour and texture attributes compared to the controls. In order to further improve the shelf-life of the minimally processed apples the same molecules were tested in combination with packaging in active modified atmosphere (7% O_2 and 0% $CO_{2)}$ and some modifications of the washing process. After the optimization, in all the samples the spoilage yeast threshold was not attained within the 35 days of storage independently on the substances supplemented. Samples treated with the combinations citral/2-*(E)*-hexenal and hexanal/2-*(E)*-hexenal showed a good retention of colour parameters during storage. Among investigated natural antimicrobials, the mixture hexanal/2-*(E)*-hexenal promoted the best retention of firmness throughout 35 days of storage. These results evidence the potentiality of dipping treatment based on these natural antimicrobials to strongly prolong the shelf-life of fresh-cut apples.

1. Introduction

Minimally processed fresh fruits represent an important component of a healthy diet and are a convenient way of increasing fresh produce consumption. Fresh-cut fruits are susceptible to microbial proliferation due to the loss of natural resistance and their high water and nutrient

content (Brackett, 1994; Rico, et al., 2007). In addition, the raw materials during processing are subjected to peeling, cutting or slicing that favour the microbial growth due to the release of nutrient and the transport of the surface microbiota on the cut surfaces (Lanciotti et al., 2003; Rojas-Grau et al., 2007). The absence of treatments able to guarantee the microbial stability, the active metabolism of fruit tissue, and the confinement of final product inside the packaging increases the growth potential of the naturally occurring microorganisms (Nguygen & Carlin, 1994 and Lanciotti et al., 2003). Due to the lack of processing steps or factors able to kill microbial contaminants, an efficient temperature control during manufacture, distribution and retailing is required for maintaining the microbiological quality and the safety of these products. However, the maintaining of the cold chain and the use of chemicals as disinfectants of raw materials are not sufficient to either eliminate or significantly delay the microbial spoilage of these products entirely and to ensure the product safety (Soliva-Fortuny, & Martín-Belloso, 2003). In fact, a wide literature shows the presence on fresh fruits and related minimally processed products of pathogenic species such as *Listeria monocytogenes*, *Salmonella* spp., *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Staphylococcus aureus* (Beuchat, 1998; Conway et al., 2000; Gunes & Hotchkiss, 2002; Alegre et al., 2010). Moreover, fresh fruits, fruit juices and minimally processed fruits have been incriminated in several outbreaks caused by *E. coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* (Powell & Luedtke, 2000; Harris et al., 2003; Abadias et al., 2011; Olaimat, & Holley, 2012; Van Boxstael et al., 2013).

Currently, several investigations have been focused on the search for natural antimicrobials able to increase the quality and safety of the minimally processed fruits (Beuchat, 1998; Allende et al., 2008; López-Gálvez et al., 2009; Vandekinderen et al., 2009; De Azeredo et al., 2011). A wide literature shows the great potential as antimicrobials in model and food systems of essential oils from citrus fruits (Fisher, & Phillips, 2008; Espina et al., 2011; Settanni et al., 2012). Moreover, the action of single constituents of these oils has been studied to identify their cell targets and the most active molecules, and to balance their intrinsic variability (Karatzas, 2000; Vazquez et al., 2001; Sado Kamdem et al., 2011; Zheng et al., 2013; Kurekci et al., 2013; Picone et al., 2013). In particular, citral (3,7-dimethyl-2-7-octadienal), is a terpenoid with 2 isomers, geranial and neral, naturally occurring in citrus essential oils and characterized by a wide spectrum antimicrobial activity both in model and real foods (Hayes & Markovic, 2002; Wuryatmo et al., 2003; Belda-Galbis et al., 2013). Citral and citron essential oil at concentration compatibles with sensorial features were able to

significantly prolong the microbial shelf-life of the fruit-based salads in syrup (Belletti et al., 2008), and the stability of fruit based soft drink (Belletti et al., 2007).

Also the antimicrobial activity of hexanal and 2-(*E*)-hexenal, which are components of the aroma of many fruits and vegetables, has been already tested in model (Gardini et al., 1997; Gardini et al., 2001; Kubo & Fujita, 2001) as well as in real systems (Lanciotti et al., 1999; Corbo et al., 2000; Lanciotti et al., 2003; Lanciotti et al., 2004). Hexanal, 2-(*E*)-hexenal, and hexyl acetate improved shelf-life and safety of minimally processed fruits (Lanciotti et al., 2004; Serrano et al., 2008). In particular, the addition of hexanal and 2-(*E*)-hexenal in storage atmosphere of fresh-cut apples resulted in a positive effect on shelf-life, due to their antimicrobial activity against naturally occurring spoilage species also when deliberately inoculated at levels of 10^3 cfu/g. Moreover, these molecules determined the enhancement of the sensorial properties, as well as the retention of the original colour of the packaged products (Lanciotti et al., 1999; Corbo et al., 2000). These aldehydes showed a great potential as antimicrobials also against pathogens such as *Salmonella* spp., *E. coli* and *Pseudeomonas aeruginosa* (Kubo et al., 2001). Little information is available on the relationship between the outgrowth of spoilage microorganisms, their volatilome, and the perception of the decay of minimally processed vegetables by consumers.

In this direction the principal aim of this work was in the first place to evaluate the effects of dipping treatments with different concentrations of hexanal, (E)-2-hexenal, citral, carvacrol and citron essential oil (EO), alone or in combination, on the shelf-life of fresh-cut apple slices stored at 6°C. Specifically, after the determination of citron EO composition, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the chosen substances against the most frequent pathogenic species in this kind of products (*L. monocytogenes*, *E. coli*, *Salmonella* spp). In addition the study of the effects of the same molecules on the shelf-life of minimally processed apples packaged in modified atmosphere, after some modifications of the washing parameters, were performed. In particular, the effects of these antimicrobials on yeast and lactic acid bacteria (LAB) cell loads, texture, colour and volatile molecule profiles were monitored during the storage at 6°C. An additional aim of this work was the identification of eventual spoilage volatile markers in relation to the natural antimicrobial used.

2. Material and methods

2.1 Natural antimicrobials

The tested compounds (hexanal, 2-*(E)*-hexenal, citral and carvacrol) were purchased from Sigma-Aldrich (Milano, Italy). Citron essential oil (EO) was obtained from Flora s.r.l. (Pisa, Italy). Citron EO and the natural tested antimicrobials were selected both for their antimicrobial activity and impact on organoleptic properties after a preliminary screening. Citron EO was preliminarily characterized by solid phase microextraction combined to gas-chromatography and mass-spectometry (GC/MS-SPME) technique to know the exactly composition of the oil (Belletti et al., 2008).

2.2 Characterization of citron essential oil (EO) by GC/MS-SPME technique

Citron EO was placed into a 10 mL vial and sealed through a PTFE/silicon septa. Three different samples were prepared. The samples were conditioned 30 min at 25°C. An SPME fiber covered by 50 mmdivinylbenzene-carboxen-poly(dimethylsiloxane)-(DVB/CARBOXEN/PDMS StableFlex) (Supelco, Steiheim, Germany) was exposed to each sample at room temperature (25 °C) for 20 min, and finally, the adsorbed molecules were desorbed in the GC for 10 min. For peak detection, an Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett–Packard, Genevra, Switzerland) and a Varian (50 m×320 µm×1.2 µm) fused silica capillary column were used.
The temperature program was 50 °C for 0 min, then heated to 230 °C at 3 °C/min, this temperature was maintained for 1 min. Injector, interface, and ion source temperatures were 200, 200, and 230 °C, respectively. Injections were performed with a split ratio of 30:1 and helium as carrier gas (1 mL/min). Compounds were identified by the use of the Agilent Hewlett–Packard NIST 98 mass spectral database.

2.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination.

For the determination of MIC values, 150 µl of BHI broth inoculated at three different levels (2, 4 or 6 log cfu/mL) of the tested pathogens (*Listeria monocytogenes* Scott A, *Salmonella Enteritidis* E5, *Escherichia coli* 555, *S. aureus* F1, *Bacillus cereus* SV90) were added to 200 µl microtiter wells (Corning Incorporated, NY, USA). 15 µL of the tested EO or natural antimicrobials, properly diluted in Brain Heart Infusion (BHI, Oxoid Ltd., Basingstoke,

United Kingdom) broth and conveyed through 96% ethanol (VWR international, PROLABO, France) were added to each well in order to obtain the required concentration of each compound in the final volume (200 µL), and with a constant amount of ethanol (1% v/v in wells). Microtiter plates were incubated at 37 °C and checked after 24 and 48 h. The MBC were determined by spotting 10 µL of each well after 48 h, onto BHI agar plates.

MIC was defined as the lowest concentration of the compound preventing visible growth of the inoculated cells after 24 h (MIC 24 h) or 48 h (MIC 48 h). The MBC was defined as the lowest concentration of the compound that caused the death of the inoculated cells, corresponding to no growth after 24 h of incubation at 37 °C of a 10 µl spot plated onto BHI agar.

2.2 Preparation of sliced apple products

Apples (*Golden delicious* sp.) were purchased at a local retailer in the same day of the analyses. The protocol used in the first experimental part is reported in Figure 3.1. Eight different treatment solutions were prepared with running water. Two contained only citral or hexanal (250 ppm); the others were mixtures of citral/hexanal (125/125 ppm), citral/2-*(E)*-hexenal (125/125 ppm), hexanal/2-*(E)*-hexenal (125/125 ppm), citral/citron EO (125/125 ppm), citron EO/carvacrol (200/50 ppm). Natural antimicrobials were conveyed through 1% (v/v) of ethanol. Control apple slices were subjected to the dipping treatment without the supplementation of natural antimicrobials. The packaged apples were stored at 6 °C and analysed until the end of shelf-life.

In the second experimental phase, an optimization of the washing protocol was carried out. Apples (*Golden delicious* sp.) were purchased at a local retailer in the same day of the analyses. They were washed with running water at 13°C for 2 min and then dried with blotting paper. After that, apples were peeled and sliced into cubes of roughly 1.5 cm^3. The citron EO and natural antimicrobials, alone or in mixture, were added to apples with the dipping (1% citric acid + 0.5% ascorbic acid). The same eight different treatment solutions of the first experimental phase were employed, at a temperature of 13°C and prepared with running water. Also in this case natural antimicrobials were conveyed through 1% (v/v) of ethanol. Control apple slices were subjected to dipping treatment without the supplementation of natural antimicrobials. The complete protocol used in this experimental phase is reported in Figure 3.2. After the treatment, apples were dried with paper and packaged in active modified atmosphere with 7% O_2 and 0% CO_2. Both control packaged in modified atmosphere and in ordinary atmosphere were considered. Apples were stored at 6 °C until the end of shelf-life.

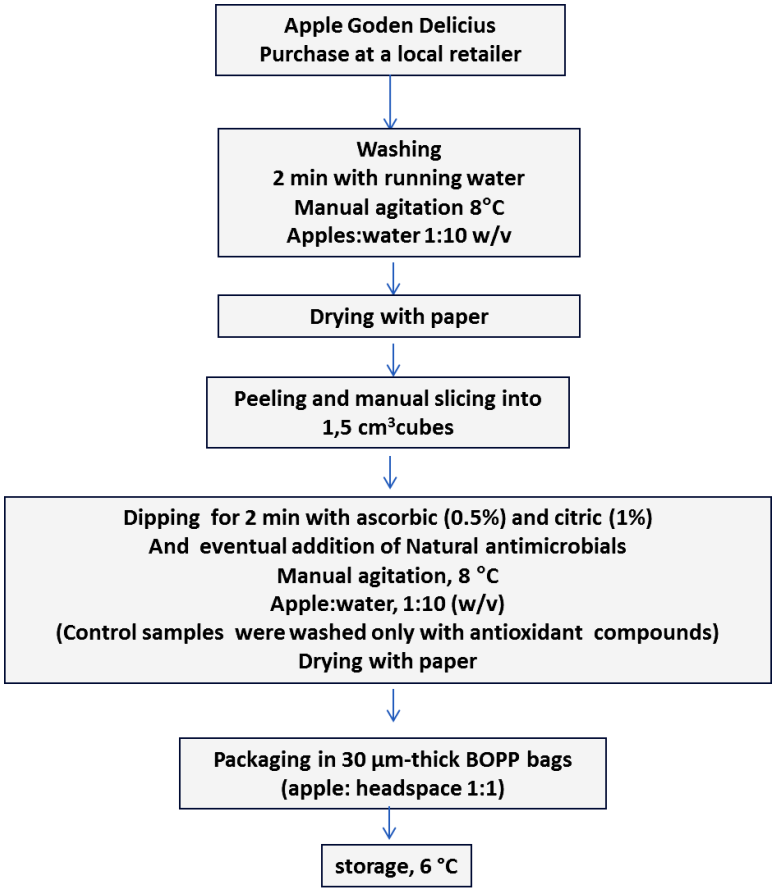


Figure 3.1- *Working protocol employed to prepare sliced apples; the addition of natural antimicrobials was performed during the dipping step, samples dipped with only citric and ascorbic acid represented the controls*

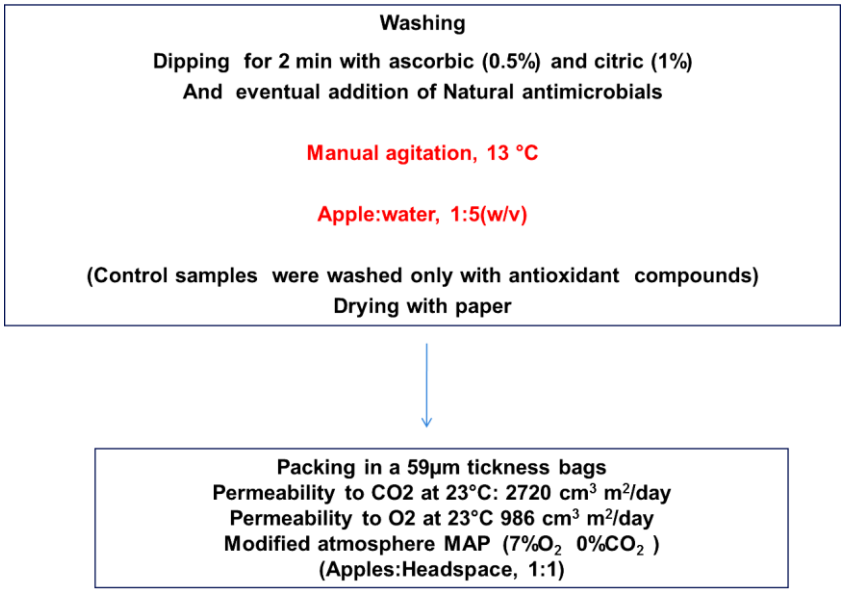


Figure 3.2- *Modifications implemented in the working protocol used in the second experimental phase; in the flow chart are reported only the modifications employed, the remaining steps were the same of the protocol reported in Figure 3.1.*

2.3 Microbiological analyses

In both the considered experimental phase, during storage, the evolution over time of LAB and yeasts was evaluated by plate counting respectively on de Man Rogosa and Sharpe Agar (MRS, Oxoid Ltd. Basingstoke, United Kingdom) with added cycloheximide (Sigma-Aldrich) and Sabouraud Dextrose Agar (SAB, Oxoid Ltd.), added to chloramphenicol (Sigma-Aldrich) respectively. After homogenization, samples were serially diluted in physiological solution (10 g of sample diluted into 90 mL of physiological water (0.9% (w/v) NaCl). For the detection of the natural occurring Listeria monocytogenes, the method suggested by McClain and Lee (1988) was followed, whereas the occurrence of Salmonella *spp*. was investigated according to the method proposed by Andrews and Hammack (1998). *Escherichia coli* was investigated on violet red bile agar (Oxoid) added to 4-methylumbelliferyl-β-D-glucuronide (Oxoid), incubating the plates at 37°C for 24 h. The potential *Staphylococcus aureus* was enumerated on Baird-Parker media (Oxoid) with added egg yolk tellurite emulsion (Oxoid) after 24 h at 37°C. In the first experimental phase, microbiological analyses were performed immediately after treatments and after 2, 3, 7, 10, 14 and 21 days of storage. In the second experimental phase, after the optimization of the washing process and modified atmosphere packaging, the analyses were performed immediately after treatments and after 3, 7, 10, 12, 14, 17, 21, 24, 28, 31 and 35 days of storage.

2.4 Volatile molecule profiles and electronic nose analyses

Apple packages were used for headspace volatile compound analysis by GC/MS-SPME technique. In the first experimental phase, for each treatment condition the samples were analysed immediately after the treatments and after 3 and 10 days of storage, while in the second experimental phase each sample was analysed immediately after the treatments and after 10 and 20 days of storage. The samples were conditioned 30 min at 37 °C; after that, for fibre and gas-chromatographic conditions, the method reported by Patrignani et al. (2013) was used. Compounds were identified by the use of the Agilent Hewlett–Packard NIST 98 mass spectral database.

Electronic nose (EN) analyses were performed on the headspace of 40 mL vials, sealed by a lid with a PTFE/silicon septa, containing 5 g of apples. EN evaluations, in the first experimental phase, were carried out immediately after the treatments and after 3 and 10 days of storage, while in the second phase after the optimization of the washing step, EN evaluations were performed immediately after treatments and after 10 and 20 days of storage.

Sample vials were conditioned before the analysis for 30 min at 37 °C. Determinations were performed with a commercial portable electric nose PEN2 (Airsense Analytics, Milano, Italy) composed of an array of 10 temperature-moderated metal-oxide sensors (MOS), a sampling system, a data acquisition system, and a data processing system. Each sensor is sensible to different kind of volatile molecules (Table 3.1). For each sample, three analysis repetitions were performed. During the analysis the response of the sensors were monitored at 1 sec intervals for an overall time of 95 sec at a flow rate of 400 mL/min. Results were obtained by comparing the signal of every sensor at every time with the minimum signal level. The signal evaluation was done following the method reported by Sado Kamden et al. (2007), in order to found out which were the most indicative signals for the evaluation of the differences among the samples.

Table 3.1- *Sensors of electronic nose, and compounds classes detected by each sensor.*

	Sensor	Compounds
s1	W1C	Aromatic
s2	W5S	generic
s3	W3C	Aromatic
s4	W6S	Hydrogenated
s5	W5C	Aromatic-Aliphatic
s6	W1S	Hydrocarbons , N_2, NH_3, SO_2, NO_2 and CO_2
s7	W1W	Sulphur organic
s8	W2S	Alcohols
s9	W2W	Sulphur chlorides
s10	W3S	Hydrocarbons-Aliphatic

2.5 Physical analyses: colour and texture

Surface colour was measured using a colour-spectrophotometer mod. Colorflex (Hunterlab, USA). Colour was measured using the CIELab scale and Illuminant D65. The instrument was calibrated with a white tile (L*98.03, a* - 0.23, b* 2,05) before the measurements. Results were expressed as L* (luminosity) and a* (red index); numerical values of a* and b* were converted into hue angle (h°), according to the following equations (McGuire, 1992):

$$h° = \frac{\tan^{-1}(b*/a*)}{2\pi} \cdot 360$$

At each storage time, 21 readings were obtained for each sample from the seven packages, measuring three slices for each package.

Firmness measurement was performed at room temperature (20±2 °C), about 1 h after removing samples from 4 °C. Penetration tests were carried out by measuring the maximum

force registered during penetration of a 6 mm diameter stainless steel cylinder for 6 mm into the apple slice tissue, using a Texture analyser mod. HD500 (Stable Micro Systems, Surrey, UK) equipped with a 5 kg load cell. Test speed was 0.5 mm/s and data were expressed in kg. At each storage time, 21 tests were performed for each sample from the seven packages, measuring three slices for each package.

2.6 Statistical analysis

For each sample, the microbiological, volatile and nose data were the mean of three different samples of three independent experiments.

The yeast cell load data were modelled according to the Gompertz equation as modified by Zwietering et al. (1990). The spoilage threshold (6 log cfu/g) can be defined as the sum of k, corresponding to the initial level of yeast after sample packaging, and A, corresponding to the maximum cellular density increase with respect to initial cell load (k).

The quantitative data obtained from metabolites determinations were used to build up a single matrix, which was submitted to a two-way hierarchical clustering analysis. A heat map, visualizing metabolite concentration was then obtained in which values are represented by cells coloured according to the Z-scores, where Z =(observed value - mean)/standard deviation (Ferrara et al., 2008; Serrazanetti, et al., 2011).

Principal component analysis (PCA) was performed using Statistica software (version 8.0; StatSoft., Tulsa, Oklahoma, USA) to obtain a visual overview of electronic nose analyses.

Regard microbiological, colour and texture data, statistical analysis was performed using Statistica software (version 8.0; StatSoft., Tulsa, Oklahoma, USA). Means were compared using one way-ANOVA followed by LSD test at $p<0.05$ level in order to monitor changes over time as well as differences between treatments.

3. Results and Discussion of the first experimental phase: effects of natural antimicrobials on minimally processed apples packaged in ordinary atmosphere

3.1 Citron essential oil composition

The composition of citron essential oil used in this study was determined throughout GC/MS-SPME analyses. This technique was chosen because a preliminary condition for the antimicrobial effects of EO is the contact between the antimicrobial molecule and the target cells (Gardini et al., 1997; Belletti et al., 2004). The contact is favoured if the molecules are in their most hydrophobic state, i.e., in their vapor phase, because this favour their

solubilisation in the cell membranes. Although the headspace composition does not correspond to the whole EO composition, its knowledge is fundamental because it gives a measure of the volatile molecules of the oil (Belletti et al., 2004). In addition, the obtaining of a volatile profile fingerprinting is fundamental to standardize the composition in terms of the most effective molecules and, consequently, to standardize antimicrobial activity of the essential oils. It is well known that the EO GC/MS-SPME profile is affected by the composition in its turn dependent on plant variety and origin, extraction modality, agronomic practices, (Nannapaneni et al. 2009). Table 3.2 shows the total area of the GC peaks and the percentage (on the basis of the relative peak area) of the identified molecules in the headspace, as well as the cumulative percentages of the classes of compounds (monoterpenes, sesquiterpenes, oxygenated monoterpenes, aliphatic alcohols, aliphatic aldehydes, esters, and ketones). This essential oil was characterized by the presence of high percentage monoterpenes such as of limonene (35.75%), β-pinene (11,03%), γ-terpinene (20.14%), p-cymene (11.46%) and α-pinene (3.06%) and oxygenated monoterpenes such as linalool (1.21%), neral (2.69%) and geranial (3.94%). It is well known that many of these terpenes can have antimicrobial activities (Dorman and Deans 2000; Belletti et al., 2004). In fact, such molecules can interact with some cellular structures causing the inhibition of cell growth or cell death. The data obtained were in accordance with those of Belletti et al. (2004, 2008). On the other hand, it is well known that that the volatile fraction of citrus essential oils is a mixture of monoterpenes, sesquiterpenes and their oxygenated derivatives including alhdeydes (citral) ketones, acids, alcohols and esters (Borgmann et al., 2004; Flamini et al., 2007).

Table 3.2 *Citron essential oil characterization*

Molecules	**Total peak area**	**Area %**
α-Pinene	38112402	3.06
Camphene	757129	0.06
β-Pinene	137322834	11.03
β-Phellandrene	26026228	2.09
β-Myrcene	17285238	1.39
α-Phellandrene	125001	0.01
α-Terpinene	689537	0.06

Limonene	445232682	35.75
β-Thujene	4555454	0.37
β-trans-Ocimene	648384	0.05
γ-Terpinene	250747986	20.14
3,8-p-Menthadiene	725860	0.06
p-Cymene	142735387	11.46
Terpinolene	3488583	0.28
(+)-(E)-Limonene oxide	3018628	0.24
Linalool	15086745	1.21
cis-β-Terpineol	166768	0.01
Linalyl propionate	58324455	4.68
α-Bergamotene	3107911	0.25
Caryophyllene	2233647	0.18
Citronellyl butyrate	305439	0.02
Neral	33540693	2.69
Terpineolo	1702439	0.14
Nerol acetate	3627589	0.29
β-Bisabolene	1823835	0.15
Geranial	49016281	3.94
Geraniale acetate	2791492	0.22

3.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) evaluation.

The MICs and the MBCs of citral, hexanal, 2-(E)-hexenal, citron EO and carvacrol against *Listeria monocytogenes* Scott A, *Escherichia coli* 555, *Salmonella enteritidis* E5, *Staphylococcus aureus* F1, and *Bacillus cereus* SV 90 were assessed after incubation at 37°C, with three levels of the target microorganisms (Table 3.3).

Table 3.3- *Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of citral, 2-(E)-hexenal, hexanal, citron oil and carvacrol against* L. monocytogenes, E. coli, S. Enteritidis, B. cereus *and* S. aureus *in relation to the inoculum level*

Listeria monocytogenes									
Cells concentration	6 log cfu/mL	6 log cfu/mL	6 log cfu/mL	4 log cfu/mL	4 log cfu/mL	4 log cfu/mL	2 log cfu/mL	2 log cfu/mL	2 log cfu/mL
MIC/MBC	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)
Citral	350	475	500	325	425	425	250	300	325
2-(*E*)-hexenal	1250	1400	>1500	1250	1400	>1500	850	1300	1400
Hexanal	>1500	>1500	>1500	1500	>1500	>1500	1350	>1500	>1500
Citron oil	>1200	>1200	>1200	>1000	>1000	>1000	500	>1000	>1000
Carvacrol	175	200	225	150	175	200	100	175	200

Escherichia coli									
Cells concentration	6 log cfu/mL	6 log cfu/mL	6 log cfu/mL	4 log cfu/mL	4 log cfu/mL	4 log cfu/mL	2 log cfu/mL	2 log cfu/mL	2 log cfu/mL
MIC/MBC	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)
Citral	>1500	>1500	>1500	>1500	>1500	>1500	>1500	>1500	>1500
2-(*E*)-hexenal	525	600	650	500	575	575	500	525	525
Hexanal	>1500	>1500	>1500	1050	>1500	>1500	700	1200	>1200
Citron oil	>1200	>1200	>1200	>1200	>1200	>1200	>1000	>1000	>1000
Carvacrol	200	200	225	200	200	200	200	200	200

Salmonella Enteritidis									
Cells concentration	6 log cfu/mL	6 log cfu/mL	6 log cfu/mL	4 log cfu/mL	4 log cfu/mL	4 log cfu/mL	2 log cfu/mL	2 log cfu/mL	2 log cfu/mL
MIC/MBC	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)
Citral	>1500	>1500	>1500	>1500	>1500	>1500	>1500	>1500	>1500
2-(*E*)-hexenal	>1500	>1500	>1500	>1500	>1500	>1500	800	1300	1500
Hexanal	>1500	>1500	>1500	1050	>1500	>1500	>1500	>1500	>1500
Citron oil	>1200	>1200	>1200	>1200	>1200	>1200	>1000	>1000	>1000
Carvacrol	200	200	250	175	175	200	175	175	200

Bacillus cereus									
Cells concentration	6 log cfu/mL	6 log cfu/mL	6 log cfu/mL	4 log cfu/mL	4 log cfu/mL	4 log cfu/mL	2 log cfu/mL	2 log cfu/mL	2 log cfu/mL
MIC/MBC	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)
Citral	300	>650	>650	300	350	350	300	300	300
2-(*E*)-hexenal	1200	1350	>1400	1200	1350	>1400	800	1250	1300
Hexanal	>1500	>1500	>1500	1500	>1500	>1500	1350	>1500	>1500
Citron oil	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000
Carvacrol	175	175	200	150	175	175	150	150	150

Staphylococcus aureus									
Cells concentration	6 log cfu/mL	6 log cfu/mL	6 log cfu/mL	4 log cfu/mL	4 log cfu/mL	4 log cfu/mL	2 log cfu/mL	2 log cfu/mL	2 log cfu/mL
MIC/MBC	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC (ppm)
Citral	500	550	550	450	500	500	250	250	250
2-(*E*)-hexenal	1200	1400	>1500	1300	1400	>1500	900	1300	1400
Hexanal	>1500	>1500	>1500	1500	>1500	>1500	1400	>1500	>1500
Citron oil	>1200	>1200	>1200	>1000	>1000	>1000	800	>1000	>1000
Carvacrol	225	275	275	200	250	250	150	200	200

Pronounced differences in the MICs and MBCs were observed in relation to the substances, the species and the inoculum level considered. Citron oil exhibited the lowest antimicrobial activity with respect to the other molecules studied. In fact, it showed MIC and MBC values higher than 1000 ppm independently from the species and the inoculation level, with the exception of the MIC values against *L. monocytogenes* and *S. aureus* inoculated at levels of 10^2 cfu/mL and recorded after 24 h. In these conditions the MIC values were respectively of 500 and 800 ppm. Belletti et al. (2008) showed a reduced effects of this oil used at a concentration ranging between 300 and 600 ppm on Gram-negative species, such as *S. enteritidis* and *E. coli* deliberately inoculated in salad fruit in syrup, but a marked inhibition toward the Gram-positive pathogen *L. monocytogenes*.

Citral showed a low antimicrobial effectiveness against the Gram negative species considered, being the MIC values always higher than 1500 ppm, independently from the inoculation level. On the contrary, Gram-positive species had MIC values ranging between 250 and 700 ppm as a function of the initial inoculation level. The effect of inoculation level is particularly evident for *B. cereus* and *S. aureus*, whose MICs and MBCs decreased from 700 and 550 ppm to 250 and 300 ppm in cultures of 10^6 and 10^2 cfu/mL respectively. The needed concentrations to obtain MICs and MBCs at high cell levels were in these cases about doubled compared with those needed to reach the same result at the lower cell level. The influence of the initial inoculum on MICs and MBCs was evident also in the presence of hexanal and 2-(E)-hexenal. These molecules showed the highest efficacy against *E. coli* and in minor extent against *S. enteritidis* while were quite ineffective against the other target microorganisms. This different response to essential oil or their components between Gram-positive and Gram-negative bacteria is already reported in the literature. Gram-negative bacteria are generally more resistant to many compounds due to the outer membrane, which acts as an efficient permeability barrier against macromolecules and hydrophobic substances (Helander et al., 1997), as well as to the high content in cyclopropane fatty acids of the inner membrane (Chang & Cronan, 1999). If enough hydrophilic, low molecular mass molecules seem to be more efficient in passing through these barriers and they may have access, throughout porin proteins, to the deeper parts of Gram-negative bacteria without any alteration to the permeability of the outer membrane (Lanciotti et al.,2003; Helander et al., 1997).

On the contrary, carvacrol showed the highest efficacy both against considered Gram-positive and Gram-negative bacteria. Actually it had MIC values ranging between 175 and 200 ppm for *S. Enteritidis* and *E. coli* and 175-275 ppm for *L. monocytogenes*, *B. cereus* and *S. aureus*. The results showed values of MIC and MBC relatively high due to the optimal microbial

growth conditions, and to the high inoculation levels used and not compatible with the product sensorial properties. However, for apple slices treatment, concentrations significantly lower with respect to MIC values were used, taking into consideration the real contamination level (pathogens were absent in 25 g of products) of the product with pathogenic species, the more stringent conditions of the real system with respect to the model one used for MIC determination, and the product sensorial acceptance.

3.3 Effects of citron EO and natural antimicrobials on the shelf-life of apple slices

During refrigerated storage, the growth of lactic acid bacteria (LAB) and yeasts was evaluated because they represent the main microbial groups involved in fresh-cut fruits spoilage (Patrignani et al., 2013). In fact for these commodities the pH value, the sugar content and the C/N ratio favour the growth of LAB, yeast and moulds. However, the increased respiration rate of the fresh-cut fruits tissue caused by endogenous wounding response rapidly consume a great part of the oxygen present in the packages, creating an environment not suitable for the growth of aerobic moulds (Belletti et al., 2008). The Gompertz parameters recorded for yeasts are shown in Table 3.4. Immediately after apple packaging, the yeast levels were under the detection limit, independently of the presence of the test compounds; for this reason K values were not reported. Yeasts showed a significant higher growth rate in the control sample reaching cell loads of 6.0 log cfu/g after about 12 days. This level of cell load can be considered as an acceptability threshold, because it corresponds to the beginning of a perceivable spoilage (Patrignani et al., 2009, 2013); higher cell concentrations can result in a visible blowing of the package. All the tested molecules, alone or in combination, significantly delayed the yeast growth. However, the most effective substances were hexanal when used at 250 ppm, citral and the mixture hexanal/2-(E)-hexenal, both used at 125 ppm. Hexanal delayed the reaching of the spoilage threshold of about 10 days with respect to the control (Table 3.4), while the yeast cell loads in samples washed with citral and the mixture of the two aldehydes never reached that limit. A similar trend has been shown by Lactic Acid Bacteria (LAB) although their growth was delayed with respect to yeasts (data not shown). In fact, after 14 days of storage at 6 °C LAB reached levels of 5.0 log cfu/g only in the control samples, while in the other samples the LAB cell loads ranged between 1.0 and 2.7 log cfu/g. The efficacy of the tested antimicrobials to prolong the shelf-life of minimally processed fruits is well documented (Patrignani et al., 2008; Belletti et al., 2008; Lanciotti et al., 2004). Belletti et al. (2008) showed that citron essential oil doubled the time needed for the degradative microflora to reach concentrations able to produce a perceivable spoilage during

storage at 9°C. A more pronounced delay of spoilage agents was obtained with citral which however evidenced some citotoxic effects on fruit tissue. Also the addition of hexanal and 2-(E)-hexenal in storage atmosphere of fresh-cut apple slices resulted in a positive effect on the product shelf-life because of the antimicrobial activity of these substances against naturally occurring spoilage species, also when deliberately inoculated at levels of 3 log cfu/g.
Moreover these molecules determined the enhancement of the aromatic properties, as well as the improvement of the original colour retention of the packed products (Lanciotti et al., 1999; Corbo et al., 2000).

Table 3.4- *Gompertz parameters of yeast cell load dynamic equations in apples, stored at 6 °C, in relation to the applied dipping.*

Samples	A	μ_{max}	λ	R	Time (days)
Control [a]	7.55	0.97	5.04	0.99	12.12
Citron oil/citral [b]	6.54	0.96	6.87	0.99	15.79
Hexanal/2-*(E)*- hexenal [c]	5.4	0.43	3.4	0.99	- [i]
Citral [d]	5.17	0.72	7	0.99	-
Citral/Hexanal [e]	8.3	0.68	7.53	0.99	17.17
Hexanal [f]	6.97	0.51	7.94	0.99	22.55
Citral/2-*(E)*- hexenal [g]	7.62	0.51	8.56	0.99	21.97
Citron oil/Carvacrol [h]	7.74	1.14	10.9	0.99	16.83

[a] Control was washed only with dipping solution (1% citric acid + 0.5% ascorbic acid)
[b] Concentration employed 125 mg L^{-1} each.
[c] Concentration employed 125 mg L^{-1} each.
[d] Concentration employed 250 mg L^{-1}.
[e] Concentration employed 125 mg L^{-1} each.
[f] Concentration employed 250 mg L^{-1}.
[g] Concentration employed 125 mg L^{-1} each.
[h] Concentration employed 200 mg L^{-1} citron oil and 50 mg L^{-1} carvarcol.
Time: the time (days) necessary to reach the cell load of 6.0 log cfu mL^{-1} chosen as spoilage threshold.
A: maximum cellular density increase with respect to the initial cell load (k) (log $cfug^{-1}$).
µmax: maximum specific growth rate ((log CFU g^{-1}) $days^{-1}$).
λ: latency time (lag time) (days).
R: correlation coefficient.
- unable to reach the spoilage value

3.4 Effects citron EO and natural antimicrobials on apple volatile molecules and electronic nose profiles

In order to evaluate the effect of the compounds taken into consideration on the volatile molecule profiles as a function of storage time, the samples were analysed by means of GC/MS-SPME and electronic nose. Although only the most significant molecules were reported in Table 3.5, the GC/MS-SPME allowed the identification of 45 molecules belonging

to different chemical classes and provided specific volatile fingerprinting in relation to the antimicrobial agent used and to the storage time advancement. The supplemented antimicrobials and their detoxification greatly affected the volatile profile composition (Table 3.5). Actually, neral, geranial, nerol and geraniol characterized the samples supplemented with citral and citral in combination with citron oil. The latter sample showed the presence of high levels of limonene and terpinene, linalyl butyrate, β-mircene. The samples treated with citral was characterized also by citronellyl acetate and β-citronellol while hexanal, 2-(E)-hexenal, hexanol, and acetic acid hexyl esters were detected in the samples supplemented with hexanal and 2-(E)-hexenal. This sample showed a remarkable abundance (in term of peak area) of 2-hexen-1ol-acetate. Hexanal and 2-(E)-hexenal showed higher levels in the control samples with respect to the samples treated with the same molecules indicating that their supplementation fastened the detoxification mechanisms adopted by tissues and naturally occurring microorganisms (Table 3.5). On the other hand, it has been demonstrated also for other aldehydes such as neral and geranial their reduction into nerol and geraniol as the first step of citral biotransformation by penicilli into lower toxicity compounds (Esmaeili & Tavassoli, 2010). Patrignani et al. (2013) showed the increase during storage of such alcohols in fruit juices supplemented with citral over the storage; the authors attributed this phenomenon to the detoxifying mechanisms of spoilage yeasts. A similar detoxifying mechanism, i.e. reduction to the respective alcohols, was shown for six carbon aliphatic aldehydes (Patrignani et al., 2008). The samples supplemented with citron EO showed the presence of high amounts of monoterpenes and oxygenated monoterpenes, whose presence is well documented in citron EO. Carvacrol and thymol methyl ether were the main volatile molecules detected in the GC/MS-profiles of the samples added with carvacrol.

Table 3.5- *Volatile aroma compounds (expressed as Area 10^{-5}) detected in apples treated with different dipping solutions during the storage time at 6°C.*

	Control [a]			Citron oil/Citral [b]			Hexanal/2-*(E)*-hexenal [c]			Citral [d]			Citral/Hexanal [e]			Hexanal [f]			Citral/2-*(E)*-hexenal [g]			Citron oil/Carvacrol [h]		
Compounds	T0	T3	T10	T0	T3	T10	T0	T3	T10	T0	T3	T10	T0	T3	T10	T0	T3	T10	T0	T3	T10	T0	T3	T10
Ethyl acetate	0.0	4.3	2.5	2.5	7.5	3.6	0.8	12.7	34.3	6.4	13.5	14.6	1.2	10.3	11.0	1.2	19.3	33.9	0.6	15.2	14.6	3.7	23.3	38.7
Acetic acid, isobutyl ester	0.6	1.2	5.6	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.2	0.6	0.0	0.0	0.5	0.0	0.0	0.0
Acetic acid, butyl ester	6.9	10.9	1.8	6.0	4.7	0.0	0.8	4.4	7.9	12.9	5.2	7.6	1.3	5.5	6.0	6.1	13.6	9.3	5.2	11.5	11.6	0.8	2.5	5.1
1-butanol, 2-methyl-acetate	11.0	11.5	12.3	5.6	4.0	0.0	1.1	1.4	4.8	23.7	11.8	5.2	4.3	5.5	1.7	15.3	27.5	3.6	5.9	10.0	2.8	2.6	4.1	4.3
Acetic acid, hexyl ester	16.4	42.2	12.6	7.3	22.0	4.2	235.1	460.8	74.0	20.6	57.2	36.8	25.0	167.3	12.2	236.5	431.6	259.3	31.3	171.6	53.5	6.9	26.0	20.3
2-Hexen-1-ol, acetate	0.0	0.0	0.0	0.0	0.0	0.0	46.4	9.5	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.1	3.0	0.2	0.0	0.0	0.0
Butanoic acid methyl esters	0.0	0.0	0.0	0.4	1.0	0.0	0.0	0.1	0.0	3.4	6.8	3.9	5.6	3.8	0.0	5.2	11.1	0.0	0.7	7.9	1.1	0.0	0.0	0.0
Citronellyl acetate	0.0	0.0	0.0	0.3	5.4	1.9	0.0	0.0	0.0	1.4	9.5	11.1	1.5	10.2	0.4	0.0	0.0	0.0	1.2	21.8	4.0	0.0	0.0	0.0
Linalyl butyrate	0.0	0.0	0.0	4.9	5.4	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	28.4	26.6	8.3
Total Esters	**34.9**	**70.0**	**34.7**	**26.9**	**50.0**	**10.1**	**284.2**	**489.0**	**122.8**	**68.5**	**104.1**	**79.2**	**38.9**	**202.6**	**31.6**	**264.3**	**503.3**	**306.6**	**58.0**	**240.9**	**88.2**	**42.4**	**82.5**	**76.6**
Hexane	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.5	0.0	0.0	0.0	0.0	0.3	0.0	6.4	0.0	0.0	0.0	0.0	0.0
β-myrcene	0.0	0.0	0.0	0.4	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.1	3.6	2.0
Limonene	0.4	1.7	1.4	38.6	28.3	2.6	0.0	0.4	0.9	1.0	1.3	0.9	1.7	0.5	0.0	0.7	0.2	0.0	0.5	1.1	0.3	213.0	203.1	124.8
α Terpinene	0.0	0.0	0.0	19.7	19.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	155.5	126.9	72.1
Cymene	0.0	0.0	0.0	13.3	3.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	35.0	66.3	55.2
β-pinene	0.0	0.0	0.0	3.1	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.7	8.0	4.4
thymol methyl ether	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	18.0	11.0
Total Hydrocarbons	**0.4**	**1.7**	**1.4**	**75.0**	**52.2**	**4.0**	**0.0**	**0.4**	**0.9**	**1.0**	**1.3**	**8.4**	**1.7**	**0.5**	**0.0**	**0.7**	**0.5**	**0.0**	**6.9**	**1.1**	**0.3**	**416.4**	**426.0**	**269.4**
Nerol	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	0.0	4.3	0.0	0.0	1.8	0.0	0.0	0.0	0.0	0.0	2.6	0.0	0.0	0.0	0.0	0.0
Geraniol	0.0	0.0	0.0	1.8	0.0	0.0	0.0	0.0	0.0	5.0	0.0	0.0	1.4	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.0
Carvacrol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	27.4	15.5	0.0
β-citronellol	0.0	0.0	0.0	39.6	58.3	33.4	0.0	0.0	0.0	63.7	144.3	81.1	44.8	64.8	4.7	0.0	0.0	0.0	42.4	72.1	21.5	6.5	6.0	0.0
Hexanol	2.5	1.7	0.0	8.4	3.0	3.8	33.1	8.4	2.6	9.5	11.0	11.4	33.3	17.8	2.6	35.2	10.2	7.6	21.0	16.3	5.6	1.5	2.4	1.5
Ethanol	0.6	0.9	0.0	12.0	10.8	6.1	11.0	8.9	8.0	14.0	12.2	10.3	11.5	10.3	4.9	14.1	12.0	8.7	10.8	11.5	7.4	14.5	14.1	9.3
Total Alcohols	**3.1**	**2.6**	**0.0**	**63.7**	**72.0**	**43.3**	**44.1**	**17.2**	**10.6**	**96.5**	**167.5**	**102.9**	**92.8**	**92.9**	**12.2**	**49.3**	**22.2**	**16.3**	**79.0**	**99.9**	**34.5**	**49.9**	**38.0**	**10.8**
Hexanal	24.6	35.6	24.0	18.2	55.5	38.5	3.9	2.7	16.5	9.9	27.7	20.0	31.5	26.1	28.8	14.7	11.3	10.4	16.3	20.2	30.2	5.1	6.3	12.5
Neral	0.0	0.0	0.0	2.9	0.0	0.0	0.0	0.0	0.0	48.7	1.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0	8.1	0.0	0.0	0.0	0.0	0.0
Geranial	0.0	0.0	0.0	3.7	0.5	0.0	0.0	0.0	0.0	48.4	1.9	0.0	3.3	0.0	0.0	0.0	0.0	0.0	8.2	0.0	0.0	0.0	0.0	0.0
2-(E)-hexenal	14.6	28.2	50.0	13.3	29.3	31.6	7.2	5.5	29.8	7.2	19.3	18.4	24.7	12.4	11.1	0.0	7.2	18.2	18.8	16.8	23.3	6.9	10.4	18.8
Total Aldehydes	**39.2**	**63.7**	**73.9**	**38.0**	**85.3**	**70.0**	**11.1**	**8.2**	**46.3**	**114.1**	**49.9**	**38.4**	**61.5**	**38.5**	**39.9**	**14.7**	**18.5**	**28.5**	**51.4**	**37.0**	**53.5**	**12.0**	**16.7**	**31.2**
Total metabolites	**77.5**	**138.1**	**110.1**	**203.6**	**259.6**	**127.4**	**339.3**	**514.8**	**180.6**	**280.1**	**322.9**	**228.9**	**194.9**	**334.5**	**83.6**	**329.0**	**544.6**	**351.4**	**195.3**	**378.9**	**176.5**	**520.6**	**563.1**	**388.1**

Legend:
[a] Control was washed only with dipping solution (1% citric acid + 0.5% ascorbic acid)
[b] Concentration employed 125 ppm each.
[c] Concentration employed 125 ppm each.
[d] Concentration employed 250 ppm
[e] Concentration employed 125 ppm each.
[f] Concentration employed 250 ppm.
[g] Concentration employed 125 ppm each.
[h] Concentration employed 200 ppm citron oil and 50 ppm carvarcol.

A multivariate analysis using a heat map was performed in order to identify the molecules able to significantly contribute to the statistical discrimination among the samples, and five small clusters were obtained. The heat map underlined the role of the EO or the natural antimicrobials in grouping the samples (Figure 3.3). In particular, the sample submitted to the treatment with citron EO/carvacrol and stored up to 5 days grouped together (Cluster 2) and α-terpinene, limonene and *p*-cymene contributed to the grouping. Hexanal clearly contributed to the formation of cluster 3 that grouped the samples added with hexanal immediately after packaging and after 10 days of storage; the sample supplemented with the mixture hexanal/2-(E)-hexenal immediately after packaging as well as the sample supplemented with citral in mixture with hexanal or 2-(E)-hexenal after 3 days of storage. The samples of this cluster were characterised by the presence of acetic acid hexyl ester. The two samples of cluster 3 supplemented with citral were also characterized by the presence of β-citronellol; the latter molecule contributed to the formation of cluster 5, grouping the samples containing citral or citron EO alone or in mixture. Neral and geranial mainly characterised the samples added with citral analysed immediately after the supplementation. The control samples were distributed in two subclusters of cluster 4 that included samples with citral/2-(E)-hexenal, hexanal and 2-(E)-hexenal and citron/citral after 10 days of storage. This cluster was characterized by the presence of 2-(E)-hexenal, hexanal and ethyl acetate. The samples supplemented with citron/citral and stored for 10 days showed the highest similarity to the controls analysed immediately after the packaging. Cluster 1 comprised the samples supplemented with hexanal and hexanal/2-(E)-hexenal after 3 days of storage, with acetic acid hexyl ester as the unique discriminating molecule. The samples supplemented with citral after 3 days of storage did not group in any cluster but was near to cluster 4 with a similarity percentage of 66.6%; these samples were characterized by the highest abundance of β-citronellol. The storage time did not contribute significantly to the clustering, probably because of the different detoxification rate and patterns of the supplemented substances, in their turns dependent on microbial composition (in terms of species and strains) and growth rate. Moreover the volatile molecule profiles reflected also the metabolisms of apple tissue. In this direction Gutierrez et al. (2009) attributed the increases of same terpenic molecules over

storage of lettuce and carrot supplemented with oregano and thyme to microbial metabolism and to the tissue synthesis throughout mevalonic acid.

The data obtained with electronic nose were subjected to a principal component analysis in order to outline the differences among the samples detected by the 10 sensors of the instrument. All the samples were mapped in the space spanned by the first two principal components PC1 versus PC2. The score and loading plot, reported in Figure 3.4, show the clustering of the samples according mainly to storage time. Three different clusters were evident in the PCA plot. The first cluster grouped the samples analysed immediately after packaging, independently of the presence of natural antimicrobials. The second group accounted for the samples stored for 10 days and samples stored for 3 days supplemented with citral or hexanal, while the third cluster contained all the remaining samples stored for 3 days. Exception was represented by the 3 day-control samples belonged to the first cluster. All the samples, except those of the cluster 3 (containing the samples analysed after 3 days of storage), were not discriminated on the basis of PC1 (57.4% of variance was captured by the first PC), while were grouped in two clusters on the basis of PC2 (26.9% of variance) that captured most of the variation among the three considered storage time. The cluster 3 differed by the other samples on the basis of PC1. In particular, the sensors 8 and 6, detecting alcohols and hydrocarbons, respectively, accounted for this clusterization. Sensor 9, a quite aspecific sensor, characterized the cluster 1 while sensors 1, 3 and 5, more responsive for aromatic compounds, defined the cluster 2.

Sado et al. (2010) in a study aimed to evaluate the sensitiveness of electronic nose to discriminate different chemical classes, showed that response of sensors 9 and 2 have a similar responsiveness while the sensors 1, 3 and 5 had an inverse responsiveness to the analysed substances. This different response could contribute to the sample clustering. The data clearly indicate that the addition of the chosen compounds did not affect significantly the electronic nose profiles. In fact, the clusterization was based mainly on storage time except for the samples added with citral or hexanal stored for 3 days that clusterized with the 10 day-stored samples. Probably in these samples this behaviour can be attributed to the delayed yeast growth, as shown by the Gompertz parameters (Table 3.4). On the other hand, the used concentrations were chosen on the basis of preliminary trials aimed to balance the antimicrobial activity and the sensorial impact of the product.

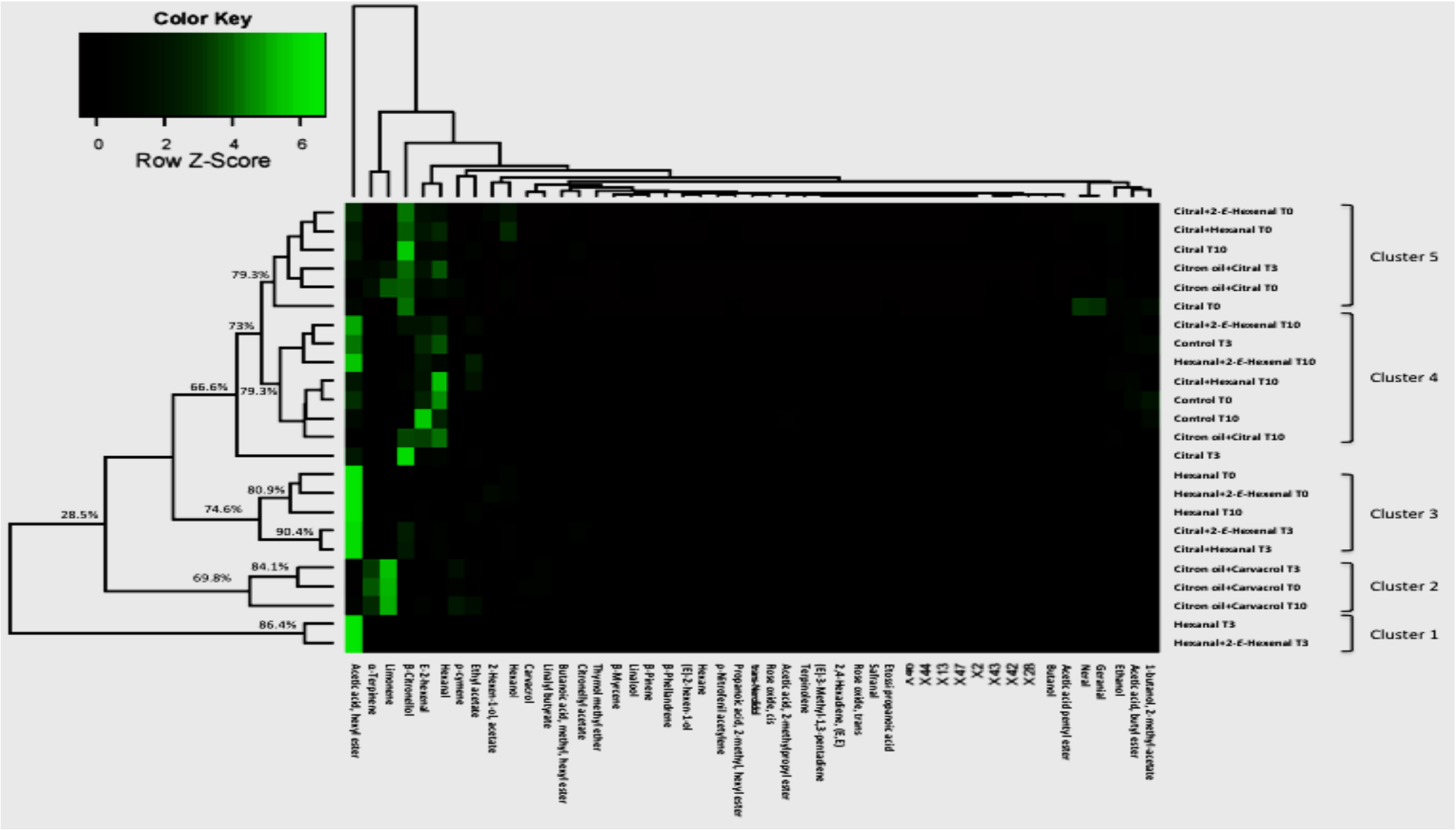


Figure 3.3- *Heat map of correlations between metabolites produced during the storage of apples treated with citron essential oil, natural antimicrobials and their combinations. Each square represents the Spearman's correlation.*

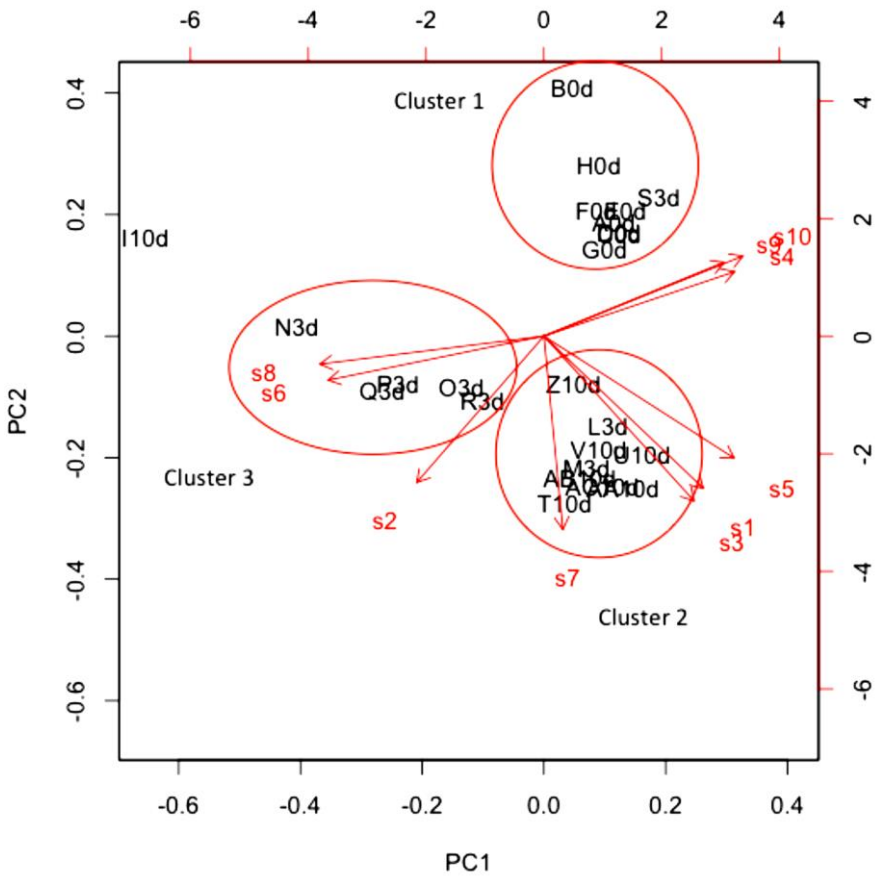


Figure 3.4- *Projection of the scores (different apples treated with citrus essential oil, natural antimicrobials and their combination) and loadings (sensors) on the factor-plane (1x2). PC1 and PC2 explained 57.4% and 26.9 of the total variance respectively.*

Legend:

Cluster 1: A0d (control 0 days), B0d (citral 0 days), C0d (hexanal 0 days), D0d (citral+hexanal 0 days), E0d (citral+citron oil 0 days), F0d (citral+2-(E)-hexenal 0 days), G0d (citron oil+carvacrol 0 days), H0d (hexanal+2-(E)-hexenal 0 days), S3d (control 3 days).

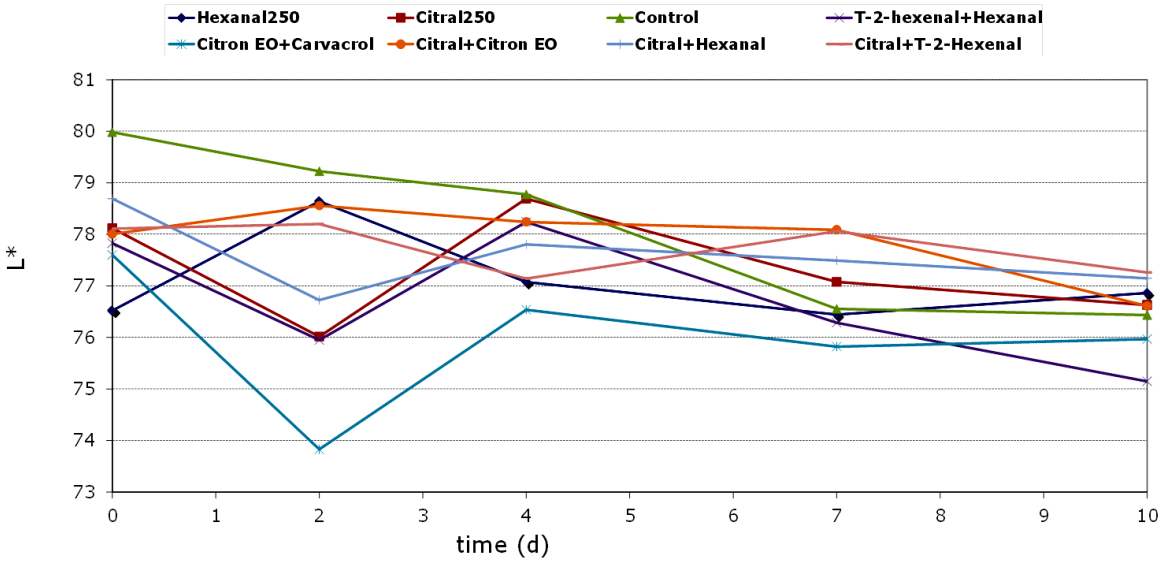
Cluster 2: T10d (citral 10 days), U10d (hexanal 10 days), V0d (citral+hexanal 10 days), Z10d (citral+citron oil 10 days), AA10d (citral+2-(E)-hexenal 10 days), AB10d (citron oil+carvacrol 10 days), AC10d (hexanal+2-(E)-hexenal 10 days), L3d (citral 3 days), M3d (hexanal 3 days).

Cluster 3: N3d (citral+hexanal 3 days), O3d (citral+citron oil 3 days), P3d (citral+2-(E)-hexenal 3 days), Q3d (citron oil+carvacrol 3 days), R3d (hexanal+2-(E)-hexenal 3 days), I10d (control 10 days).

3.5 Effects of Citron EO and natural antimicrobials on colour and texture of fresh-cut apple slices

As reported in Figure 3.5a, dipping treatments with essential oil and natural antimicrobials promoted immediately a modification of the achromatic component of fresh-cut apples colour, corresponding to a decrease of L* in the range of 1.5-3.5 units. According to Fltcher

(1999), the human eyes can recognize ΔL* differences higher than three units; the L* decrease promoted by the treatments investigated is around this value. Until the fourth day of storage, control sample showed the highest L* values, with a progressively decreasing trend until the end of the experiment, as a consequence of enzymatic browning advancement. After four days of storage, among investigated treatments, citral/citron EO, citral/2-(E)-hexenal, citral/hexanal and citral 250 ppm evidenced the highest levels of L*, showing a positive effect on the inhibition of L* decrease. Treatments with hexanal 250 ppm, citral 250 ppm, citral/citron EO and citral/2-(E)-hexenal caused an immediate increase on the red index (a*) of apple slices colour (Figure 3.5b), while hexanal/2-(E)-hexenal, citron EO/carvacrol and citral/hexanal samples showed initial values of a* very similar to control sample. After two days of storage, all treated samples showed significantly higher values of a* compared to the control, reaching similar values at the fourth day of refrigeration. Among investigated treatments, during the second part of storage, citral/hexanal permitted to maintain the lowest a* values on apple slices surface. As far as hue angle (h°) (Figure 5c), treatments with citrus EO/carvacrol and citral/hexanal seemed not to influence this parameter, but after two days of storage control sample showed a h° value very similar to the initial one, while all treated samples evidenced a fast decrease in the first part of the storage period. From the fourth day to the end of the experiment, the decreasing trend of h° was very similar for all the samples investigated excluded sample citral/hexanal, that showed the highest h° values after respectively height and ten days of storage.



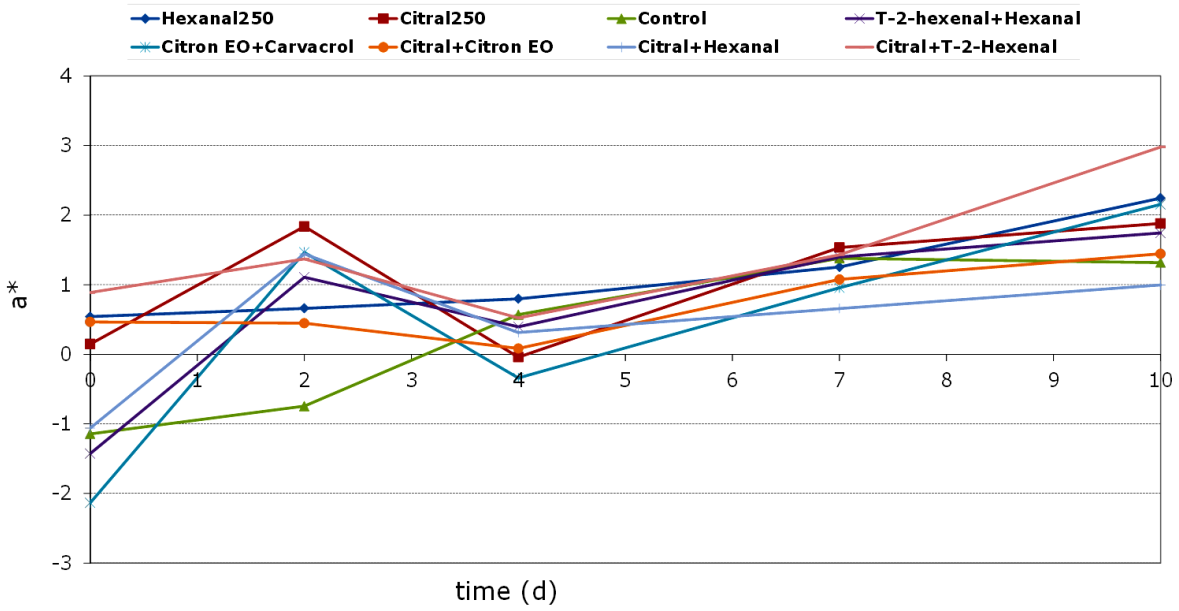


Figure 3.5- *Evolution of luminosity, L*; (3.5a), and red index, a*, (3.5b) of apples treated with citron essential oil, natural antimicrobials and their combinations during storage time. The variability coefficients were ranged between 2 and 5%*

The initial browning caused by citral is in accordance with the data of Belletti et al. (2008) who observed citotoxic effect on apple slices in fruit salad in syrup, when the terpenic molecule was used at concentration of 125 ppm . Probably in our experimental conditions, the negative effects of citral was reduced when in combination with hexanal. On the other hand, the positive effect of hexanal on apple colour maintenance has been already observed (Lanciotti et al., 1999; Corbo et al., 2000). To the conversion of hexanal to hexanol was attributed the key to understanding its effect on browning delay. In fact, the aliphatic alcohols are regarded as inhibitors of polyphenol oxidase (Valero et al., 1990). As a consequence of dipping treatment, only sample citral 250 ppm showed higher value of firmness compared with the control, while the treatment with citral/citron EO caused the maximum softening. Citral 250 ppm effect was lost after just two days of storage (Figure 3.6). As expected, generally during storage, apple slices firmness decreased for all samples investigated in a very similar way. Among them, only the sample hexanal/2-(E)-hexenal maintained higher firmness values compared with the control for all the storage period investigated.

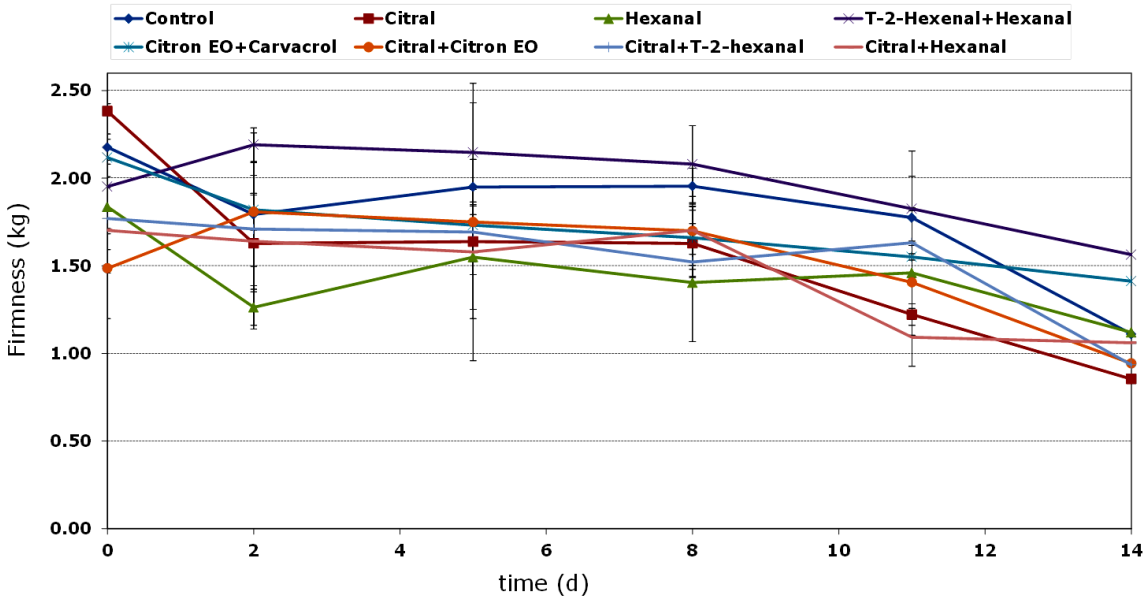


Figure 3.6- *Evolution of firmness (kg.g) of apples treated with citron essential oil, natural antimicrobials and their combinations during storage time. Data are the mean of three different samples.*

4. Results and Discussion of the second experimental phase: effects of natural antimicrobials on minimally processed apples packaged in modified atmosphere

4.1 Microbiological results

During the refrigerated storage (6°C), the growth of yeasts and lactic acid bacteria (LAB) was monitored due to their dominant role in the spoilage of minimally processed fruits (Patrignani et al., 2013). In Figure 3.7 the yeast cell loads recorded over 35 days of storage at 6°C are shown. In all the apples the yeast cell loads remained below 5 log cfu/g within the 35 days of storage independently on the substance or mixture of substances supplemented. After 35 days of storage, the mixtures 2-(*E*)-hexenal/hexanal, hexanal/citral and citron EO/citral were the most effective to delay the yeast growth compared to the other samples ($p < 0.05$). On contrary, citron/carvacrol mixture was the less effective one; from the sixth days of storage, the yeast cell loads of these samples were significantly higher ($p < 0.05$) compared to the other samples at the same time of storage.

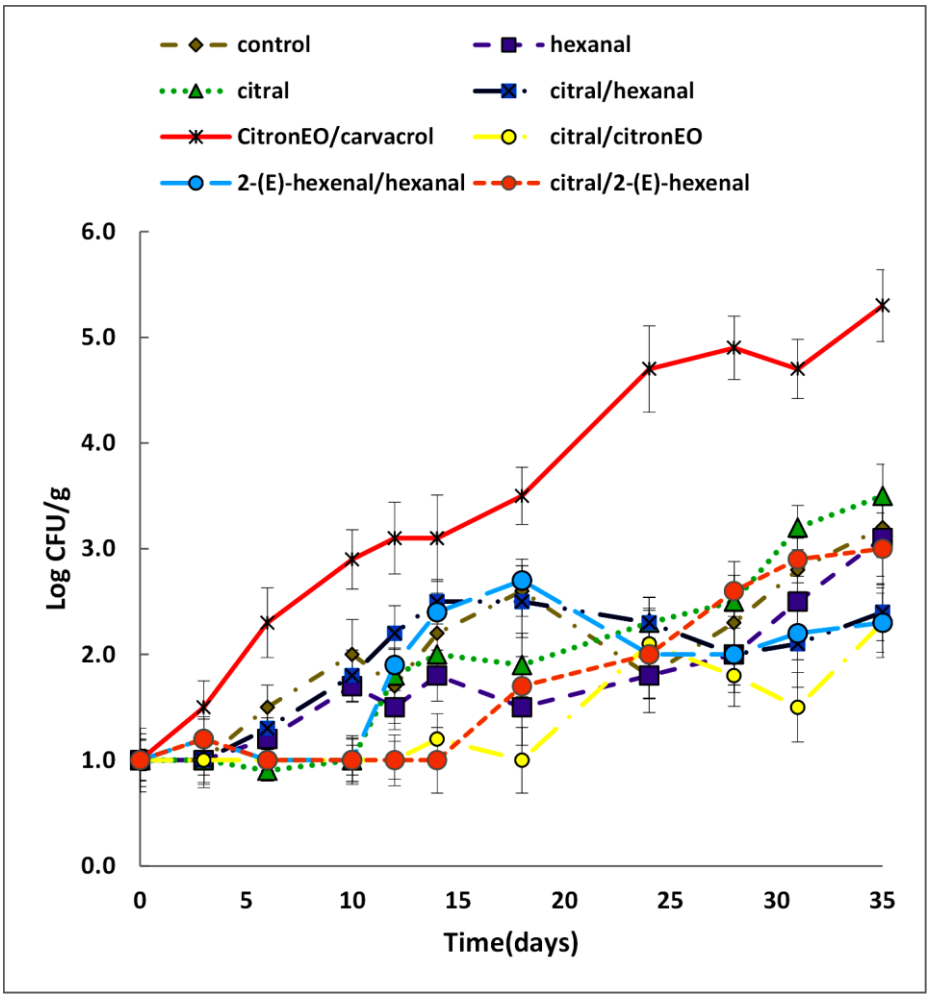


Figure 3.7- *Evolution of the load of yeasts, in sliced apples packaged in MAP, in relation to the addition of citron essential oil and natural antimicrobials during the storage time.*

Lactic Acid Bacteria (LAB) showed a behaviour similar to yeasts but with decreased growth potential. In fact, after 35 days of storage at 6 °C in all the apple samples the LAB cell loads ranged between 1.0 and 3.7 log cfu/g without significant differences. On the other hand, yeasts were favoured with respect to LAB by the high sugar content and the C/N ratio of the system (Patrignani et al., 2013). The efficacy of the antimicrobials used to decrease yeasts and LAB growth in minimally processed fruits is well documented (Lanciotti et al., 1999; Corbo et al., 2000; Lanciotti et al., 2004; Belletti et al., 2008; Patrignani et al., 2008). The use of modified atmosphere further increased the efficacy of these antimicrobials against the main spoilage agents of minimally processed fruits. On the other hand, it is well known that modified atmosphere (MAP) technology is largely used for minimally processed fruits to control both product respiration and ethylene production resulting in product of high organoleptic quality (Sandhya, 2010). Concerning the pathogens, *L. monocytogenes* and *Salmonella* spp. were never found while *E. coli* and *S. aureus* were always under the detection

limits (1 Log cfu/g) also after 35 days of storage, independently on the natural antimicrobial supplementation.

4.2 *Effects EO and natural antimicrobials on apple colour and texture*

Among the investigated dipping treatments, only the mixture citral/citron EO caused an immediate significant ($p<0.05$) decrease of the achromatic component of apple cubes, as reported in Figure 3.8a, while the other treatments showed values very close to the control ($p>0.05$). During storage, all samples underwent a progressive but slight decrease of L* value; in particular, after 20 days control sample showed a value of about 3 units lower compared to the initial one. Sample treated with carvacrol/citron EO did not differ from the control in the first part of the storage period but after 14 days it showed a sharp decrease of L*, while samples treated with hexanal and the combinations citral/2-*(E)*-hexenal, hexanal/2-*(E)*-hexenal, and citral/hexanal showed L* values very close to the initial ones, until the 35th day of storage, as shown also by Figure 3.8a.

Dipping treatment did not seem to have an immediate marked effect on a* value compared to the control sample, as reported in Figure 3.8b. The kinetics of this parameter showed a similar behaviour to the one of L* value. Samples treated with hexanal, citral/2-*(E)*-hexenal, citral/hexanal and hexanal/2-*(E)*-hexenal showed a good retention of this parameter during storage, while citral treatment caused an increase compared to the control. This negative effect has already been reported by Belletti et al. (2008), who observed a cytotoxic effect of citral causing browning on apple slices. As far as colour retention is concerned, the combination citral/citron EO showed a positive interaction, particularly in the second part of storage. The b* parameter values did not show significant modification over storage (data not shown).

It is worth noting that the major phenomenon limiting the shelf-life of fresh-cut apples is enzymatic browning caused by polyphenol oxidase (PPO) enzymes that, after peeling and cutting operations, come in contact with their substrate promoting their oxidation, with a consequent decrease in L* and increase in a* values of fresh apple colour.

According to Lanciotti et al. (1999) and Corbo et al. (2000) dipping treatments with hexanal delayed enzymatic browning, inhibiting polyphenol oxidase (PPO) activity in apples particularly if associated to modified atmospheres. These authors suggested two possible mechanisms to explain this effect: the conversion of hexanal to hexanol, that can act as PPO inhibitor according to Valero et al. (1990) and/or an inhibitory effect of hexanal on phenylalanine ammonia-lyase (PAL), an enzyme that can be activated by tissue disruption

and responsible for the biosynthesis of polyphenol substrate of PPO. In the present study, a positive interaction between 2-*(E)*-hexenal with both hexanal and citral was observed. In fact, as shown in Figure 3.8b, the samples 2-*(E)*-hexenal/citral, 2-*(E)*-hexenal/hexanal and hexanal/citral, showed the better retention ($p<0.05$) the a* value after 35 days of storage.

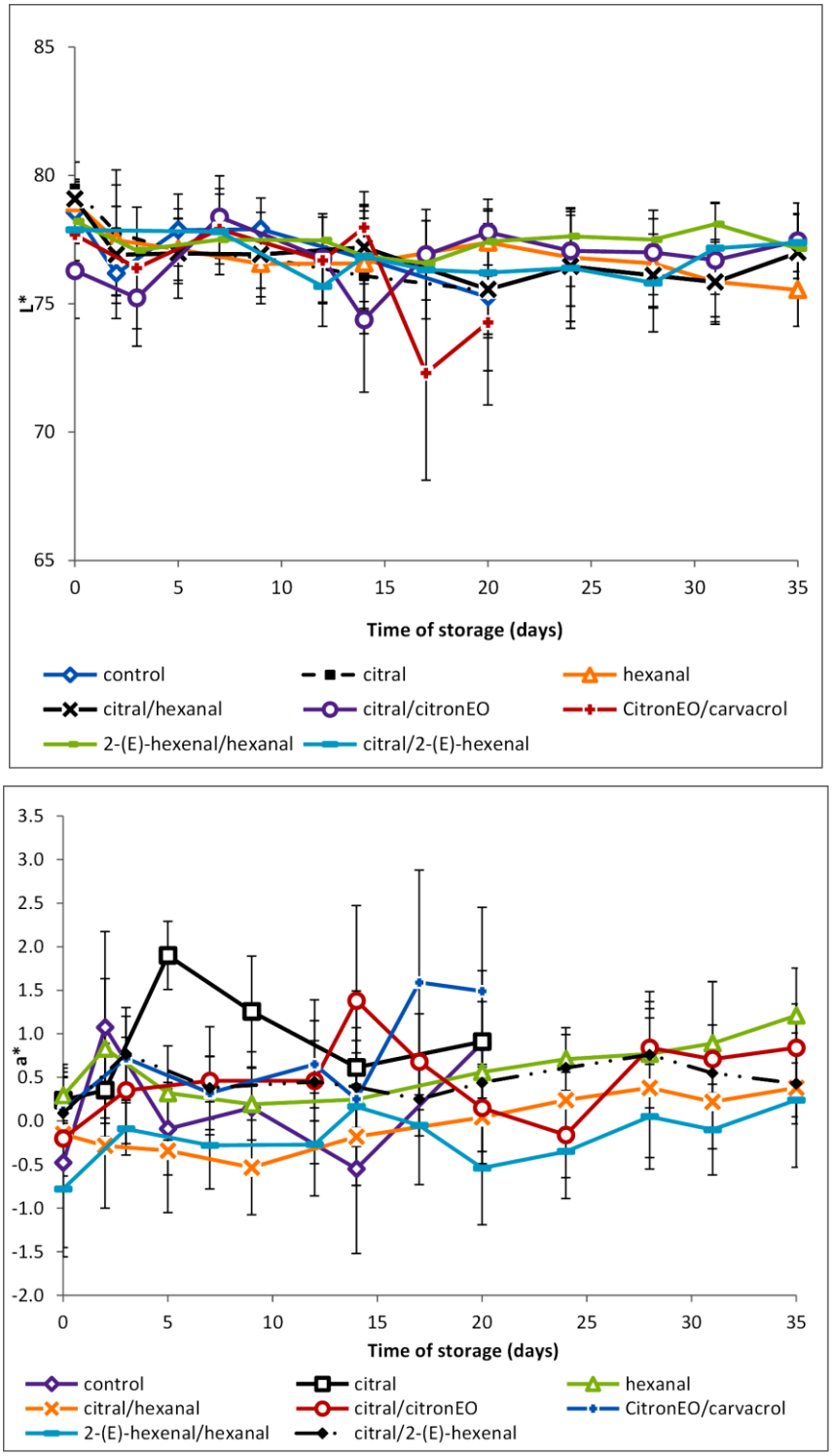


Figure 3.8 a, b- *Evolution of luminosity, L*, of apples treated with natural antimicrobials, their combinations and packaged in MAP, during storage time.*

Recently, Gao et al. (2014) suggested that the antioxidant activity reported for the essential oils may reduce occurrence of browned polymers responsible for the browning in mushrooms. Although the role of these compounds on browning phenomena and more generally on quality parameters is still unclear, the positive interaction between EOs and MAP observed by Lanciotti et al (1999) on fresh cut apples, has been also reported on table grapes and on sweet cherries (Serrano et al., 2005; Valero et al., 2006; Guillén et al., 2007).

Figure 3.9 reports apple sample firmness evolution during storage. Immediately after dipping, citral/2-*(E)*-hexenal, hexanal/2-*(E)*-hexenal, treated samples showed a firmness values significantly higher ($p<0.05$) compared to the control. As expected, during storage, control samples underwent a decrease in firmness; a similar behaviour was observed in citral, citral/citron EO and carvacrol/citron EO treated samples. Samples dipped in hexanal and citral/hexanal showed a decrease in firmness immediately after the treatment but, following, a good retention ($p<0.05$) for 19 days of storage. Among investigated samples, hexanal/2-*(E)*-hexenal and citral/2-(*E*)-hexenal promoted the best retention of firmness throughout 35 days of storage ($p<0.05$). For samples showing the worst colour retention, texture analyses were performed only until the 20th day of storage at 6°C.

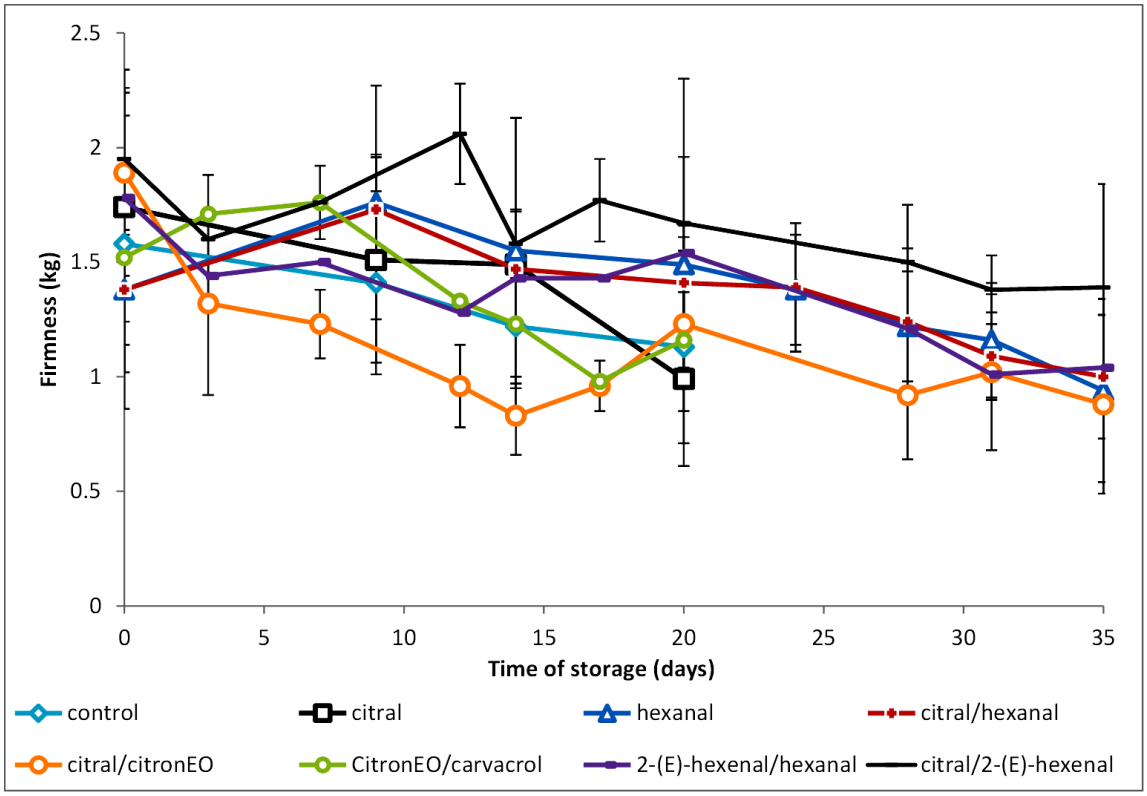


Figure 3.9- *Evolution of firmness (kg.g) of apples treated with citron essential oil, natural antimicrobials and their combinations during storage time.*

Physical parameter analyses showed that treatments including 2-*(E)*-hexenal in combination with either hexanal or citral and with modified atmosphere packaging promoted a better retention ($p<0.05$) of the initial characteristics of the product compared to control samples. The positive effect was maintained until 35 days of storage, evidencing the potentiality of dipping treatment based on these substances in order to obtain long storage fresh-cut apples. However further researches are needed in order to better understand the mechanism of action of these molecules on enzymatic reactions and/or cellular modifications, that result in browning and loss of firmness.

4.3 Effects of citron EO and natural antimicrobials on apple volatile molecule and electronic nose profiles

To evaluate the effects of the compounds taken into consideration on the volatile molecule profiles in relation to storage time, the samples were analysed by means of GC/MS-SPME and electronic nose. The samples were analysed until 20 days due to the colour quality loss of several samples (*i.e.* control samples and those supplemented with citral, citron EO/carvacrol). Although only the most significant molecules were reported in Table 3.6, the GC/MS-SPME allowed the identification of 39 molecules belonging to different chemical classes and provided specific volatile fingerprinting, in relation to the concentration and composition of the antimicrobials used, to the storage time and to the tissue detoxification mechanisms. In fact, high percentages of monoterpenes such as limonene, γ-terpinene, β-pinene and β-mircene and oxygenated monoterpenes such as linalool, neral and geranial characterized the samples supplemented with citron EO, in combination with citral. On the other hand, a wide literature shows that the volatile fraction of citrus EOs is a mixture of monoterpenes, sesquiterpenes and their oxygenated derivatives including aldehydes (citral), ketones, acids, alcohols and esters (Flamini et al., 2007; Smith-Palmer et al., 2001; Belletti et al., 2004; Belletti et al., 2008).

Neral, geranial, nerol, geraniol, citronellyl acetate and β-citronellol characterized the samples supplemented with citral analysed immediately after packaging. However, neral and geranial peak areas decreased in the samples added with citral alone or in combination with citron EO due to their transformation for the interaction with apple and microbial enzymes, while ethyl acetate and ethanol increased remarkably during the storage. On the other hand, it has been demonstrated that the reduction of neral and geranial into nerol and geraniol is the first step of citral biotransformation by *Penicilli* into lower toxicity compounds (Esmaeili & Tavassoli,

2010). The high level of nerol and geraniol in the samples immediately after packaging, indicated that the apple tissues immediately respond to the chemical stress applied producing the corresponding alcohols. Also Patrignani et al., (2013) showed the increase during storage of such alcohols in fruit juices supplemented with citral over the storage, and attributed this phenomenon to the detoxifying mechanisms of spoilage yeasts. A similar detoxifying mechanism, i.e. reduction to the respective alcohols, was shown for six carbon aliphatic aldehydes (Patrignani et al., 2008).

Immediately after packaging the samples supplemented with citron EO/carvacrol presented, in addition to high percentages of monoterpenes such as limonene, *γ*-terpinene, *β*-pinene and *β*-mircene, and carvacrol, high level of 1-butanol,2-methylacetate, acetic acid ethylester and linalyl butyrate. After 3 days of storage at 6°C, a significant increase of ethyl acetate and ethanol associated to the remarkable decrease of carvacrol was observed.

Hexanal, 2-*(E)*-hexenal, hexanol, and acetic acid hexyl esters were detected in the samples supplemented with hexanal/2-*(E)*-hexenal. The samples supplemented with hexanal alone, 2-(*E*)-hexenal/citral and 2-(*E*)-hexenal/hexanal showed a remarkable abundance (in term of peak area) of 2-hexen-1ol-acetate. Hexanal and 2-*(E)*-hexenal showed higher levels in the control samples with respect to the samples supplemented with the same molecules, indicating that their supplementation fastened the detoxification mechanisms adopted by tissues and naturally occurring microorganisms, increasing the peak area of derived alcohols. In the samples supplemented with hexanal/citral, a remarkable increase of hexanol was observed while nerol and geraniol were not detected.

Table 3.6- *Volatile aroma compounds (expressed as Area 10^{-5}) detected in apples treated with different dipping solutions and packaged in MAP during the storage time at 6°C.*

	Control [a]			Hexanal [b]			Citral [c]			Citron EO/Carvacrol [d]			Citral/Citron EO [e]			Citral/2-(E)-Hexenal [f]			Hexanal/2-(E)-Hexenal [g]			Citral/Hexanal [h]		
Compounds	T0	T10	T20	T0	T10	T20	T0	T10	T20	T0	T10	T20	T0	T10	T20	T0	T10	T20	T0	T10	T20	T0	T10	T20
Ethyl acetate	1.0	14.1	30.7	3.8	11.5	13.9	6.4	14.0	24.3	0.7	28.1	23.0	1.7	24.3	26.6	1.1	16.9	12.2	0.8	17.1	17.1	0.0	17.8	12.3
Acetic acid, butyl ester	3.1	4.2	3.1	2.8	1.1	1.7	12.9	1.4	1.4	1.1	1.4	0.5	4.4	3.1	2.4	2.3	2.5	2.0	0.0	1.2	1.9	1.6	3.9	2.4
Acetic acid, pentyl ester	0.0	0.6	0.3	0.5	0.0	0.0	1.9	0.0	0.0	0.0	0.2	0.0	0.6	0.4	0.3	0.6	0.4	0.2	0.0	0.1	0.2	0.0	0.6	0.3
1-butanol, 2-methyl-acetate	28.6	26.5	23.5	2.6	1.1	0.7	23.7	1.2	2.1	6.5	6.9	2.1	12.8	15.4	9.1	6.1	6.2	2.8	0.3	1.1	1.7	7.6	20.6	10.7
Acetic acid, hexyl ester	8.3	19.7	4.9	235.1	54.5	57.3	20.6	2.5	3.2	9.4	9.5	1.8	13.9	14.8	13.1	42.7	63.5	33.3	88.3	82.0	48.1	32.6	142.6	95.4
2-Hexen-1-ol, acetate	0.0	0.0	0.0	46.4	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	50.1	0.5	0.0	39.4	1.0	0.2	0.0	0.0	0.0
Citronellyl acetate	0.0	0.0	0.0	0.0	0.0	0.0	1.4	0.7	0.6	0.0	0.0	0.0	0.0	1.6	0.5	0.0	1.2	0.2	0.0	0.0	0.0	0.0	3.9	1.2
Linalyl butyrate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.4	4.2	5.3	1.9	3.6	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
propanoic acid methyl esters	0.0	0.0	0.0	0.8	0.0	0.0	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	1.4	0.0	1.0	0.0	0.0	0.0	0.0
butanoic acid methyl esters	0.0	0.0	0.0	0.0	0.0	0.2	3.4	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.1	0.0	1.0	2.2	0.0	1.0	0.2	0.0	1.6	1.5
Total Esters	**41.0**	**65.1**	**62.5**	**292.0**	**68.2**	**73.9**	**72.3**	**19.8**	**31.6**	**27.1**	**50.3**	**33.3**	**35.4**	**63.2**	**54.4**	**103.0**	**93.0**	**54.4**	**128.8**	**104.5**	**69.4**	**41.9**	**191.1**	**123.8**
β-myrcene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.9	5.1	5.5	1.2	2.5	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Limonene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	123.8	145.3	151.8	51.4	80.2	82.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-Terpinene	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.6	0.9	0.0	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1
γ-Terpinene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.5	16.8	17.3	4.6	8.4	7.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Terpinolene	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.4	0.8	1.1	0.0	0.8	0.0	0.0	0.9	0.3	0.0	0.6	0.0	0.0	1.5	0.0
Cymene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	2.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-pinene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.4	8.7	9.6	2.9	4.5	4.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Terpens	**3.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.6**	**0.0**	**0.0**	**0.0**	**155.1**	**179.2**	**188.2**	**60.1**	**96.7**	**97.4**	**0.0**	**0.9**	**0.3**	**0.0**	**0.6**	**0.0**	**0.0**	**1.7**	**0.1**
Thymol methyl ether	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.1	12.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Ethers	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**14.1**	**12.6**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
Nerol	0.0	0.0	0.0	0.0	0.0	0.0	4.3	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Geraniol	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Carvacrol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.6	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

β-citronellol	0.0	0.0	0.0	0.0	0.0	0.0	63.7	39.9	22.4	0.0	0.0	0.0	8.8	14.0	10.7	4.3	12.9	16.3	0.0	0.0	0.0	7.0	13.6	13.0
Hexanol	0.0	1.5	0.4	33.1	18.6	21.6	9.5	1.6	0.9	1.1	0.8	1.4	1.7	0.9	1.4	4.7	11.2	13.4	12.7	16.6	13.1	15.6	5.2	7.2
Ethanol	1.3	6.7	15.1	8.4	12.4	18.3	14.0	14.0	17.3	7.4	13.4	22.2	6.6	8.7	13.6	6.4	11.4	15.5	7.7	12.4	16.7	6.9	5.3	7.9
Butanol	0.0	0.9	0.7	0.5	0.2	0.7	1.9	0.3	0.4	0.0	0.0	0.0	1.2	1.0	1.0	0.7	0.6	0.9	0.3	0.7	0.5	1.2	0.4	0.4
2-methyl-, 1-Butanol	1.1	1.3	1.3	0.0	0.0	0.2	1.0	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.7	0.6	0.0	0.1	0.3	0.9	0.4	0.6
2-hexenol (E)	0.0	0.0	0.0	2.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
Linalool	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	0.4	0.8	1.5	1.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Alcohols	**2.4**	**10.4**	**17.5**	**44.9**	**31.2**	**40.7**	**99.4**	**57.2**	**41.3**	**18.4**	**14.5**	**25.1**	**19.7**	**25.8**	**27.3**	**19.1**	**36.8**	**46.8**	**22.8**	**29.8**	**30.7**	**31.7**	**24.9**	**29.1**
Hexanal	11.9	6.3	4.0	3.9	2.5	4.9	9.9	3.3	3.3	7.0	2.6	3.2	10.9	3.0	2.2	5.9	2.5	3.5	5.7	3.5	3.4	9.1	2.9	2.9
Neral	0.0	0.0	0.0	0.0	0.0	0.0	48.7	0.2	0.0	0.0	0.0	0.0	1.4	0.0	0.0	2.5	0.0	0.0	0.0	0.0	0.0	11.8	0.0	0.0
Geranial	0.0	0.0	0.0	0.0	0.0	0.0	48.4	0.6	0.0	0.0	0.0	0.0	1.0	0.0	0.0	2.9	0.0	0.0	0.0	0.0	0.0	9.4	0.0	0.0
2-hexenal (E)	4.3	0.5	0.0	7.2	0.0	0.3	7.2	0.0	0.0	3.1	0.0	0.0	5.2	0.2	0.1	4.7	0.0	0.1	5.2	0.1	0.4	3.5	0.0	0.0
Total Aldehydes	**16.2**	**6.8**	**4.0**	**11.1**	**2.5**	**5.2**	**114.1**	**4.1**	**3.3**	**10.1**	**2.6**	**3.2**	**18.4**	**3.2**	**2.3**	**16.1**	**2.5**	**3.6**	**10.9**	**3.6**	**3.8**	**33.8**	**2.9**	**2.9**
	62.5	**82.3**	**84.0**	**348.0**	**101.9**	**120.4**	**285.8**	**81.1**	**76.2**	**210.7**	**260.8**	**262.4**	**133.6**	**188.9**	**181.5**	**138.1**	**133.1**	**105.0**	**162.5**	**138.6**	**103.8**	**107.3**	**220.6**	**156.0**

[a] Control was washed only with dipping solution (1% citric acid + 0.5% ascorbic acid)
[b] Concentration employed 250 ppm.
[c] Concentration employed 250 ppm.
[d] Concentration employed 200 ppm citron oil and 50 ppm carvarcol.
[e] Concentration employed 125 ppm each.
[f] Concentration employed 125 ppm each.
[g] Concentration employed 125 ppm each.
[h] Concentration employed 125 ppm each.

A multivariate analysis using a heat map was performed in order to identify the molecules able to significantly contribute to the statistical discrimination between the samples and five small clusters were obtained. The heat map underlined the role of the EO or the natural antimicrobials in grouping the samples (Figure 3.10). In particular, the samples added with citron EO/carvacrol and stored up to 20 days grouped together (Cluster 1) and limonene, ethanol, ethyl acetate and α-terpinene contributed to the grouping. The samples supplemented with hexanal alone or in combinations with the considered antimicrobials grouped in cluster 2 and 5, with the exception of the samples supplemented with hexanal alone immediately after packaging. Acetic acid hexyl esters, hexanol, ethanol and ethyl acetate characterized cluster 5, while to the formation of cluster 2 contributed acetic acid hexyl esters, 2-hexenol acetate, hexanol, ethyl acetate and ethanol. The samples added with citral and analysed immediately after packaging did not cluster with the other samples due to the presence of neral, geranial and β-citronellol. Differently, the samples added with citral and analysed after 10 and 20 days of storage grouped with all the control samples in cluster 4 due to the presence of ethanol, ethyl acetate, β-citronellol and 1-butanol,2-methyl acetate.

Cluster 3 grouped the samples added with citron EO/citral due to the high levels of limonene. The very limited microbial growth in all the samples, independently on the antimicrobial supplementation, did not permit the identification of eventual spoilage volatile markers. In fact, also in the control samples yeasts and LAB attained at the end of storage 2.3 and 3.7 log cfu/g, respectively. Such cell loads were not able to allow the accumulation of microbial spoilage markers. With exception of the samples added with hexanal/citral, analysed immediately after packaging, that did not cluster with any other samples, the storage time did not contribute significantly to the grouping, probably due to the different detoxification rate and patterns of the supplemented substances.

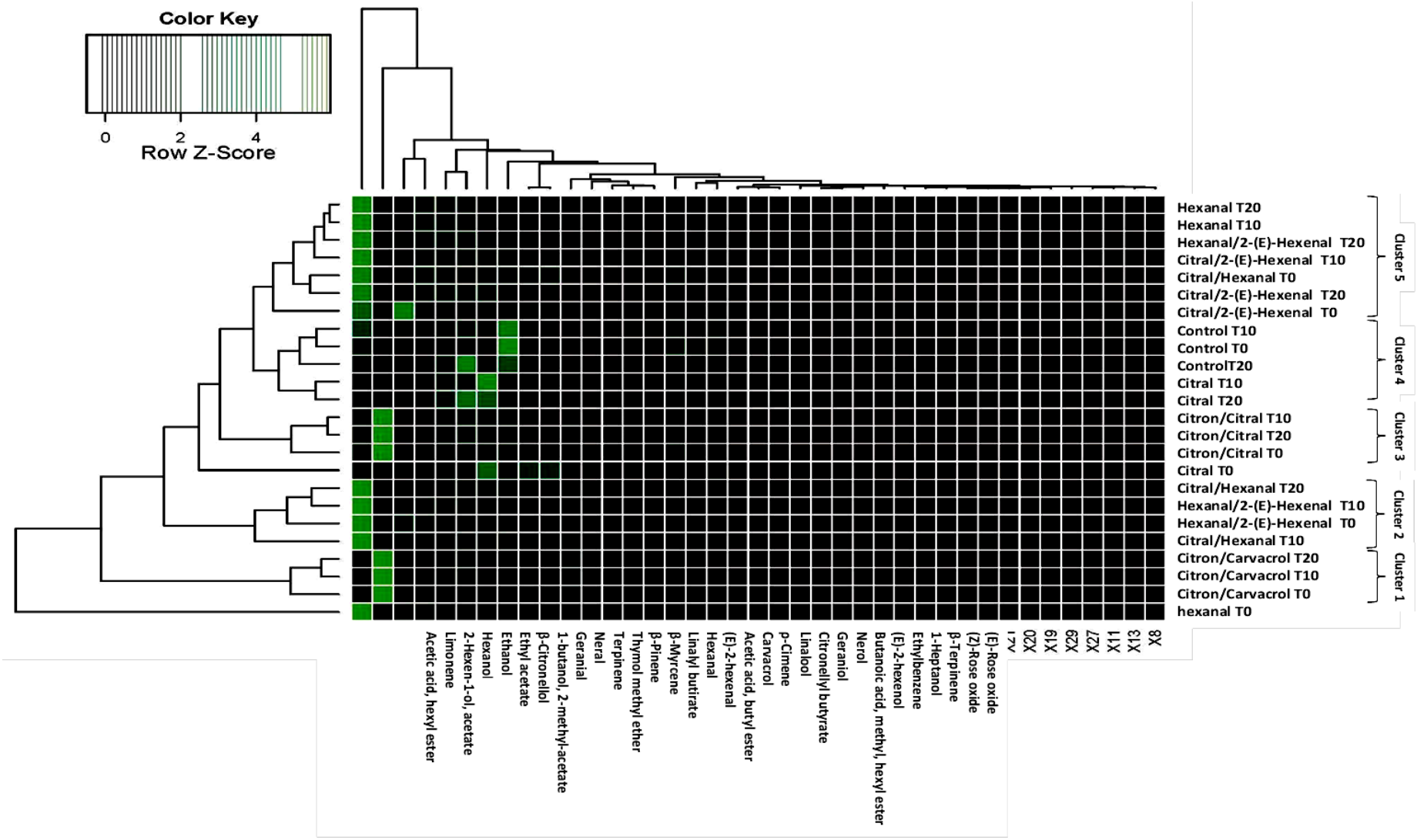
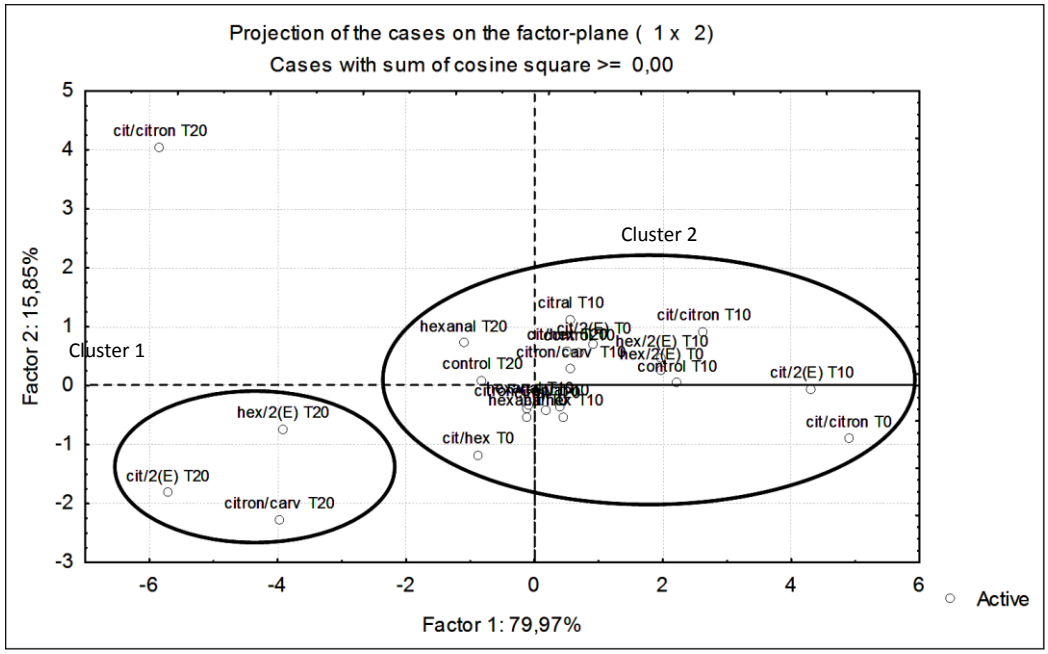


Figure 3.10- *Heat map of correlations between metabolites produced during the storage of apples treated with citron essential oil, natural antimicrobials and their combinations and packaged in MAP. Each square represents the Spearman's correlation.*

The electronic nose data were subjected to a principal component analysis in order to outline the differences among the samples detected by the 10 sensors of the instrument. All the samples were mapped in the space spanned by the first two principal components PC1 versus PC2. The score and loading plot, reported in Figure 3.11a and 3.11b, showed the clustering of the samples according mainly to storage time, differently from heat map that clustered samples mainly on the base of added antimicrobials.



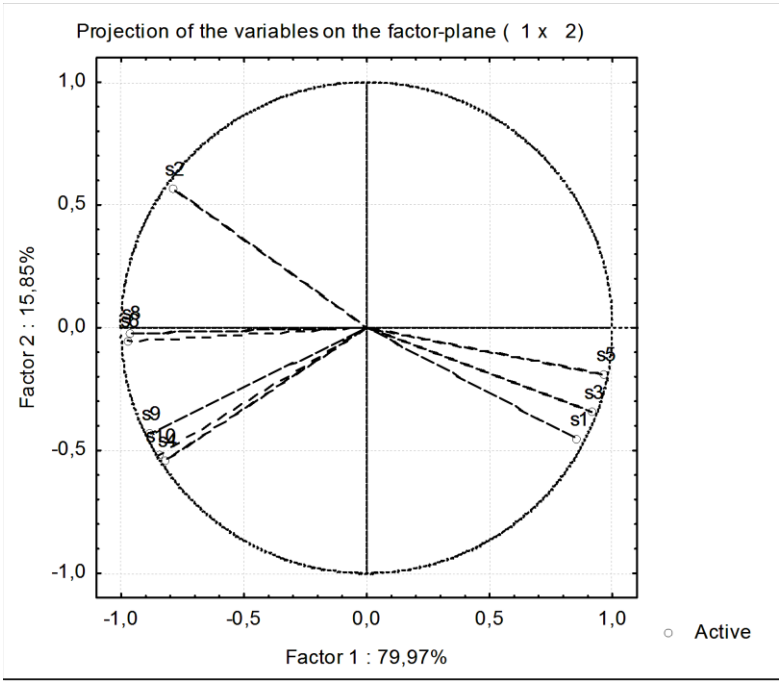


Figure 3.11 a, b- *Projection of the cases (6a, different apples treated with citron EO, natural antimicrobials and their combination and packaged in MAP) and loadings (6b sensors) on the factor-plane (1x2). PC1 and PC2 explained 79.97% and 15.85 of the total variance respectively.*

This can be attributed to the minor sensitiveness of the electronic nose with respect to GC-MS-SPME to the natural antimicrobials and their detoxification products. In fact, two different clusters were evident in the PCA plot, while the samples supplemented with citral/citron EO resulted separated from the other both on the basis of PC1 (explaining 74.36% of variance) and PC2 (explaining 14.92% of the variance). The first large cluster grouped the samples supplemented with 2-(*E*)-hexenal/hexanal, 2-(*E*)-hexenal/citral carvacrol/citron EO after 20 days of storage. These samples were grouped on the basis of sensors 9, 10 and 4. The second cluster grouped all the other samples that in their turns were well separated along the PC1 able to explain 74.36% of the variance. The sensors 1, 3 and 5, more responsive for aromatic compounds, defined this cluster. Only the sample added with citral/citron EO after 20 days of storage was not included in the other clusters, and it was well separated from the first cluster along PC2, and from the second cluster both on the basis of PC1 and PC2. This sample was separated from the others on the basis of sensor 2.

4. Conclusion

The effectiveness of citron oil and natural antimicrobial compounds to delay the spoilage agents of minimally processes apples packaged in ordinary atmosphere was demonstrated in this research. The antimicrobials considered delayed the reaching of the yeast spoilage threshold in a range of 3-10 days with respect to the controls. Among the tested conditions, citral and hexanal+2-*(E)*-hexenal were the most effective to inhibit the yeast growth that did not attain the spoilage threshold within 21 days of storage. Although all the compounds used determined a specific GC/MS-SPME volatile molecule profile, they did not affect the electronic nose profiles of the samples that clusterized mainly on the basis of storage time. Exceptions were represented by the samples added with citral or hexanal stored for 3 days that clusterized with 10 day-stored samples, demonstrating that during this storage time no significant modifications appeared in the electronic nose profiles of these samples. Physical analysis results showed that generally until fourth-seven days of storage control sample better maintained its initial colour and texture characteristics. The beneficial effects of dipping with essential oils solutions become noticeable in the second part of the storage period, suggesting the potential use of these treatments for long storage fresh-cut apples stabilization.

In addition the results obtained showed that the shelf-life of minimally processed apples can be significantly prolonged by combined use of natural antimicrobials in the dipping solution and packaging in modified atmosphere. However in these conditions the shelf-life of the products is quite unaffected by microbial growth, independently on the addition of natural

antimicrobials such as hexanal, citral, 2-(*E*)-hexenal, citron EO and carvacrol, alone or in combination. In fact, the end of shelf-life was determined by changes mainly in colour and texture. However, among the tested natural antimicrobials, 2-(*E*) hexenal in combination with citral or hexanal allowed the prolongation of the product shelf-life up to 35 days, without detrimental effects on safety. The addition of such molecules in the dipping solution of fresh sliced apples in combination with MAP permitted the retention of quality parameters such as colour, texture and volatile profiles detected throughout electronic nose. In fact, on the basis of PCA analysis the electronic nose data grouped the samples on the basis of storage time showing that the volatile profiles, perceived by the instrument that mimes the human nose (Wilson, & Baietto, 2009), were quite unaffected by the added antimicrobials. These results are very promising and with great applicative potential for the minimally processed vegetable manufacturing.

Acknowledgment

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CHAPTER 4

Natural antimicrobials to prolong the shelf-life of minimally processed lamb's lettuce

Abstract

The health benefits of increasing intake of fruits and vegetables are well recognized and their consumption is strongly promoted. Food industry offer a broad range of packaged fresh-cut vegetables that merging “health” and “convenience” features. However, in recent years the number of foodborne outbreaks linked to minimally processed vegetables has increased dramatically. Currently, the safety and shelf-life of minimally processed vegetables is based on few tools such as modified atmosphere packaging and maintaining of refrigeration chain. Chlorine is the most common decontaminant used in these products, however, at the concentration used it is quite ineffective in reducing pathogens on vegetables. In addition chlorine-based compounds formation of potentially harmful chlorinated by-products such as trihalomethanes. These drawbacks of chlorinate sanitizers have stimulated the investigation on alternatives. Plant essential oils (EOs) and their components have been investigated as natural sanitizer alternative to chlorine to control of foodborne pathogens and spoilage bacteria associated with minimally processed vegetables because Generally Recognized as Safe and endowed with a wide antimicrobial activity. In fact, The antimicrobial activity of oregano, thyme EOs and their main components carvacrol and thymol against variety of microorganisms is well documented both in model and real food system. In this perspective the main aim of this study was to evaluate the efficacy of oregano and thyme EOs as well as carvacrol in comparison with chlorine for lamb’s lettuce decontamination addressing the control of spoilage and pathogenic species and improving shelf-life of these products. To reach this goal, preliminarily the two EO were characterized by GC-MS-SPME and the MIC values of the antimicrobials considered against *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enteritidis* were determined. Following, the effects of the antimicrobials used as alternative to chlorine in the washing solution of lamb’s lettuce were evaluated on mesophilic aerobic bacteria, yeasts, LAB, color parameters and volatile molecule profiles detected by GC-MS-SPME. In addition, in a second experimental phase, the study of the effects of thyme and oregano EOs on the shelf-life of minimally processed lamb’s lettuce packaged in artificial ordinary atmosphere, after some modifications of the washing parameters, were performed. In particular, the effects of these antimicrobials on total mesophilic aerobic bacteria and lactic acid bacteria (LAB) cell loads, texture, color and volatile molecule profiles were monitored during the storage at 6°C.

1. Introduction

Today consumers are more conscious of the rapport between nutrition and health. The health benefits of increasing intake of fruits and vegetables are well recognized and their consumption is strongly promoted. In fact, due to their high levels of nutrients, vitamins, minerals and fibers, they are considered an essential part of the world's population's diet (World Health Organisation (WHO) 1998; Olaimat & Holley, 2012; Goodburn & Wallace 2013). Food industry and the retail market anticipated this market trend and offer a broad range of packaged fresh-cut vegetables that merging "health" and "convenience" features could be an excellent alternative to raw materials (Allende et al., 2006). However, in recent years the number of foodborne illness outbreaks linked to raw and minimally processed vegetables has increased dramatically (Warriner et al., 2009; Olaimat & Holley, 2012; Gudburn & Wallace 2013; Van Boxstael et al. 2013). In fact, the minimally processing and lacking of thermal treatment before eating enhance the risk due to pathogen contamination (Berger et al., 2010). A wide literature shows that *Aeromonas hydrophila*, *Bacillus cereus*, *Clostridium* spp., *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *Campylobacter* spp., *Yersinia enterocolitica* are frequently associated with illness outbreaks related to consumption of fresh produce (Aruscavage et al., 2006; Beuchat, 2002; Buck et al., 2003; Rangel et al., 2005; Sivapalasingam et al., 2004; Olaimat and Holley 2012; Gudburn and Wallace 2013). However, *Salmonella* and *E. coli* O157:H7 are reported as the main cause of foodborne illness associated with fresh produce (Buck et al., 2003; FDA, 1998; Warriner et al., 2009). On the other hand microbial contamination, with spoilage and pathogenic species can arise during the different steps from farm-to-consumer (production, harvest, processing, wholesale storage, transportation or retailing and handling in the home) and this contamination can occur from environmental, animal or human sources (FDA, 2001; WHO/FAO, 2008). In addition, the release tissue damages and the release of nutrient due to cutting, slicing or peeling as well as and the confinement of final product inside the packaging enhance the microbial growth (Lanciotti et al. 2003; Harris et al., 2003). Currently, the safety and shelf-life of minimally processed vegetables is based on few tools such as modified atmosphere packaging and maintaining of refrigeration chain (Gomez-Lopez et al. 2007; Alegre et al. 2010; Siddiqui et al. 2011). However washing with sanitizing solutions is usually the only step during production of minimally processed vegetables by which the number of pathogenic and spoilage microorganisms can be reduced (Sao José & Vanetti 2012). Nodaway, chlorine is the most common decontaminant used in the minimally processed vegetable industry (Tirpanalan et al. 2011; Joengen, 2005), although is use is prohibited in

some European countries such as the Netherlands, Sweden, Germany and Belgium (Rico et al. 2007; Gil et al. 2009). However, at the concentration normally used (50-200ppm) it does not achieve more than a 1-2 log reduction in bacterial populations and it is quite ineffective in reducing pathogens on vegetables (Oliveira et al. 2012; Gil et al. 2009). In addition chlorine-based compounds are corrosive, cause skin and respiratory tract irritation and reacts with the organic matter present in the water and, as a consequence, formation of potentially harmful chlorinated by-products such as trihalomethanes (Selma et al. 2008; Sao Josè and Vanetti, 2012; Lopez Galvez 2010; 2012). In addition some literature reports show that emerging pathogens are more resistant to chlorinated compounds raising further concerns about the effectiveness and the use of chlorine in the minimally processed food industry (Allende et al., 2008; Alvaro et al., 2009).

These drawbacks of chlorinate sanitizers have stimulated the investigation on the efficiency of non-traditional sanitizers (hydrogen peroxide, peroxyacetic acid and ozone) and other alternative technologies such as physical treatments (UV-C light, ultrasound and gamma rays) (Rivera et al. 2011; Rico et al. 2007, Artès-Hernandez et al. 2009; Alegria et al. 2009; Gil et al. 2009). Also plant essential oils (EOs) and their components have been investigated as natural sanitizer alternative to chlorine to control of foodborne pathogens and spoilage bacteria associated with minimally processed vegetables because Generally Recognized as Safe and endowed with a wide antimicrobial activity (Gutierrez, et al. 2008a; 2008b; 2009; Gunduz et al. 2010). The in vitro antimicrobial activity of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) EOs and their main components carvacrol and thymol against variety of Gram-positive, Gram-negative bacteria, yeasts and molds is well documented (Dorman and Deans 2000; Marino et al., 2001; Burt 2004; Sagdic et al., 2003; Viuda-Martos et al., 2007). In addition their efficacy has already be experienced in several real foods including meat (Skandamis & Nychas, 2001; Tsigarida et al., 2000; Boskovic et al. 2013), fish products (Kykkidou et al. 2009; Sagdic & Ozturk 2014) and dairy products (Lucera et al., 2012; Govaris et al. 2011; Shan et al. 2011). However, there are very limited studies that investigate the antimicrobial efficacy of these natural antimicrobials alone or in combinations with other hurdles on fresh produce (Gutierrez et al. 2008b; Gutierrez et al., 2009; Sellamuthu et al. 2013).

In this perspective the main aim of this work was to evaluate the efficacy of oregano and thyme EOs as well as of carvacrol in comparison with chlorine for lamb's lettuce decontamination addressing the control of spoilage and pathogenic species and improving shelf-life of the minimally processed products. To reach this goal, preliminarily the two EO

were characterized by GC-MS-SPME and the MIC values of the antimicrobials considered against *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enteritidis* were determined. Following the effects of the antimicrobials used as alternative to chlorine in the washing solution of lamb's lettuce were evaluated on mesophilic aerobic bacteria, yeasts, LAB, color parameters and volatile molecule profiles detected by GC-MS-SPME. In addition, in a second experimental phase, the study of the effects of thyme and oregano EOs on the shelf-life of minimally processed lamb's lettuce packaged in artificial ordinary atmosphere, after some modifications of the washing parameters, were performed. In particular, the effects of these antimicrobials on total mesophilic aerobic bacteria and lactic acid bacteria (LAB) cell loads, texture, color and volatile molecule profiles were monitored during the storage at 6°C.

2. Material and Methods

2.1 Natural antimicrobials

Thyme and Oregano EOs were obtained from Flora s.r.l. (Pisa, Italy). Carvacrol was purchased from Sigma-Aldrich (Milano, Italy). The EOs and the natural antimicrobial used were selected both for their antimicrobial activity and impact on organoleptic properties.

2.2 Characterization of thyme and oregano essential oil by GC/MS-SPME technique

One mL of oregano and thyme EOs were placed into a 10 mL vial and sealed through a PTFE/silicon septa. Three different samples were prepared. The samples were conditioned 30 min at 25°C. An SPME fiber covered by 50 µm divinylbenzene-carboxen-poly (dimethylsiloxane) (DVB/CARBOXEN/PDMS StableFlex) (Supelco, Steiheim, Germany) was exposed to each sample at room temperature (25°C) for 20 min, and finally, the adsorbed molecules were desorbed in the GC for 10 min. For peak detection, an Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett–Packard, Geneva, Switzerland) and a Varian (50 m×320 µm×1.2 µm) fused silica capillary column were used.

The temperature program was 50°C for 0 min, then heated to 230 °C at 3°C/min, this temperature was maintained for 1 min. Injector, interface, and ion source temperatures were 200, 200, and 230°C, respectively. Injections were performed with a split ratio of 30:1 and helium as carrier gas (1 mL/min). Compounds were identified by the use of the Agilent Hewlett–Packard NIST 98 mass spectral database.

2.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination.

For the determination of MIC values, 150 µl of BHI broth inoculated at three different levels (2, 4 or 6 log CFU/mL) of the tested pathogens (*Listeria monocytogenes* Scott A, *Salmonella Enteritidis* E5, *Escherichia coli* 555, *S. aureus* F1, *Bacillus cereus* SV90), belonging to the DISTAL Department, were added to 200 µl microtiter wells (Corning Incorporated, NY, USA). Fifty µl of the tested EO or natural antimicrobials, properly diluted in Brain Heart Infusion (BHI, Oxoid Ltd., Basingstoke, United Kingdom) broth and conveyed through 96% ethanol (VWR international, PROLABO, France), were added to each well so as to obtain the required concentration of each compound in the final volume of 200 µl, with a constant amount of ethanol (1% v/v in wells). Microtiter plates were incubated at 37°C and checked after 24 and 48 h. The MBC were determined by spotting 10 µL of each well after 48 h, onto BHI agar plates.

MIC was defined as the lowest concentration of the compound preventing visible growth of the inoculated cells after 24 h (MIC 24 h). The MBC was defined as the lowest concentration of the compound that caused the death of the inoculated cells and therefore no growth after 24 h of incubation at 37°C of a 10 µl spot plated onto BHI agar.

2.4 Preparation of lamb's lettuce products

Lamb's lettuce (*Valerianella locusta* sp.) was purchase at a local retailer in the same day of the experiment. To prepare the lettuce, the withered leaves and roots were removed. Different washing solutions were prepared with running water at concentration of 250 ppm for thyme and oregano EO alone; 125/125 ppm for the combinations thyme/oregano EO and oregano EO/carvacrol; 120 ppm for chlorine. Natural antimicrobials were conveyed through 1% (v/v) of ethanol. Control samples were represented by the product washed with 120 p of chlorine, without the supplementation of natural antimicrobials. Prepared lettuce was immersed and gently agitated into appropriate washing solution for 2 min and with a ratio product/water of 1:10 (w/v). The employed complete process protocol is reported in Figure 4.1. After the treatment, lettuce was spin dried and packaged into 59µm-thick BOPP bags (permeability CO_2 at 22°C: 2720 cm^3/m^2/day, permeability O_2 at 22 °C: 986 cm^3/m^2/day) with 25 g of product and a ratio apples/headspace of 1:1. Samples were stored at 6°C until the end of shelf-life. In the second experimental phase only samples added with thyme and oregano EOs alone

at a concentration of 250 ppm as well as the control added with 120 ppm of chlorine were employed. The working protocol was modified as reported in Figure 4.2. After the treatment, lettuce was spin dried and packaged in artificial ordinary atmosphere atmosphere into 59µm-thick BOPP bags (permeability CO_2 at 22°C: 2720 cm^3/m^2/day, permeability O_2 at 22 °C: 986 cm^3/m^2/day) with 25 g of product and a ratio apples/headspace of 1:1. Samples were stored at 6°C until the end of shelf-life.

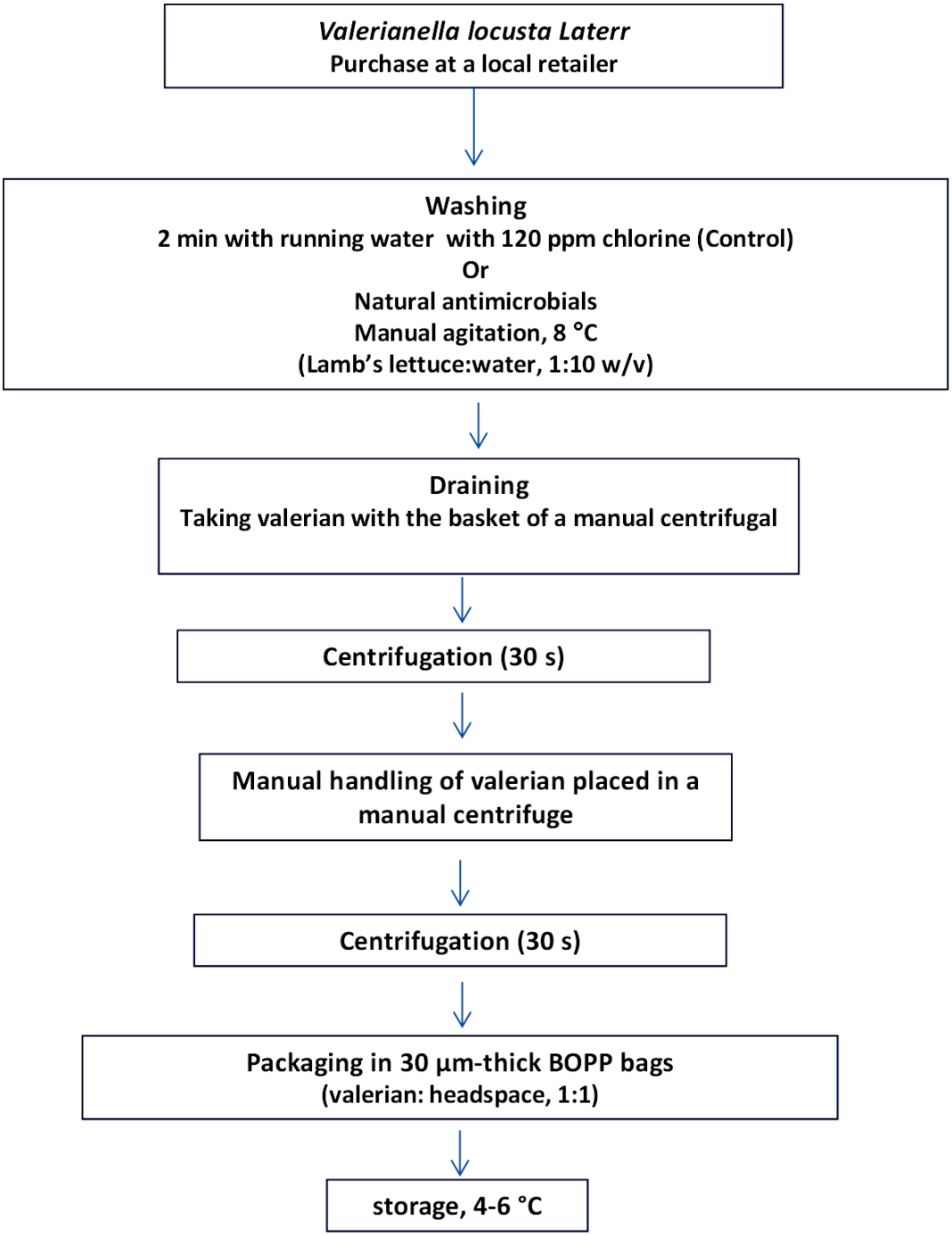

Figure 4.1- *Working protocol employed to prepare lamb's lettuce; the addition of natural antimicrobials was performed during the washing step, samples washed with chlorine represented the controls*

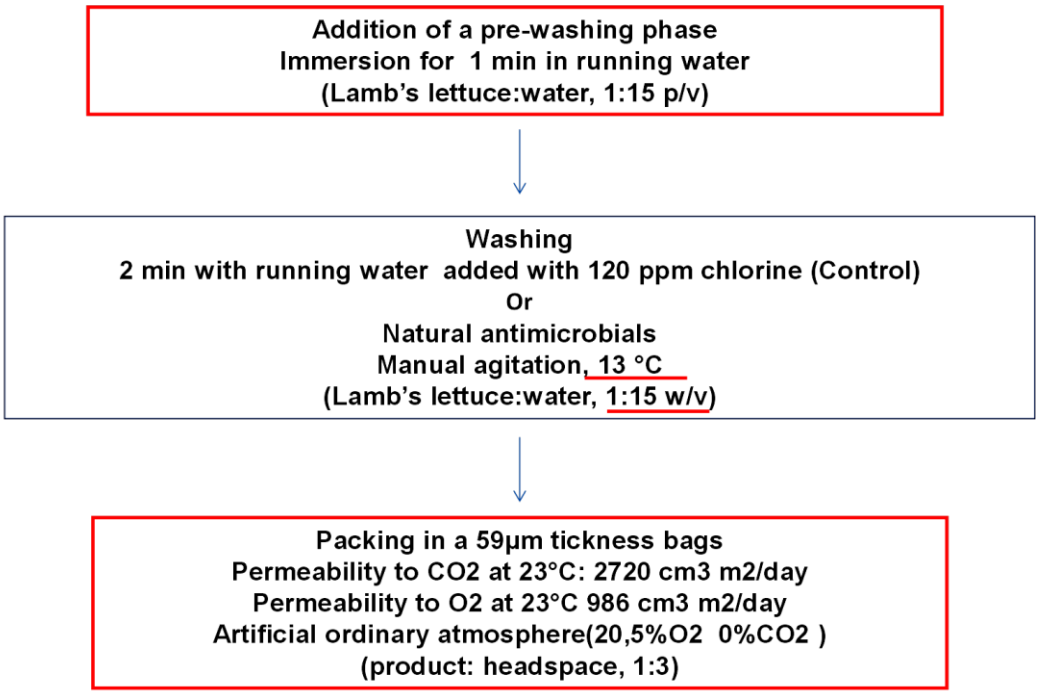

Figure 4.2- *Modifications implemented in the working protocol used in the second experimental phase; in the flow chart are reported only the modifications employed, the remaining steps were the same of the protocol reported in Figure 1.*

2.5 Microbiological analyses

During storage, the evolution over time of mesophylic aerobic bacteria, lactic acid bacteria and yeasts, was evaluated by plate counting respectively on Plate Count Agar (PCA, Oxoid Ltd., Basingstoke, United Kingdom), Man Rogosa and Sharpe Agar (MRS, Oxoid Ltd., Basingstoke, United Kingdom) and Sabouraud Dextrose Agar (SAB, Oxoid Ltd., Basingstoke, United Kingdom), respectively. After homogenization, samples were serially diluted in physiological solution (10 g of sample diluted into 90 mL of physiological water (0.9% (w/v) NaCl). For the detection of the natural occurring *Listeria monocytogenes*, the method suggested by McClain and Lee (1988) was followed, whereas the occurrence of *Salmonella* spp. was investigated according to the method proposed by Andrews and Hammack (1998). *Escherichia coli* was investigated on violet red bile agar (Oxoid) added to 4-methylumbelliferyl-β-D-glucuronide (Oxoid), incubating the plates at 37°C for 24h. *Staphylococcus aureus* was enumerated on Baird-Parker media (Oxoid) with added egg yolk tellurite emulsion (Oxoid) after 24 h at 37°C. In the first experimental phase the analyses were performed immediately after treatments and after 2, 3, 6 and 8 days of storage. In the second experimental phase, after the optimization of the washing process and artificial ordinary atmosphere packaging, the analyses were performed immediately after treatments and after 1, 2, 3, 6, 9, 11 and 14 days of storage.

2.6 Volatile molecule profiles analyses

Lettuce bags, containing 25 g of products, were used for headspace volatile compound analysis by GC/MS-SPME technique. In the first experimental phase, for each treatment condition samples were analyzed immediately after treatments and after 3 and 7 days of storage, while in the second experimental phase each sample was analyzed immediately after the treatments and after 3 and 10 days of storage.

The samples were conditioned 30 min at 37°C. The same type of SPME fiber used for the characterization of oregano and thyme EO, was exposed to each sample at room temperature (25°C) for 40 min, and finally, the adsorbed molecules were desorbed in the GC for 10 min. An Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett–Packard, Geneva, Switzerland) and a Varian (50m×320µm×1.2µm) fused silica capillary column were used for peak detection. The temperature program was 50 °C for 0 min, then heated to 230°C at 3°C/min, this temperature was maintained for 1 min. Injector, interface, and ion source temperatures were 200, 200, and 230°C, respectively. Injections were performed with a split ratio of 30:1 and helium as carrier gas (1 mL/min). Compounds were identified by the use of the Agilent Hewlett–Packard NIST 98 mass spectral database.

2.7 Physical analyses: color and withering index

Surface colour was measured using a color-spectrophotometer mod. Colorflex (Hunterlab, USA). Color was measured using the CIELab scale and Illuminant D65. The instrument was calibrated with a white tile (L*98.03, a* - 0.23, b* 2,05) before the measurements. Results were expressed as L* (luminosity) and a* (red index);

At each storage time, 21 readings were obtained for each sample from the seven packages, measuring three slices for each package.

To evaluate the withering phenomena during storage, a geometric index was created. Perimetral sections of about 4 cm were cut from the leaves and placed on a tweezer mounted on a vertical stand. Images were acquired on black background with a distance objective-leaf of about 8 cm. The angle created by the two leaf sections and the fixing point was measured (Figure 4.3) and used as Withering Index.

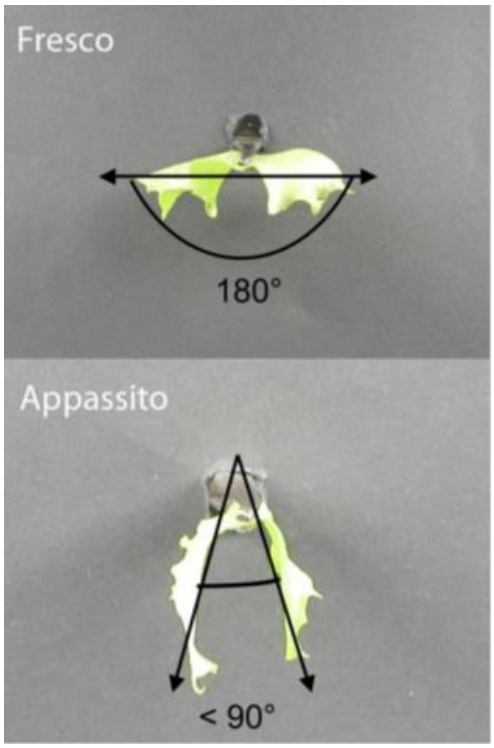

Figure 4.3- *Example of images acquired to evaluate the withering index of Valerianella locusta leaves.*

2.8 Statistical analysis

For each sample, the microbiological and volatile data were the mean of three different samples. Regarding microbiological data, statistical analysis was performed using Statistica software (version 8.0; StatSoft., Tulsa, Oklahoma, USA). Means were compared using ANOVA followed by LSD test at $p<0.05$ level in order to monitor changes over time as well as differences between treatments.

Principal component analysis (PCA) was performed using Statistica software (version 8.0; StatSoft., Tulsa, Oklahoma, USA) to obtain a visual overview of electronic nose analyses.

3. Results of the first experimental phase: effects of natural antimicrobials on minimally processed lamb's lettuce packaged in ordinary atmosphere

3.1 Characterization of thyme and oregano essential oils throughout GC-MS-SPME

Thyme and oregano EOs were characterized throughout GC-MS-SPME analyses. Table 4.1 reports the total area of the GC peaks and the percentage (on the basis of the relative peak area) of each compound present in the headspace of the oregano and thyme EO, as well as the cumulative percentages of the different classes of compounds (monoterpenes, sesquiterpenes, oxygenated monoterpenes, aliphatic alcohols, aliphatic aldehydes, esters, and ketones). The

volatile profile of oregano EO was characterized by the presence of 34 identified molecules belonging to different chemical classes, while that of thyme showed 41 identified molecules. Carvacrol, thymol, *p*-cymene, α-pinene, caryophyllene and gamma terpinene accounted for about 88 and 83 % of the total peak area of oregano and thyme EO, respectively. Oregano EO showed also high levels of and α-terpinene (3.89%) while thyme had higher level of linalool (2.04%), limonene (1.08%) and β-thujene (1.01%).

Table 4.1- *Oregano and thyme EOs characterization*

Thyme EO			Oregano EO		
Molecules	**Total peak area**	**Area %**	**Molecules**	**Total peak area**	**Area %**
α-pinene	64161013	4.83	α-pinene	29616709	3.43
camphene	25751016	1.94	camphene	3431254	0.4
β-pinene	8433414	0.63	β-pinene	1664439	0.19
β-phellandrene	91377	0.01	3-carene	1693569	0.2
3-carene	2933646	0.22	β-myrcene	22216747	2.57
β-myrcene	35761675	2.69	α-phellandrene	2456150	0.28
α-phellandrene	4773632	0.36	α-terpinene	33644575	3.89
α-terpinene	51235593	3.85	limonene	8547408	0.99
limonene	13389082	1.01	β-thujene	5079423	0.59
β-thujene	14346138	1.08	ϒ-terpinene	72693569	8.41
ϒ-terpinene	203560670	15.31	*p*-cymene	309885246	35.86
p-cymene	374581749	28.17	terpinolene	3166331	0.37
terpinolene	4470401	0.34	Ylangene	1739261	0.2
cis-β-terpineol	3278286	0.25	α-cubebene	7410171	0.86
α-cubebene	1572027	0.12	β-bourbonene	3635866	0.42
β-bourbonene	899816	0.07	linalol	322546	0.04
linalol	27058205	2.04	caryophyllene	49654406	5.75
bornyl acetate	796292	0.06	(+)-aromadendrene	1836300	0.21
timol methil ether	6150134	0.46	carvone	122563	0.01
caryophyllene	90878013	6.84	α-caryophyllene	1293689	0.15
(+)-aromadendrene	846134	0.06	ϒ-Muurolene	2910065	0.34
allo-Aromadendrene	403729	0.03	α-terpineol	140525	0.02
β-farnesene	390745	0.03	borneol	1637263	0.19
β-gurjunene	339524	0.03	copaene	311791	0.04
nerol acetate	274055	0.02	β-farnesene	1325112	0.15
α-caryophyllene	1874589	0.14	α-Muurolene	237200	0.03
ϒ-Muurolene	1271159	0.1	δ-Cadinene	3114797	0.36
α-terpineol	592502	0.04	ϒ-cadinene	1189108	0.14
borneol	6007370	0.45	anetol	529966	0.06
ϒ-Terpinene	464250	0.03	Calamenene	525772	0.06
copaene	152918	0.01	*p*-Cymen-8-ol	244657	0.03
α-Muurolene	471346	0.04	*p*-timol	1092970	0.13
(+)-carvone	159069	0.01	timol	41459717	4.8
δ-Cadinene	2369546	0.18	carvacrol	249347302	28.85
ϒ-cadinene	1201383	0.09			
Calamenene	602444	0.05			
p-Cymen-8-ol	388408	0.03			
timol acetate	163468	0.01			
p-timol	2137097	0.16			
timol	154571998	11.63			
carvacrol	220709539	16.6			

3.2 Evaluation of MIC and MBC values

The MICs and the MBCs of the oregano EO, thyme EO and carvacrol against *Listeria monocytogenes* Scott A, *Salmonella Enteritidis* E5, *Escherichia coli* 555, *S. aureus* F1, in relation to inoculation levels of the target microorganisms were assessed after incubation at 37°C (Table 4.2). The data showed the great antimicrobial activity of the 3 substances against all the chosen target organisms. The Gram-positive species considered resulted more sensitive to oregano and thyme EO with respect to Gram-positive ones. The Gram-negative species showed, with inoculation level of 2 log cfu/mL, MBC values at 48h lower or equal than 250 ppm. However they increased with the inoculation level. Carvacrol showed a higher effectiveness with respect to the two EOs showing MIC values at inoculation level of 6 log cfu/mL lower than 275 ppm independently on the species. Concerning thyme and oregano EOs, the positive effect of inoculation level on MIC and MBC values of all the target microorganisms, was evident; also for carvacrol there was the same effect with the exception of *E.coli* and *Salmonella*.

Table 4.2- *Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oregano EO, thyme EO and carvacrol against* L. monocytogenes, E. coli, S. Enteritidis *and* S. aureus *in relation to the inoculum level*

Carvacrol						
cell concentration	**6 log cfu/mL**	**6 log cfu/mL**	**4 log cfu/mL**	**4 log cfu/mL**	**2 log cfu/mL**	**2 log cfu/mL**
MIC/MBC	**MIC 24h (mg/L)**	**MBC (mg/L)**	**MIC 24h (mg/L)**	**MBC (mg/L)**	**MIC 24h (mg/L)**	**MBC (mg/L)**
Listeria	175	225	150	200	100	200
Escherichia coli	200	225	200	200	200	200
Salmonella	200	250	175	200	175	200
S. aureus	225	275	200	250	150	200

Oregano EO						
cell concentration	**6 log cfu/mL**	**6 log cfu/mL**	**4 log cfu/mL**	**4 log cfu/mL**	**2 log cfu/mL**	**2 log cfu/mL**
MIC/MBC	**MIC 24h (mg/L)**	**MBC (mg/L)**	**MIC 24h (mg/L)**	**MBC (mg/L)**	**MIC 24h (mg/L)**	**MBC (mg/L)**
Listeria	175	225	175	225	125	150
Escherichia coli	350	350	300	325	250	250
Salmonella	325	350	300	325	250	250
S. aureus	275	300	250	250	225	250

Thyme EO						
cell concentration	**6 log cfu/mL**	**6 log cfu/mL**	**4 log cfu/mL**	**4 log cfu/mL**	**2 log cfu/mL**	**2 log cfu/mL**
MIC/MBC	**MIC 24h (mg/L)**	**MBC (mg/L)**	**MIC 24h (mg/L)**	**MBC (mg/L)**	**MIC 24h (mg/L)**	**MBC (mg/L)**
Listeria	325	500	275	400	200	225
Escherichia coli	475	475	400	400	350	350
Salmonella	400	475	325	350	250	300
S. aureus	350	475	300	425	225	250

3.3 Effects of natural antimicrobials on lamb's lettuce microbial spoilage

On the basis of the MIC values and the preliminary evaluation of sensorial acceptability, thyme EO (250 ppm), oregano EO (250), a mixture of thyme/oregano (125/125 ppm) and a mixture of oregano/carvacrol (125/100 ppm) were chosen to be used as alternative to chlorine in the washing solution of lamb's lettuce. Lamb's lettuce washed with chlorine solution (120 ppm) was used as controls. The products were then packaged and stored at 6°C. During the refrigerated storage the evolution of mesophylic aerobic bacteria, lactic acid bacteria and yeasts was performed. Also the presence of pathogenic species such as *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli* and *Staphylococcus aureus* was evaluated the end of refrigerated storage. The results obtained for mesophylic aerobic bacteria are shown in Table 4.3. No significant differences with respect to control samples were evidenced. In fact, the behavior of cell loads of mesophylic aerobic bacteria was similar in the different samples independently on the washing solution used. No significant differences were observed for yeasts and lactic acid bacteria whose cell loads (data not shown) remained lower than 3.0 log cfu/g during the 8 d of storage at 6°C. Concerning the pathogens, *L. monocytogenes* and *Salmonella* spp. were absent in 25 g of products while *E. coli* and *S. aureus* were always under the detection limits (1 log cfu/g) after 8 days of storage independently on the whishing solution used.

Table 4.3- *Mesophylic aerobic bacteria count on lamb's lettuce treated with EOs or chlorine*

	day 0	day 2	day 3	day 6	day 8
Control[1]	5.1±0.32a	6.3±0.41ab	6.4±0.21ab	6.5±0.45ab	5.9±0.47ab
Thyme EO[2]	5.6±0.41ab	6.5±0.25ab	6.8±0.29ab	6.8±0.57ab	5.9±0.41ab
Oregano EO[3]	5.6±0.23a	7.2±0.66b	7.3±0.52b	6.6±0.44ab	5.4±0.61a
Oregano EO/Thyme EO[4]	5.6±0.27ab	7.2±0.43b	7.5±0.31b	5.9±0.47ab	5.0±0.43a
Carvacrol/Oregano EO[5]	5.7±0.35ab	6.9±0.22ab	6.8±0.24ab	6.6±0.38ab	6.4±0.42ab

Counts are expressed in Log cfu/mL (+/− standard deviation). Means followed by different letters are significantly different ($p<0.05$)

[1] Control was washed with 125 ppm of chlorine

[2] Concentration employed 250 ppm.

[3] Concentration employed 250 ppm.

[4] Concentration employed 125 ppm each.

[5] Concentration employed 125 ppm each.

3.4 Effects of natural antimicrobials on volatile molecule profiles

The effects of the substances used in the washing solution instead of chlorine on volatile molecule profiles of lamb's lettuce were studied analysing samples during storage at 6°C by GC-MS-SPME. This method allowed the identification of 41 molecules belonging to different chemical classes and to obtain specific volatile fingerprinting in relation to antimicrobial used in the washing solution. In fact, the natural antimicrobial used affected significantly the product volatile molecule profiles. The most significant molecules detected are reported in Table 4.4.

Table 4.4 *Volatile aroma compounds (expressed as Area 10^{-5}) detected in lamb's lettuce treated with different solutions during the storage time at 6°C*

	Control[a]			Oregano[b]			Thyme[c]			Oregano/Thyme[d]			Oregano/Carvacrol[e]		
Compounds	T0	T3	T7	T0	T3	T7	T0	T3	T7	T0	T3	T7	T0	T3	T7
Isovaleric acid, ethyl ester	1.1	0	0	1.7	2.1	0	2.2	0	12.7	0	0	11.8	1.2	0	15.2
Isovaleric acid, 2-methylbutyl ester	0	0	0	0	0	1.9	0	0	1.9	0	1.7	3.3	0	0	4.2
Total Esters	**1.1**	**0**	**0**	**1.7**	**2.1**	**1.9**	**2.2**	**0**	**14.6**	**0**	**1.7**	**15.1**	**1.2**	**0**	**19.4**
Hexane	1.2	0	0	0	0	0	0.5	0.7	0	0	0	2.1	0	0.7	0
E-3-Octene	4.4	0	0.9	2.7	1.2	0	1.3	0	1.3	1.1	0	0	1.6	1.1	0.8
β-myrcene	0	0	0	12.8	1.9	0.5	13.9	3.8	1.2	12.6	2.4	0.4	1.0	0	0
α-terpinene	0	0	0	13.2	11.1	9.4	3.3	3.8	1.7	7.1	8.9	0.7	11.1	3.6	0
dodecane	7	5.8	5.8	6.7	7.1	8.3	4.6	7.3	6.1	6.5	6.2	8.5	6.5	2.7	8.5
Butane, 1-chloro-3-methyl-	0.8	1	0.9	0	0	0	0	0	0	0	0	0	0	0	0
Limonene	0	0	0	3.9	3.5	2.4	16.7	14.8	13	35.9	39.6	21.8	3	1.2	1.3
γ-terpinene	0	0	0	75.2	74.7	59.1	251.9	247.3	221.1	186.7	195.4	71.1	54.5	21	23.1
p-Cymene	0	0	0	33.3	30.4.6	25.3.7	345.5	328.1	280.5	31.4.1	31.6.8	141.3	28.3.8	10.5.7	87
Decane, 2,5,9-trimethyl-	2	2.1	2	0	0	0	0	0	0	0	0	0	0	0	0
Terpinolene	0	0	0	5.5	5.8	5.5	5.5	6	5.5	5.9	6.1	5.2	4	1.3	3.9
Total Hydrocarbons	**15.4**	**9**	**9.6**	**45.3**	**40.9.9**	**33.8.9**	**64.3.2**	**61.1.7**	**53.0.4**	**56.9.9**	**57.5.5**	**25.1.1**	**37.4.4**	**13.7.3**	**12.4.6**
1-Penten-3-one, 4-methyl-	1.9	0	1.3	1.2	0.9	1.2	0	0	0.8	0.9	0.8	0.8	0.8	0	1.3
Total Ketones	**1.9**	**0**	**1.3**	**1.2**	**0.9**	**1.2**	**0**	**0**	**0.8**	**0.9**	**0.8**	**0.8**	**0.8**	**0**	**1.3**
Ethanol	0	0.7	2.3	10.3	10.2	4.8	16.9	13.6	16.8	8.9	4.5	16.8	9.6	6.7	13.6
1-Hexanol	1.6	20	18.7	8.8	10.3	8.8	3.9	2.4	4.9	3.5	7.3	3.2	9.7	14.1	20.7
3-Hexen-1-ol	7.2	9.6	10.3	4.3	6.9	9.7	7.7	5.3	1.0	6.5	8.4	6.8	6.5	6.9	9.5
1-Hexanol, 2-ethyl-	0.7	1.1	0.7	0.3	0.6	0.2	0	0	0	0	0	0	0	0	1.2
Phenylethyl Alcohol	3.6	3.7	4.3	3.7	4.9	4.6	3	3.5	3.4	5.3	3.7	7.4	5.2	2.9	4.9
Thymol	0	0	0	0.5	0	0	62.6	14.3	0	16.2	3	0	0	0	0
Carvacrol	0	0	0	29.2	1.9	1.6	4.9	0	0	33	6.2	1.8	2.8	1.8	2.2
Total Alcohols	**27.5**	**35.1**	**36**	**57.2**	**34.9**	**29.7**	**99**	**39.1**	**35.1**	**73.4**	**33**	**36**	**59**	**32.5**	**52.1**
2-Hexenal, (E)-	33.1	19.8	9.1	23.6	12.5	1.5	18.1	14.8	0.2	22.9	1.3	17.8	26.5	12.9	0
Total Aldehydes	**33.1**	**19.8**	**9.1**	**23.6**	**12.5**	**1.5**	**18.1**	**14.8**	**0.2**	**22.9**	**1.3**	**17.8**	**26.5**	**12.9**	**0**
	79.1	**63.9**	**56**	**536.7**	**460.2**	**373.2**	**762.5**	**665.6**	**581**	**667.1**	**612.2**	**320.8**	**461.8**	**182.6**	**197.4**

[a] Control was washed with 125 ppm of chlorine
[b] Concentration employed 250 ppm.
[c] Concentration employed 250 ppm.
[d] Concentration employed 125 ppm each.
[e] Concentration employed 125 ppm each.

Immediately after packaging the main components of thyme and oregano EO were also the main components detected in the head space of samples with these EOs added in the washing solution instead of chlorine. Moreover, carvacrol, thymol, p-cymene, α-pinene, caryophyllene and γ-terpinene remained the main components also during the storage, even if the percentages of some of them decreased probably due to the microbial and vegetable tissue detoxification mechanisms. In particular, thymol and carvarvacrol were the molecules subjected to the highest decrease during the storage.

To better evaluate the effects of antimicrobials used on product volatile molecule profiles the GC-MS-SPME data were subjected to a Principal Component Analysis (PCA). All the samples were mapped in the space spanned by the first two principal components PC1 versus PC2 that explain 47.6 and 22.6% of the variance. The score and loading plot, reported in Figure 4.4a and 4.4b, show the clustering of the samples according mainly to storage time. In fact, the samples were grouped in 4 clusters. The first cluster, well separated by the others on the basis of PC1 that explain about 50% of variance, was composed by the control samples independently on storage time. Control samples were grouped on the basis of 2-(*E*)-hexenal and butane 1-chloro-3-methyl, the latest, probably a deriving from chlorine added during the washing process. The other clusters were separated from each other along PC2, that accounted for 22.6% of variance. The second cluster included all the samples washed with natural antimicrobials, alone or in combination, immediately after packaging. This cluster was grouped mainly by the molecules present in the EOs added, as carvacrol, thymol and b-myrcene, and that showed a rapid decrease of their amount during the storage. The third cluster grouped all the samples treated with thyme, oregano or carvacrol after 3 days of storage. In this case, the samples were characterized by *p*-cymene, γ-terpinene and limonene. These molecules were components of the EOs added, and their amount was quite stable during storage. In the last cluster (cluster 4) were grouped all the samples after 8 days of storage except the control and the sample added with oregano EO/carvacrol after 8 days of storage. This cluster was characterized by the presence of isovaleric acid 2-methylbutyl-ester and isovaleric acid, ethyl ester. These molecules are produced through detoxification mechanisms adopted by vegetable tissues and naturally occurring microorganisms. Only the samples added with oregano EO/carvacrol after 8 days of storage were not included in the other clusters, and they were well separated from the other samples mainly on the basis of PC2.

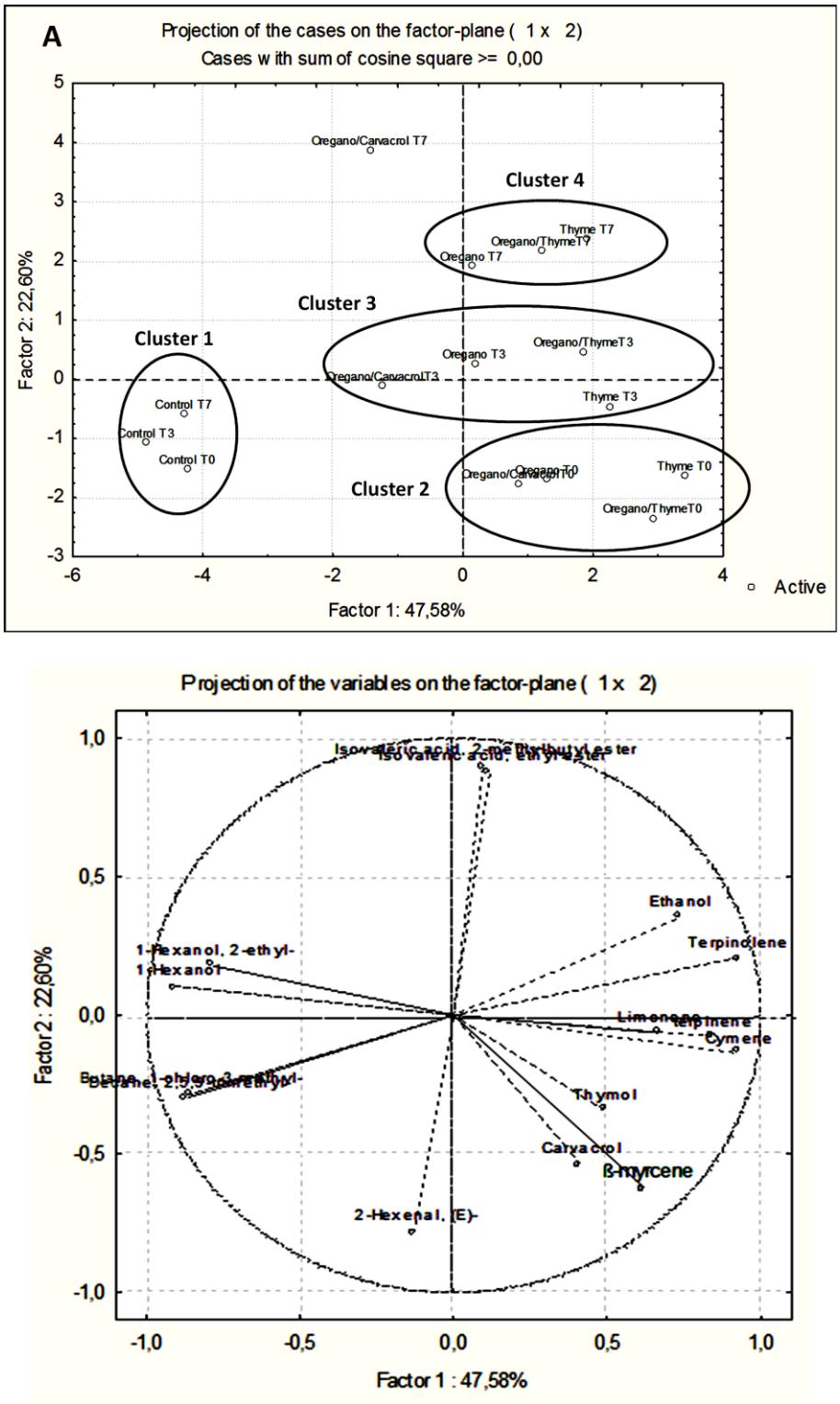

Figure 4.4 a, b *Projection of the cases (3a, samples treated with different natural antimicrobials at different times of storage) and loadings (3b molecules detected by GC/MS/SPME) on the factor-plane (1x2). PC1 and PC2 explained 47.58% and 22.60% of the total variance respectively*.

3.5 Effects of natural antimicrobials on color and withering index

During the storage at 6°C the changes of colour indices of lamb's lettuce in relation to dipping solution used were monitored. As evidenced by Table 4.5, relative to L, a and b values, after 3 days of storage no significant differences were observed among control and treatments, while after 5 days the mixture oregano/carvacrol induced a significant decrease of the product color quality, while thyme and oregano used at concentration of 250 ppm and their mixture showed performances not significantly different from chlorine. In addition, thyme used alone analogously to chlorine, did not affect negatively the retention of turgidity of the products (Figure 4.5) during 5 days of storage at 6°C. On the contrary, a significant turgidity loss was observed after 3 days in samples dipped in thyme-oregano Eos and after 5 days in samples dipped in oregano and oregano-carvacrol EOs.

Table 4.5- *Color parameters of lamb's lettuce treated with EOs or chlorine, immediately after treatments and after 3 and 5 days of storage*

	L*			a*			b*		
	day 0	day 3	day 5	day 0	day 3	day 5	day 0	day 3	day 5
Control[1]	40.4 ±2.0	40.5 ±1.8	37.4 ±3.3	-9.9 ±1.3	-8.9 ±0.5	-9.0 ±0.9	21.3 ±3.6	19.1 ±1.3	18.7 ±2.3
Thyme EO[2]	40.8 ±3.5	41.0 ±1.9	39.4 ±3.1	-9.5 ±0.9	-9.4 ±0.7	-8.5 ±0.9	20.1 ±3.6	20.7 ±2.6	17.7 ±3.6
Oregano EO[3]	41.7 ±2.0	40.3 ±2.4	38.4 ±2.7	-9.8 ±1.1	-8.9 ±0.7	-8.9 ±1.4	21.0 ±2.6	19.2 ±1.9	19.2 ±3.1
Oregano EO/Thyme EO[4]	41.3 ±3.1	38.9 ±1.9	37.6 ±2.4	-9.7 ±0.7	-9.1 ±0.7	-8.1 ±0.9	21.2 ±2.5	20.2 ±1.9	17.9 ±1.9
Carvacrol/Oregano EO[5]	40.5 ±1.3	41.3 ±3.6	34.6 ±2.4	-8.9 ±1.0	-9.3 ±0.9	-7.4 ±1.3	19.0 ±2.4	20.3 ±2.9	15.0 ±1.8

Values are reported with +/− standard deviation.

[1] Control was washed with 125 ppm of chlorine
[2] Concentration employed 250 ppm.
[3] Concentration employed 250 ppm.
[4] Concentration employed 125 ppm each.
[5] Concentration employed 125 ppm each.

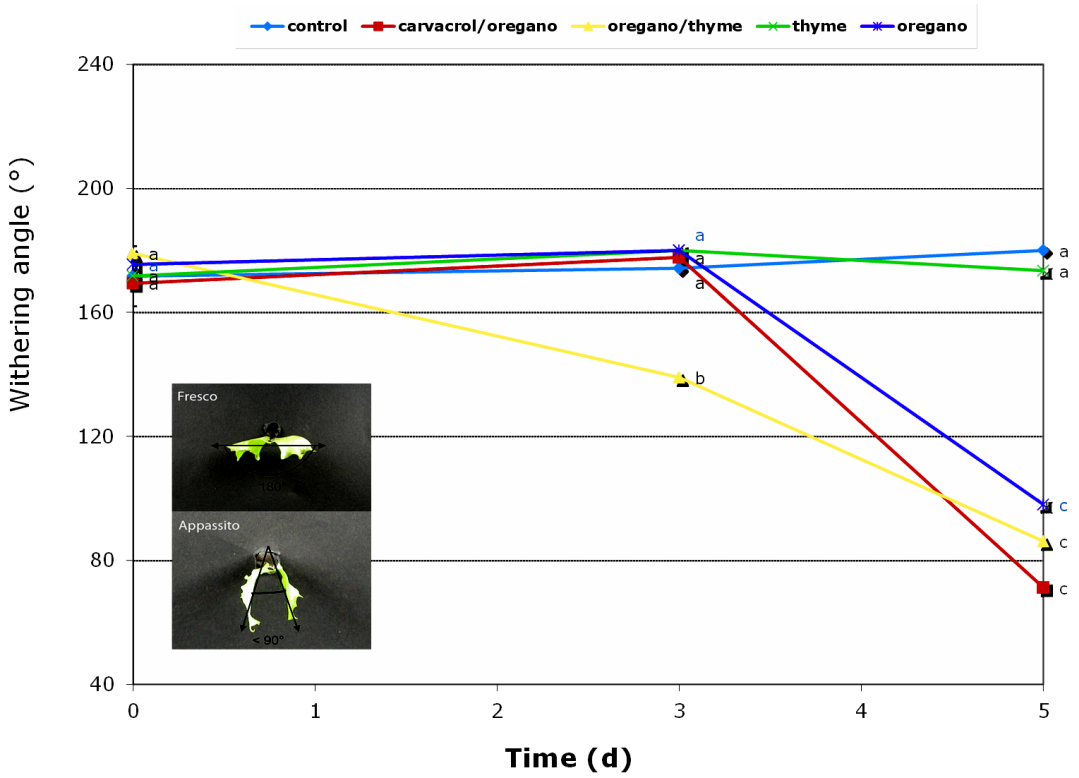

Figure 4.5- *Whitening index determined after 3 and 5 days of storage, it represent an index of the freshness of the products. Means followed by different letters are significantly different (p<0.05).*

5. Results of the second experimental phase: effects of selected natural antimicrobials on minimally processed lamb's lettuce after optimization of the washing process and packaged in artificial ordinary atmosphere

5.1 Effects of natural antimicrobials on lamb's lettuce microbial spoilage

On the basis of the results of the first experimental phase, only thyme EO (250 ppm) and oregano EO (250 ppm), were considered to be used as alternative to chlorine in the washing solution of lamb's lettuce. Also in this experiments, lamb's lettuce washed with chlorine solution (120 ppm) was used as controls. As reported in material and methods, an optimization of the washing process was carried out by performing a pre-washing with tap water at 8 °C for 1 min, increasing the temperature of washing solution (from 8 to 13°C) and augmenting the ratio between product and washing solution. The products after washing were packaged in artificial ordinary atmosphere and stored at 6°C. During the refrigerated storage the evolution of mesophylic aerobic bacteria, lactic acid bacteria and yeasts was performed. Also the presence of pathogenic species such as *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli* and *Staphylococcus aureus* was evaluated the end of refrigerated

storage. The results obtained for mesophylic aerobic bacteria are shown in Figure 4.6. No significant differences with respect to control samples were showed. In fact, the behavior of cell loads of mesophylic aerobic bacteria was similar in the different samples independently on the washing solution adopted. No significant differences were observed for yeasts and lactic acid bacteria whose cell loads ranged between 2 and 3 log CFU/g during all the storage period. Concerning the pathogens, *L. monocytogenes* and *Salmonella* spp. were absent in 25 g of products while *E. coli* and *S. aureus* were always under the detection limits (1 log CFU/g) after 14 days of storage independently on the whishing solution used.

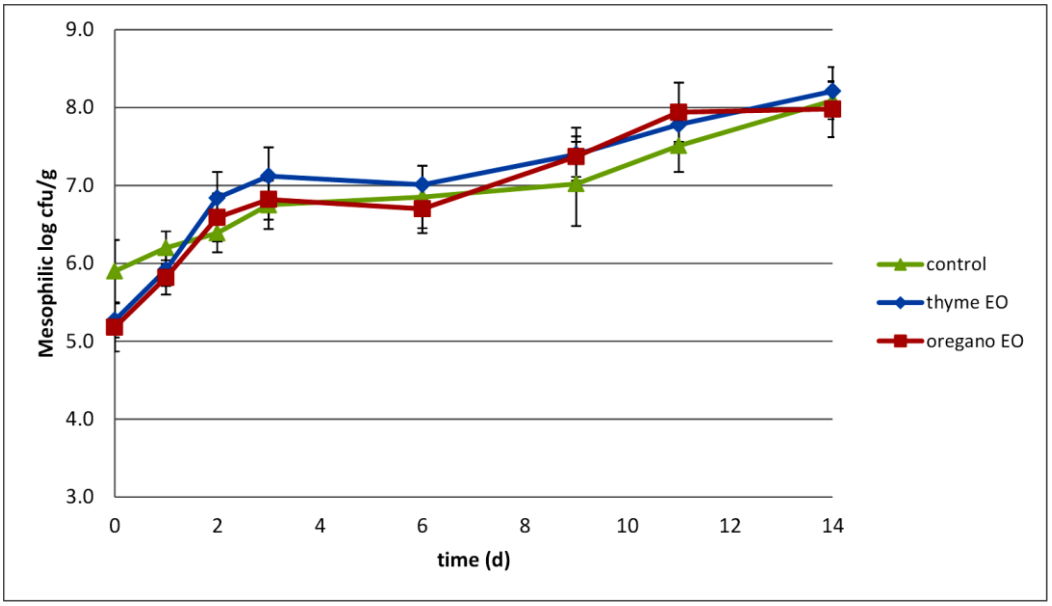

Figure 4.6- *Evolution of the load of mesophilic, in lamb's lettuce added with essential oils and/or bioactive components during storage at 6°C*

5.2 Effects of natural antimicrobials on volatile molecule profiles

The effects of thyme and oregano used in the washing solution instead of chlorine on volatile molecule profiles of lamb's lettuce were studied by GC-MS-SPME analysing samples during storage at 6°C. The most significant molecules detected are shown in Table 4.6. Immediately after packaging the main components of thyme and oregano EO were also in this case the main components detected in the head space of treated samples. Moreover, *p*-cymene, α-terpinene, caryophyllene and γ-terpinene were the main volatile molecules also during the storage and showed a behaviour similar to that observed in the first experimental phase.

Table 4.6- *Volatile aroma compounds (expressed as Area 10^{-5}) detected in the optimization of the washing process of lamb's lettuce treated with different solutions during the storage time at 6°C*

	Control			Oregano			Thyme		
Compounds	T0	T3	T10	T0	T3	T10	T0	T3	T10
dimethylsulfide	0.0	0.9	1.9	0.0	0.8	3.2	0.0	0.0	1.1
4-Hexen-1-ol, acetate	2.0	2.0	1.4	1.4	1.1	1.0	0.0	0.8	0.0
Butanoic acid,3-methyl,3-methylbutyl	2.6	3.6	4.1	3.7	5.4	12.5	2.5	4.2	11.2
Heptanoic acid ethyl ester	0.0	0.0	0.0	7.1	7.2	6.8	10.8	20.1	16.6
Butanoic acid, 3-methyl- ethyl ester	0.0	0.0	0.0	89.2	52.7	48.4	93.5	180.1	71.8
cis-3-Hexenyl isovalerate	2.6	3.6	4.8	2.2	2.8	5.7	2.7	2.7	10.2
Total Esters	**7.2**	**10.1**	**12.2**	**103.6**	**70.0**	**77.5**	**109.5**	**207.9**	**110.8**
1-octene	15.4	10.5	6.9	5.6	3.7	4.6	5.8	4.9	3.0
β-myrcene	0.0	0.0	0.0	42.5	11.1	0.7	26.7	22.6	2.0
α-terpinene	0.0	0.0	0.0	30.6	28.0	15.7	16.4	18.6	11.2
dodecane	15.7	18.5	22.8	15.0	22.5	18.4	8.7	12.0	14.4
Limonene	0.0	0.0	0.0	7.1	6.3	4.1	8.3	8.6	6.2
β-thujene	0.0	0.0	0.0	8.3	6.3	3.7	7.4	6.2	3.5
γ-terpinene	0.0	0.0	0.0	126.0	144.5	91.1	153.0	158.9	123.8
ρ-Cymene	0.0	0.0	0.0	280.6	288.2	204.2	432.3	522.8	375.6
Bicyclo[3.1.1]hept-2-ene-2-methanol,	2.7	5.9	6.2	3.2	8.2	0.0	2.7	5.3	8.6
caryophyllene	0.0	0.0	0.0	13.7	30.7	11.8	24.0	16.7	12.7
Total Hydrocarbons	**33.9**	**34.9**	**36.0**	**532.5**	**549.5**	**354.3**	**685.1**	**776.6**	**561.1**
Ethanol	3.2	2.9	3.3	14.7	19.1	6.1	17.2	22.2	9.7
Benzyl alcohol	2.0	2.3	2.6	2.3	3.2	1.3	1.6	1.2	0.0
1-Hexanol	3.0	3.1	3.2	2.8	3.5	5.5	2.2	2.6	5.5
3-hexen-1-ol	27.7	26.1	25.1	18.7	23.3	21.9	13.0	12.4	17.3
2-Hexen-1-ol	1.0	0.8	0.6	1.2	0.9	0.8	0.0	0.5	0.6
5-Hepten-2-ol, 6-methyl	0.0	2.2	4.7	3.2	4.6	4.3	2.1	3.1	3.8
Phenylethyl Alcohol	24.3	26.2	29.9	27.2	38.3	20.7	19.9	23.6	23.1
Thymol	0.0	0.0	0.0	1.2	0.7	0.0	78.7	2.5	1.6
Carvacrol	0.0	0.0	0.0	47.4	16.7	4.1	3.6	0.0	0.0
Total Alcohols	**61.3**	**63.6**	**69.4**	**118.7**	**110.3**	**64.6**	**138.3**	**68.1**	**61.6**
Benzaldehyde	4.6	5.0	4.9	4.9	8.4	1.9	2.6	3.9	1.5
1,3,5-Heptatriene, (EE)	0.0	2.8	3.7	1.8	4.0	0.0	1.9	3.5	3.4
2-Hexenal, (E)-	33.7	12.5	7.4	19.9	13.5	5.7	9.1	6.1	4.1
Total Aldehydes	**38.3**	**20.3**	**15.9**	**26.6**	**25.9**	**7.5**	**13.6**	**13.6**	**9.0**
Total metabolites	**140.6**	**128.9**	**133.5**	**780.2**	**755.0**	**504.0**	**946.6**	**1066.1**	**742.5**

The PCA of the GC-MS-SPME data showed that all the samples were mapped in the space spanned by the first two principal components PC1 versus PC2 that explain 48.05 and 30.34% of the variance. The score and loading plot, reported in Figure 4.7a and 4.7b, show the clustering of the samples according to substances used. Also the storage time affected, although in minor extent, the clustering of the samples. In fact, three clusters were evident: the first included the control samples that, however, resulted well separated along PC2 in relation to the storage time; the second cluster included the samples treated with thyme and oregano analyzed immediately and after 3 d of storages. The samples treated with oregano resulted well separated along PC2. The third cluster grouped the samples treated with OEs analyzed after 10 d of storage at 6°C.

The variable factor coordinates for the first two factors, reported in Figure 6b, shows the molecules that contributed significantly to the clustering of the samples.

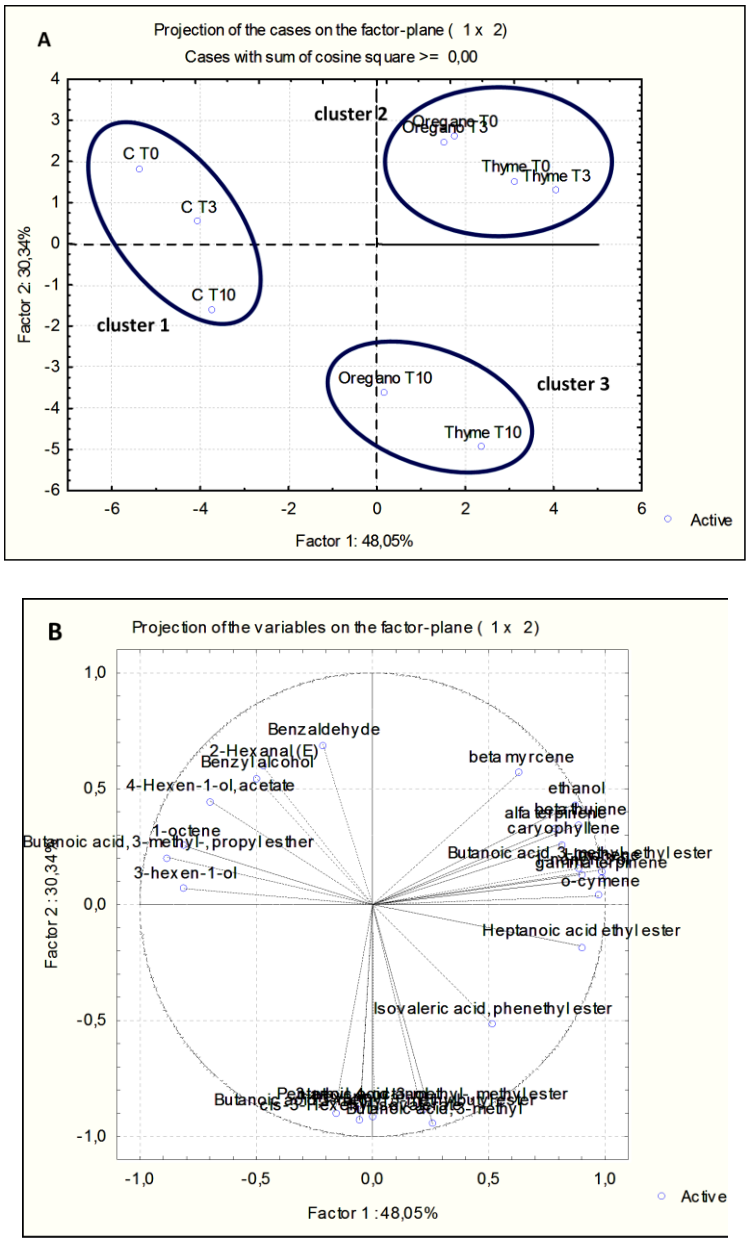

Figure 4.7 a, b- *Projection of the cases (6a, samples treated with different natural antimicrobials at different times of storage) and loadings (6b molecules detected by GC/MS/SPME) on the factor-plane (1x2). PC1 and PC2 explained 48.05% and 30.34% of the total variance respectively*.

The samples were analyzed with electronic nose during the storage at 6°C.The data were subjected to a principal component analysis in order to outline the differences among the samples detected by the 10 sensors of the instrument. All the samples were mapped in the space spanned by the first two principal components PC1 versus PC2. The score and loading plot, reported in Figure 4.8a and 4.8b, showed the clustering of the samples according mainly to storage time and in minor extent on the basis of the added antimicrobial, differently from PCA obtained with GC-MS-SPME data that clustered samples mainly on the basis of added EO. This can be attributed to the minor sensitiveness of the electronic nose with respect to

GC-MS-SPME to the natural antimicrobials and their detoxification products. In fact, three different clusters were evident in the PCA plot, while the control samples analyzed immediately after packaging resulted separated from the other both on the basis of PC1 (explaining 56,47% of variance) and PC2 (explaining 24,75% of the variance). The first cluster grouped the samples supplemented with EOs immediately after packaging. These samples were grouped on the basis of sensors 5 and 7. The second cluster grouped all the samples analyzed after 3 d of storage independently on the EO supplementation due to the sensors 1 and 3. The cluster 3 included the samples analyzed after 10 d of storage and the sensors responsible for the clustering were sensors 4, 9 and 10.

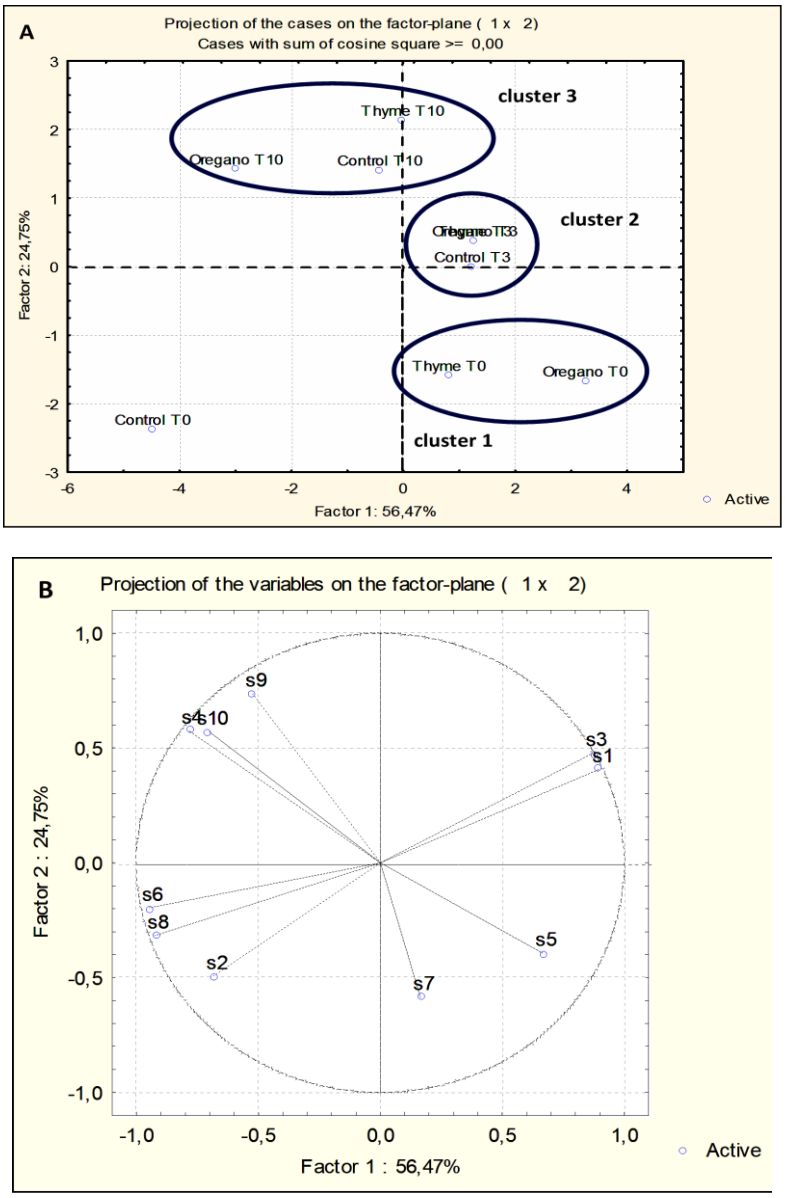

Figure 4.8a, b *Projection of the cases (4.8a, samples treated with different natural antimicrobials at different times of storage) and loadings (4.8b electronic nose sensors) on the factor-plane (1x2). PC1 and PC2 explained 56.47% and 24.75% of the total variance respectively*

5.3 Effects of natural antimicrobials on color and withering index

During the storage at 6°C the changes of colour indices of lamb's lettuce in relation to dipping solution used were monitored. As evidenced by Figure 4.9a and 4.9b, relative to L* and a* values, during storage, no significant differences were observed among control and treatments, only after 2 days of storage a decrease of luminosity in treated samples was detected. Anyway, after 12 days of storage the L* and a* values of treated samples were similar to the controls and to the initial values. In addition, thyme and oregano used did not affect negatively the retention of turgidity of the products (data not shown) during 12 days of storage in modified atmosphere. In fact a slight decrease of the withering index was observedt after 8 days of storage, and was similar to those of the controls.

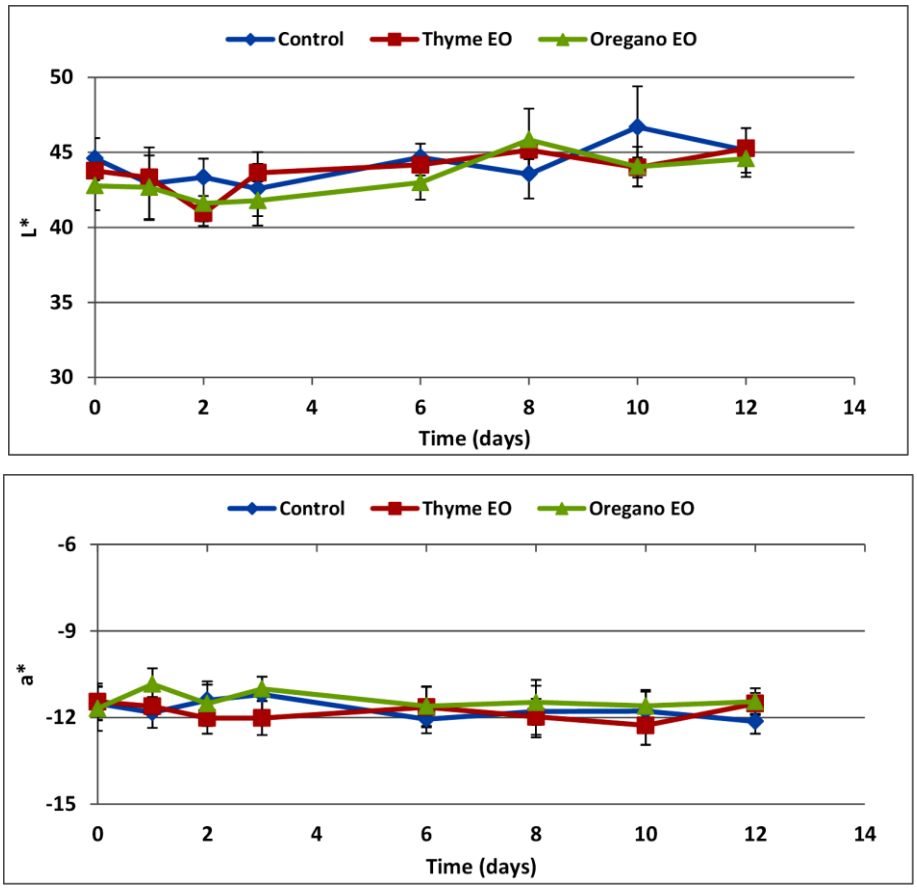

Figure 4.9a, b- *Evolution of luminosity, L*; (4.9a), and red index, a*, (4.9b) of lamb's lettuce treated with chlorine (120 ppm), or thyme EO(250 ppm) or oregano EO (250ppm) and stored at 6°C.*

4. Discussion

Thyme and oregano EOs were characterized by GC-MS-SPME technique because it has been applied increasingly often in the EO analyses (Richter & Schellenberg 2007; Klimankova et al. 2008; Zawirska-Wojtasiak & Wasowicz 2002; Mazida et al. 2005; Belletti et al. 2004; Ndagijimana et al. 2004; Fuselli et al., 2007; Wojtowicz et al. 2010). In addition it evaluates the volatile molecules in the vapor phase of the oil (Belletti et al. 2004) and the preliminary

condition for the antimicrobial effects of EO is the contact between the antimicrobial molecule and the target cells (Gardini et al. 1997; Belletti et al. 2004). The contact is favored if the molecules are in their vapor phase, that correspond to in their most hydrophobic state, because this improve their partition in the microbial cell membranes. In addition, this techniques provides a volatile profile fingerprinting fundamental to standardize both the EO composition in terms of the most effective molecules and their antimicrobial activity. In fact, the EO composition, and consequently the volatile molecule profile, can notably vary with plant variety and origin, extraction modality, agronomic practices, etc (Nannapaneni et al. 2009; Figuereido et al., 2008). The volatile molecule profiles of thyme and oregano EOs were in agreement with those of literature (Ortega-Nieblas et al. 2011; Cosentino et al., 1999; Juven et al., 1994). In fact, although the GC-MS-SPME profile is affected by age, season and developmental state, the literature indicates p-cymene, carvacrol, thymol and γ -terpinene as the main components in oregano and thyme EOs (Johnson et al. 2004; Richter & Schellenberg 2007). A wide literature attribute to carvacrol and to monoterpenes the great antibacterial activity of the considered EOs (Burt, 2004; Dorman & Deans, 2000; Elgayyar et al., 2001; Gutierrez et al., 2008a; Oussalah et al., 2006; 2007; Belletti et al. 2004). In fact, such molecules can interact with some cellular structures causing the inhibition of cell growth or cell death. However according to Caccioni et al. (1998) to evaluate the antimicrobial activity of an EO it is fundamental to use an holistic approach due to synergistic or antagonistic actions among the different EO components.

The MIC and MBC values showed that the natural antimicrobials taken into consideration had great *in vitro* antimicrobial activity against the target chosen microorganisms, although affected by inoculation levels. The effects of inoculation level on MIC and MBC are well known and in agreement with literature (Belletti et al., 2008; Sado Kamden et al., 2011). Also the higher values of MBC with respect to MIC ones are in agreement with literature (da Silva Hughes et al. 2013; Shen et al. 2014). The data showed that the Gram-positive species were more sensitive to thyme and oregano EOs. The different response to EO or their components among Gram-positive and Gram-negative bacteria is already reported in the literature. Gram-negative bacteria are generally resistant to many compounds due to the outer membrane, which acts as an efficient permeability barrier against macromolecules and hydrophobic substances (Helander et al. 1997; Nazzaro et al. 2013), as well as to the high content in cyclopropane fatty acids of the inner membrane (Chang & Cronan 1999; Gardini et a. 2009; Patrignani et al. 2008). In addition, Gram-negative bacteria like *S. enteritidis* have efflux pumps, which are the first defense of bacteria in harsh environment, allowing them to

selectively extrude specific toxic compounds (Yow et al., 2012; Shen et al. 2014). More efficient to pass these barriers seems quit hydrophilic low molecular mass molecule that may have access, throughout porin proteins, to the deeper parts of Gram-negative bacteria without any alteration to the permeability of the outer membrane (Nikaido, 1996; Helander et al., 1997; Lanciotti et al. 2003b).

Carvacrol showed a similar antimicrobial activity against the Gram-positive and Gram-negative species considered. On the other hand several studies indicated that the antimicrobial activity of carvacrol is related, in addition to the permeabilization and depolarization the cytoplasmic membrane, to its ability to break up the outer membrane properties inducing morphology modification (Helander et al.1998; Di Pasqua et al. 2007; La Storia et al. 2011; Lambert et al. 2001; Veldhuizen et al. 2006; Xu et al. 2008; Cristani et al. 2007, Picone et al 2013; Ait-Ouazzou et al. 2013). The inhibition of membrane bound ATPases and the dissipation of pH gradientdue to the treatment with carvacrol were demonstred both in Gram-positive and Gram-negative bacteria (Picone et al. 2013; Gill & Holley, 2006a; 2006b; Ben Arfa et al. 2006).

The GC MS-SPME analyses and the Principal Component Analysis (PCA) showed that the antimicrobials used in the washing solution affected the volatile profile of the products, and the storage time showed a significant effects, indeed the volatile molecule profiles changed over time. In fact, during the storage β-myrcene, carvacrol, 2-(*E*)-hexenal decreased their abundance probably due to the detoxification mechanisms of vegetable tissue and microorganisms present. With except to the control, all the other samples grouped in relation to the time of storage. The color and turgidity data showed that after 5 days of storage, only the mixture oregano/carvacrol induced a significant decrease of the product color quality, while thyme and oregano used at concentration of 250 ppm and their mixture showed performances not significantly different from chlorine. Moreover, the addition of thyme EO did not affect the turgidity of lamb's lettuce after 5 days of storage. This results are in agreement with Gutierrez et al. (2009) that did not find any significant differences in color and textural parameters of lettuce dipped in oregano and oregano-thyme EOs.

The data concerning the optimization of the production process showed that if it is possible to increase further the shelf-life of the product, improving the washing process, introducing a pre-washing step, increasing at 13°C the temperature of the washing solution, increasing the ratio product/washing water to 1:15, and packaging into artificial ordinary atmosphere. In fact, it is well known that the increase of temperature results in the increase of the vapor pressure of volatile molecules composing the essential oils and consequently their affinity for

the cell membranes, main and primary target of antimicrobials (Gardini et al., 1997). In fact, while in the first experimental phase chlorine and the natural antimicrobial showed the same reduction of the naturally occurring microbial population, in the second trial thyme and oregano reduced the cell loads of mesophilic aerobic bacteria of about 1 log cfu/g more than the chlorine solution. However, the differences decreased during the storage and after 3 d no significant differences was observed between control and treated samples. On the other hand some literature reports show that the reduction of the naturally occurring microbiota can favor the growth of numerically less represented and competitive species, including pathogenic species, suggesting the use of biocontrol agents (Bracket, 1992; Schuenzel & Harrison, 2002). In our experimental conditions the initial reduction of the naturally occurring microbiota due to the use of EOs did not affects negatively the safety of the products. In fact, the pathogenic species, most frequently associated to minimally processed vegetables, such as *L. monocytogenes*, *E. coli*, *S. enteritidis* and *S. aureus* were not detected also after 14 d of storage at 6°C. Also de color and the withering data showed that the treatments applied can guarantee the maintenance of the main quality parameters affecting the consumer choice. In fact, by improving the washing process, the products washed with thyme and oregano, similarly to chlorine, were able to maintain good color and turgidity attributes during over 12 days of storage at 6°C. In addition, on the basis of PCA analysis the electronic nose data grouped the samples on the basis of storage time showing that the volatile profiles of the products after the optimization process, perceived by the instrument that mimes the human nose (Wilson, & Baietto, 2009), were quite unaffected by the added antimicrobials. Also the sensorial analysis confirmed that the organoleptic features of the Lamb's lettuce treated with oregano and thyme instead of chlorine was not significantly. Considering that EOs such as thyme and oregano EOs are not only considered powerful antimicrobials but also have antioxidant features (Misharina et al., 2009; Graßmann et al., 2000; Bakkali et al., 2008), they could be used not only to increase safety and shelf-life but also to increase the health benefits of the products. Although these results are very promising and with great applicative potential for the minimally processed vegetable manufacturing, a consumer test seem necessary to better evaluate the consumer acceptance of this innovative minimally processed lettuce.

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CHAPTER 5

Lactic acid bacteria and natural antimicrobials to improve safety and shelf-life of minimally processed sliced apples and lamb's lettuce

Abstract

Fruits and vegetables are strongly recommended in the human diet due to their content in vitamins, antioxidants, minerals and dietary fibers. However, outbreaks of food-borne disease associated to the consumption of fresh and minimally processed fruits and vegetables have increased dramatically since the 1970s. In fact, a wide literature shows the presence of pathogenic species on fresh produce and related minimally processed products. The application of decontamination methods is the most important tool to guarantee the safety and shelf-life of minimally processed products. Several chemical sanitizers have been employed to decontaminate raw materials. However, a wide literature showed, in addition to their potential toxicity, their inability to completely eradicate or kill microorganisms on fresh produce. These reasons have stimulated the research of alternative methods to decrease minimally processed fruits and vegetables decay and increase the product safety. The use of protective cultures, and especially lactic acid bacteria (LAB), has been proposed for their potential application in minimally processed fruits and vegetables. However, the application of bioprotective cultures at industrial level for commercial products is scarce because satisfactory conditions under laboratory settings are unable to guarantee the success under real processing and distribution conditions. In this perspective, the main aims of this research were i) to isolate, identify and characterize LAB from minimally processed fruits and vegetables and select some strains to be used as biocontrol agents in the same products; ii)to evaluate the effects of the selected strains on the shelf-life and safety features of minimally processed apples and lamb's lettuce; iii)to evaluate the combined effects of the most effective biocontrol agents and natural antimicrobials such as hexanal, 2-(E)-hexenal, citral and thyme EO on minimally processed lamb's lettuce and apple safety and shelf-life. The results showed that the use of the strains *Lactobacillus plantarum* CIT3 and V7B3, respectively, on apples and lettuce provided encouraging results regarding the safety and shelf life of minimally processed products considered.

1. Introduction

Fruits and vegetables are strongly recommended in the human diet due to their content in vitamins, antioxidants, minerals and dietary fibers. They are generally consumed fresh, minimally processed, pasteurized or cooked by boiling in water or microwaving. Heat treatments, although they increase product safety and shelf-life, decrease the nutritional properties and sensorial features of the raw materials, while fresh produce and minimally

processed products are characterized by a very short shelf-life since subjected to rapid microbial spoilage (Zia-ur-Rehman et al., 2003; Zhang & Hamauzu, 2004; Di Cagno et al. 2008). In addition, outbreaks of food-borne disease associated to the consumption of fresh and minimally processed fruits and vegetables, primarily due to *Escherichia coli* O157:H7, *Salmonella* spp and *Listeria monocytogenes*, have increased dramatically since the 1970s (Harris et al., 2003; CDC, 2007; Powell & Luedtke, 2000; Abadias et al., 2011; Olaimat & Holley, 2012; Van Boxstael et al. 2013). In fact, a wide literature shows the presence on fresh produce and related minimally processed products of pathogenic species such as *Listeria monocytogenes*, *Salmonella* spp., *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Staphylococcus aureus* (Beuchat, 1998; Conway et al., 2000; Gunes & Hotchkiss, 2002; Alegre et al., 2010; Francis et al., 1999; Ilic et al., 2008; Froder et al., 2007). Nowadays, modified atmosphere packaging and refrigeration are the most utilized tools to improve shelf-life of minimally processed fruits and vegetables delaying microbial growth and physiological degradation of vegetable tissues (King et al. 1991; Gomez-Lopez et al. 2007; Alegre et al. 2010; Siddiqui et al. 2011). The application of decontamination methods is another tool to reduce the microbial cell loads of the raw materials with positive effects on product safety and shelf-life (Rico et al., 2007; Gomez-Lopez et al., 2008; Manzocco et al., 2011; Ramos et al., 2013). Presently, chlorine is the most broadly used among the washing and sanitizing agents available for fresh produce. However, a wide literature shows that it has a limited antimicrobial efficacy, allowing, at the permitted concentrations, 1–2 log reductions in the bacterial population of raw materials, associated to the production of potentially toxic substances (Beuchat, 1998; Brackett, 1999; Abadias et al., 2008). Also other disinfectants such as hydrogen peroxide, organic acids and ozone have been used to reduce the natural occurring microorganisms of raw fruits and vegetables (Beuchat, 1998; EU Scientific Comitee on Food, 2002; Alegre et al. 2013). However, a wide literature showed, in addition to their potential toxicity, their inability to completely eradicate or kill microorganisms on fresh produce (Koseki & Itoh, 2001; Park et al., 2001; Alegre et al. 2013). The washing procedures are able to remove from raw fruits and vegetables only a part of spoilage or pathogenic microbial cells; the remaining part can survive to the sanitizing agents attached to the surfaces of raw material (Allende et al., 2008; Sapers et al., 2001; Takeuchi & Frank, 2001). Peeling, slicing, and shredding of fresh produce stimulate the growth of survived microorganisms transferring them to inner tissues and releasing nutrients (Lanciotti et al. 2003a; King & Bolin, 1989). In addition, the reduction of naturally occurring population throughout washing and sanitization can reduce the competition for space and nutrients against human pathogenic

species (Brackett, 1992; Schuenzel & Harrison, 2002). The consumer concern about chemical synthetic additives, perceived as negative for human health and environmental pollution (Ayala-Zavala et al., 2008; Roller & Lusengo, 1997), has stimulated the research of alternative methods to decrease minimally processed fruits and vegetables decay and increase the product safety. The use of protective cultures has been proposed for their potential application in minimally processed fruits and vegetables (Schillinger et al., 1996; Bennik et al., 1999; Rodgers, 2001). Protective cultures of lactic acid bacteria (LAB) to increase safety and shelf-life have been developed in last decades (Vescovo et al. 1996; Bennik et al. 1999; Leroy et al. 2003; Palmai & Buchanan 2002). For example Torriani et al. (1997) and Scolari and Vescovo (2004) showed the potential of a strain of *Lactobacillus casei* to increase the safety of minimally processed vegetables due to the inhibition of *Aeromonas hydrophila*, *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes*. Selected strains of *Pseudomonas syringae*, *Pseudomonas graminis*, *Gluconobacter asaii*, *Candida* spp., *Dicosphaerina fagi* and *Metschnikowia pulcherrima* showed great potential as biocontrol agents in minimally processed fruits due to their ability to antagonize under laboratory conditions several foodborne pathogens (Leverentz et al., 2006; Abadias et al., 2009; Trias et al., 2008a, 2008b; Alegre et al., 2012, 2013). However, the application of bioprotective cultures at industrial level for commercial products is scarce because satisfactory conditions under laboratory settings are unable to guarantee the success under real processing and distribution conditions (Trias et al., 2008a; Abadias et al., 2009). However, some Authors showed that microorganisms isolated from the same commercial type of products can better succeed to control spoilage end pathogenic microorganisms (Vescovo et al. 1996; Breidt & Flemming 1997; Reina et al., 2006; Rodgers, 2008). In this perspective the main aims of this research were: i) to isolate, identify and characterize lactic acid bacteria from minimally processed fruits and vegetables and select some strains to be used as biocontrol agents in the same products; ii) to evaluate the effects of the selected strains on the shelf-life and safety features of minimally processed apples and lamb's lettuce; iii) evaluate the combined effects of the most effective biocontrol agents and the natural antimicrobials selected in during the first experimental phase (chaper 3 and 4) on minimally processed lamb's lettuce and apple safety and shelf-life.

2. Material and Methods

2.1 Isolation and identification of lactic acid bacteria from minimally processed apples and lamb's lettuce

Samples of commercial slides apples and minimally processed lamb's lettuce were obtained from a local market. Ten grams of each vegetable was suspended in 90 mL of sterile sodium chloride (0.9%, w/v) solution and homogenized with a Stomacher for 2 min at room temperature. Serial dilutions were made, plated on MRS agar (Oxoid Ltd., Basingstoke, England), and incubated at 30 °C for 48– 72 h under anaerobic conditions, for isolating presumptive mesophilic lactic acid bacteria. In the case of lamb's lettuce, an enrichment onto MRS broth of 24 h at 30°C was necessary in order to isolate lactic acid bacteria. Serial dilutions of the enrichment cultures were then plated on MRS agar. Different colonies, possibly with different morphology, were isolated from the MRS plates. Gram-positive, catalase-negative, non-motile rods and cocci were cultivated in MRS broth at 30 °C for 24 h, and restreaked onto MRS agar. Stock cultures were stored at −20 °C in 10% (v/v) glycerol.
Genomic DNA from each strain of presumptive lactic acid bacteria was extracted as described by Balcàzar et al. (2007). Thirty-nine representative isolates were identified by RAPD-PCR (primer M13) and sequencing of the 16S rRNA region by following the protocol reported by De Angelis et al. (2006).

2.2 Phenotypic characterization and evaluation of antagonistic activity of identified LAB

The identified strains were characterized on the basis of the capability to grow in different environmental conditions such as different temperatures (4, 8, 15 and 30°C), different levels of sodium chloride (2, 4 and 6%), high concentrations of sucrose (20%) and low pH values (3.5, 4.0 and 4.5). The strains, grown overnight, were inoculated at a level of approximately 5 log cfu/mL in tubes with 10 mL of MRS broth for the evaluation of growth at different temperatures or supplemented with the selected concentrations of NaCl or sucrose. Regarding to the conditions at low pH values, glacial acetic acid was used to reach the selected pH values. The inoculated tubes (5 repetitions for each condition) were stored at the expected temperatures, while in case of the addition of NaCl, sucrose and different pH values, the tubes were stored at 30°C. The growth of the strains was evaluated on the basis of the optical density at 600nm (OD_{600}) using a spectrophotometer UV-1204 (Shimadzu, Kyoto, Japan). If

the growth was not observed, the viability of the strains was verified by counting without any dilution on MRS agar plates.

For the evaluation of the ability of the identified LAB to antagonize the pathogenic strains *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enteritidis*, the method reported by Schillinger and Lucke (1989) was followed.

2.3 Preparation of minimally processed apples and lamb's lettuce added of the selected LABs

In a first experimental phase, on the basis of the preliminary results the strains M3B6, CIT3 and V4B4 and V7B3 were chosen as potentially biocontrol agents for minimally processed apples and lamb's lettuce, respectively. The protocols used to prepare apples and lamb's lettuce minimally processed products were the same reported in chapter 3 (Figure 3.2) and 4 (Figure 4.2) for the process optimization. The chosen LABs were inoculated at level of about 7 log cfu/mL in dipping and washing solutions, for apple and lamb's lettuce, respectively. In some conditions, *Listeria monocytogenes* and *Escherichia coli* were inoculated in the washing or dipping solutions at levels ranging between 2.5 to 3.5 log cfu/g. Six different conditions were considered for apples and seven for lamb's lettuce (Table 5.1). After the treatments, apples were dried with paper, packaged in active modified atmosphere with 7% O_2 and 0% CO_2 and stored at 6 °C until the end of shelf-life. After the different treatments, lettuce was spin dried and packaged in artificial ordinary atmosphere, and then stored at 6°C until the end of shelf-life.

Table 5.1- *Conditions tested on apples and lamb's lettuce; the inoculation of the selected LABs was at a level of 7 log cfu/mL in dipping or washing solutions; when provided by the experimental plan, the pathogenic microorganisms were inoculated at a level ranged between 2.5 to 3.5 log cfu/mL in dipping or washing solutions. The products were dipped (apples) or washed (lettuce) for two minutes.*

Tested Conditions	
Apples	**Lamb's lettuce**
1. dipping (0.5% ascorbic acid; 1.0% citric acid	1. washing water+ *Lb. Casei* (V4B4)
2. dipping+ pathogens (*L. monocytogenes, E. coli*)	2. washing water+ *Lb. plantarum* (V7B3)
3. dipping+ *Lb. Casei* (M3B6)	3. washing water+ pathogens (*L. monocytogenes, E. coli*)
4. dipping+ *Lb. Plantarum* (CIT3)	4. washing water+*Lb. Casei* (V4B4)+ pathogens (*L. monocytogenes, E. coli*)
5. dipping+ *Lb. Plantarum* (CIT3)+pathogens (*L. monocytogenes, E. coli*)	5. washing water+*Lb. Plantarum* (V7B3)+ pathogens (*L. monocytogenes, E. coli*)
6. dipping+ *Lb. casei* (M3B6)+pathogens (*L. monocytogenes, E. coli*)	6. washing water+Chlorine 120ppm
	7. washing water+Chlorine 120ppm+pathogens (*L. monocytogenes, E. coli*)

In a second phase, the combination of natural antimicrobials and lactic acid bacteria were tested on apples and lamb's lettuce. On the basis of the results obtained in chapter 3 and 4 and in the first experimental part of this chapter, the *Lb. plantarum* CIT3 for apples and V7B3 for lamb's lettuce were selected to be used in combination with 2-(E)-hexenal/hexanal and

citral/2-(E)-hexenal in apple dipping and with thyme essential oil in lamb's lettuce washing solution. The same protocols used in chapter 3 (Figure 3.2) and 4 (Figure 4.2) for the optimization of the processes of apples and lamb's lettuce were used to produce minimally processed apples and lettuce. Also in this phase, challenge tests with *Escherichia coli* and *Listeria monocytogenes* were performed. When provided by the experimental plan, the pathogenic microorganisms were inoculated in the washing or dipping solution at levels ranging between 3.5 to 4.5 log cfu/g. The supplementation of the biocontrol agents and/or natural antimicrobials and/or pathogens occurred in the dipping or in the washing solution for apples and lamb's lettuce, respectively. All the conditions employed in this experimental phase, both for apples and lettuce, are reported in Table 5.2.

Table 5.2- *Conditions tested on apples and lamb's lettuce; the inoculation of the selected LABs were at a level of 7 log cfu/mL; the pathogenic microorganisms were inoculated at a level ranged between 3.5 and 4.5 log cfu/g. Thyme EO was added at a concentration of 250ppm; while 2-(E)-hexenal/hexanal and citral/2-(E)-hexenal were used at 125ppm for each compound. LABs and/or antimicrobial compounds and/or pathogens were supplemented in the dipping and washing solution for apples and lamb's lettuce, respectively.*

Tested Conditions	
Apples	**Lamb's lettuce**
1. dipping (0.5% ascorbic acid; 1.0% citric acid	1. washing water+Chlorine 120ppm
2. dipping+2-(E)-hexenal/hexanal	2. washing water+Chlorine 120ppm+pathogens (*L. monocytogenes, E. coli*)
3. dipping+2-(E)-hexenal/hexanal+pathogens (*L. monocytogenes, E. coli*)	3. washing water+*Lb. Plantarum* (V7B3)
4. dipping+2-(E)-hexenal/hexanal+pathogens+*Lb. Plantarum* (CIT3)	4. washing water+*Lb. Plantarum* (V7B3)+ pathogens (*L. monocytogenes, E. coli*)
5. dipping+2-(E)-hexenal/hexanal+*Lb. Plantarum* (CIT3)	5. washing water+*Lb. Plantarum* (V7B3)+ pathogens+thyme EO
6. dipping+2-(E)-hexenal/citral	6. washing water+*Lb. Plantarum* (V7B3)+thyme EO
7. dipping+2-(E)-hexenal/citral+pathogens (*L. monocytogenes, E. coli*)	7. washing water+thyme EO
8. dipping+2-(E)-hexenal/citral+pathogens +*Lb. Plantarum* (CIT3)	8. washing water+ pathogens (*L. monocytogenes, E. coli*)+thyme EO
9. dipping+2-(E)-hexenal/citral+*Lb. Plantarum* (CIT3)	9. washing water+ pathogens (*L. monocytogenes, E. coli*)
10. dipping+ pathogens (*L. monocytogenes, E. coli*)	

The samples, packaged in active modified atmosphere with 7% O_2 and 0% CO_2 and in artificial ordinary atmosphere for apples and lettuce, respectively, were stored at 6°C until the end of the shelf-life.

2.4 Microbiological analyses

During the refrigerated storage, the evolution of LAB, yeasts and mesophylic aerobic bacteria was evaluated by plate counting, respectively, on de Man Rogosa and Sharpe Agar (MRS, Oxoid Ltd. Basingstoke, United Kingdom) with added cycloheximide (Sigma-Aldrich), Sabouraud Dextrose Agar (SAB, Oxoid Ltd.), added to chloramphenicol (Sigma-Aldrich) and Plate Count Agar (PCA, Oxoid Ltd., Basingstoke, United Kingdom). After homogenization, samples were serially diluted in physiological solution (10 g of sample diluted into 90 mL of

physiological water (0.9% (w/v) NaCl). The lactic acid bacteria were incubated at 37°C for 48h; yeasts and mesophylic aerobic bacteria were incubated at 30°C for 48h. The detection of the inoculated pathogens *Listeria monocytogenes* and *Escherichia coli* was evaluated by plate counting on Listeria Selective Agar Base (LSO, Oxoid) added to Selective Listeria Supplement (Oxoid) and Violet Red Bile Agar (Oxoid) added to 4-Methylumbelliferyl-β-D-glucuronide (Oxoid), respectively. The incubation was performed at 37°C for 24h. In the first experimental phase, microbiological analyses were performed immediately after treatments and after 2, 5, 7, 9, 13 and 16 days of storage in the case of apples, while for lamb's lettuce immediately after treatments and after 2, 5, 7 and 9 days of storage. In the second experimental phase, the analyses were performed immediately after treatments and after 3, 7, 10, 13, 15, 22, and 27 days of storage for apples and immediately and after 2, 5, 7, 12 and 15 days of storage for lamb's lettuce.

3. Results and Discussion

3.1 Identification and characterization of lactic acid bacterial from minimally processed apples and lamb's lettuce

Commercial apple and lamb's lettuce were analyzed in order to isolate lactic acid bacteria (LAB). Apple samples showed LAB cell loads ranging between 2 and 4 log cfu/g while lamb's lettuce samples had LAB counts lower than 1 log cfu/g. Consequently, for lettuce samples, an enrichment procedure was necessary to isolate LAB. A total of 15 and 55 strains were isolated respectively from lamb's lettuce and apple samples. Gram-positive, catalase-negative, non-motile cocci and rods, oxidase negative able to grow on MRS agar, randomLy isolated from the highest plate dilution of each products or enrichments, were identified by partial sequencing of the 16S rRNA. The following species were identified for minimally processed apples: *Leuconostoc mesenteroides* (10 isolates), *Lactobacillus plantarum/pentosus* (14 isolates), *Weissella soli* (2 isolates), *Lactobacillus casei/paracasei/rhamnosus* (6 isolates), *Lactococcus lactis* (1 isolate). *Lactobacillus casei/paracasei/rhamnosus* (1 isolate) and *Lactobacillus plantarum* (5 isolates) were identified on lamb's lettuce samples.

The LAB identified were screened for bacteriocin production using an agar overlay spot test method and to antagonize *Salmonella enteritidis*, *Listeria monocytogenes* and *Escherichia coli*. None of the identified strains showed the ability to produce bacteriocin. However, some of them were able to inhibit the pathogenic species considered. The most promising, as reported in Table 5.3, were two *Leuconostoc mesenteroides* strains (M5B7 and M19B25), two

strains of *L. plantarum* (CIT3 and ESA3) and the strain of *L. paracasei* M3B6 isolated from minimally processed apples; among the strains isolated from minimally processed lamb's lettuce, two strains of *Lactobacillus casei/paracasei* (V4B4 and V4B5) and *Lactobacillus plantarum* V7B3 have shown the best performances.

Table 5.3- *Inhibitory activity of the identified strains on* Escherichia coli, Salmonella enteritidis *and* Listeria monocytogenes.

		Inhibition of indicator strains[a]		
Strain	**Identification**	***E.coli***	***Salmonella***	***Listeria***
CE+CA 1	*L. plantarum / L. pentosus*	++	+	+
CE+CA 2	*L. plantarum / L. pentosus*	-	-	++
CE+CA 4	*L. plantarum / L. pentosus*	+	-	+
CE+CA 5	*L. plantarum / L. pentosus*	-	+	-
CI+CE 2	*L. pentosus / L. plantarum*	+	+	-
CIT1	*Lactobacillus plantarum*	++	-	-
CIT2	*L. plantarum / L. pentosus*	++	++	-
CIT3	*Lactobacillus plantarum*	++	+++	+++
ESA+T2 1	*L. plantarum / L. pentosus*	-	-	+
ESA+T2 2	*L. plantarum / L. pentosus*	++	+	+
ESA1	*Lactobacillus plantarum*	+	++	-
ESA2	*L. plantarum / L. pentosus*	++	+	+
ESA3	*Lactobacillus plantarum*	++	+++	++
M10G19	*Lactococcus lactis subsp. Lactis*	+	-	+
M11G8	*Leuconosto mesenteroides*	-	-	-
M16B21	*Leuconostoc mesenteroides*	-	+++	+
M16B22	*Leuconostoc mesenteroides*	++	-	-
M17B23	*Leuconostoc mesenteroides*	++	+	+
M17G20	*Leuconostoc mesenteroides*	+	-	-
M19B24	*Leuconostoc mesenteroides*	-	-	+
M19B25	*Leuconostoc mesenteroides*	+	+++	++
M2B2	*Leuconostoc mesenteroides*	-	+	+
M2G1	*Weissella soli*	-	+	++
M2G2	*Weissella soli*	+++	+	+++
M2P2	*Lactobacillus plantarum*	-	-	-
M3B6	*Lactobacillus paracasei*	+	+++	+++
M3G4	*Lactobacillus casei/paracasei*	-	++	+++
M3G5	*Lactobacillus casei/paracasei*	-	-	-
M5B7	*Leuconostoc mesenteroides*	++	++	++
M5B8	*Leuconostoc mesenteroides*	-	++	+++
M7B16 1	*Lactobacillus paracasei*	-	++	+++
M7B16 2	*Lactobacillus casei/paracasei*	++	+	-
M8B18	*Lactobacillus casei/paracasei*	-	-	++
V 7	*Lactobacillus casei/paracasei/*	-	-	-
V4B4	*Lactobacillus casei/paracasei*	+	+++	+++
V4B5	*Lactobacillus plantarum/acidophilus*	++	++	+++
V4B6	*Lactobacillus plantarum*	-	+++	++
V7B3	*Lactobacillus plantarum*	++	++	+++
V9	*L. plantarum / L. pentosus*	-	-	-

[a] Symbols: -No inhibition zone; +small inhibition zone (0.5 to 1 mm); ++medium inhibition zone (1 to 2 mm); +++large inhibition zone (>2mm)

The identified strains were characterized also for their physiological features, such as the ability to grow at low temperature, low pH and aw values, able to give a competitive advantage in real systems. The results, reported in Table 5.4, showed that all the strains were able to grow at 30°C within 24h, the major part of them grew within 5-9 days at 15°C while, at 4°C, only 3 strains were able to growth within 7 days. None of the strains tested was inhibited by 2 and 4% NaCl and 20% sucrose attaining cell loads higher than 8 log cfu/mL within 48 h of incubation at 30°C. In the presence of 6% NaCl at 30°C, all the stains, except three, grew within 5 days attaining cell load levels higher than 8 log cfu/mL. Low pH values resulted in the most stringent hurdles. In fact, all the strains were unable to grow at pH 3.5 within 20 days of incubation at 30°C. However, at pH 4.0 and 4.5 15 strains grew within 48 h.

Table 5.4- *Characterization of the identified lactic acid bacteria strains for their ability to growth in different conditions*

		Temperature				NaCl[a]			pH[b]			Sucrose[c]
Strain	**Identification**	**30°C**	**15°C**	**8°C**	**4°C**	**2%**	**4%**	**6%**	**4.5**	**4**	**3.5**	**20%**
M2B4	*Leuconostoc mesenteroides*											
M5B7	*Leuconostoc mesenteroides*											
M5B8	*Leuconostoc mesenteroides*											
M16B22	*Leuconostoc mesenteroides*											
M19B24	*Leuconostoc mesenteroides*											
M19B25	*Leuconostoc mesenteroides*											
M17G20	*Leuconostoc mesenteroides*											
M11G8	*Leuconosto mesenteroides*											
M16B21	*Leuconostoc mesenteroides*											
M17B23	*Leuconostoc mesenteroides*											
M3G4	*Lactobacillus casei/paracasei*											
M7B16 2	*Lactobacillus casei/paracasei*											
M8B18	*Lactobacillus casei/paracasei*											
M3G5	*Lactobacillus casei/paracasei*											
M3B6	*Lactobacillus paracasei*											
M7B16 1	*Lactobacillus paracasei*											
M10G19	*Lactococcus lactis subsp. Lactis*											
M2G1	*Weissella soli*											
M2G2	*Weissella soli*											
M2P2	*Lactobacillus plantarum*											
ESA1	*Lactobacillus plantarum*											
ESA3	*Lactobacillus plantarum*											
CIT1	*Lactobacillus plantarum*											
CIT3	*Lactobacillus plantarum*											
ESA2	*L. plantarum / L. pentosus*											
CIT2	*L. plantarum / L. pentosus*											
CED+CARV1	*L. plantarum / L. pentosus*											
CED+CARV2	*L. plantarum / L. pentosus*											
CED+CARV4	*L. plantarum / L. pentosus*											
CED+CARV5	*L. plantarum / L. pentosus*											
CIT+CEDRO 2	*L. pentosus / L. plantarum*											
ESA+T2 1	*L. plantarum / L. pentosus*											
ESA+T2 2	*L. plantarum / L. pentosus*											
V9	*L. plantarum / L. pentosus*											
V4B6	*Lactobacillus plantarum/acidophilus*											
V7	*Lactobacillus plantarum*											
V7B3	*Lactobacillus plantarum*											
V4B5	*Lactobacillus casei/paracasei*											
V4B4	*Lactobacillus casei/paracasei/*											

Legend: [a,b and c]) The samples were incubated at 30°C

3.2 Effects of selected LAB, used as biocontrol agents, on microbiological quality of minimally processed apples and lamb's lettuce

On the basis of this preliminary results, the strains M3B6 and CIT3 and V4B4 and V7B3 were chosen as potentially biocontrol agents for minimally processed apples and minimally processed lamb's lettuce, respectively. They were inoculated at a level of about 7 log cfu/mL in dipping and washing solutions, for apple and lamb's lettuce, respectively.

In order to assess the ability of the selected biocontrol agents to antagonize pathogenic microorganisms in real products and under real process conditions, during the dipping or washing phase, in some conditions *Listeria monocytogenes*, and *Escherichia coli* were inoculated.

As evidenced from the Figure 5.1, the lactic acid bacteria used as biocontrol agents remained viable during the whole storage period considered, independently on the presence or the absence of the deliberately inoculated pathogenic microorganisms. In fact, in the samples added with the biocontrol agents the loads of lactic acid bacteria were always higher than 6 log cfu/g. After 16 days of storage at 6 °C, all samples inoculated with the biocontrol agents, showed LAB loads higher than 7 log cfu/g of product. By contrast, in the control samples not containing added biocontrol agents, the loads of lactic acid bacteria were at the end of storage less than 3 log cfu/g of product.

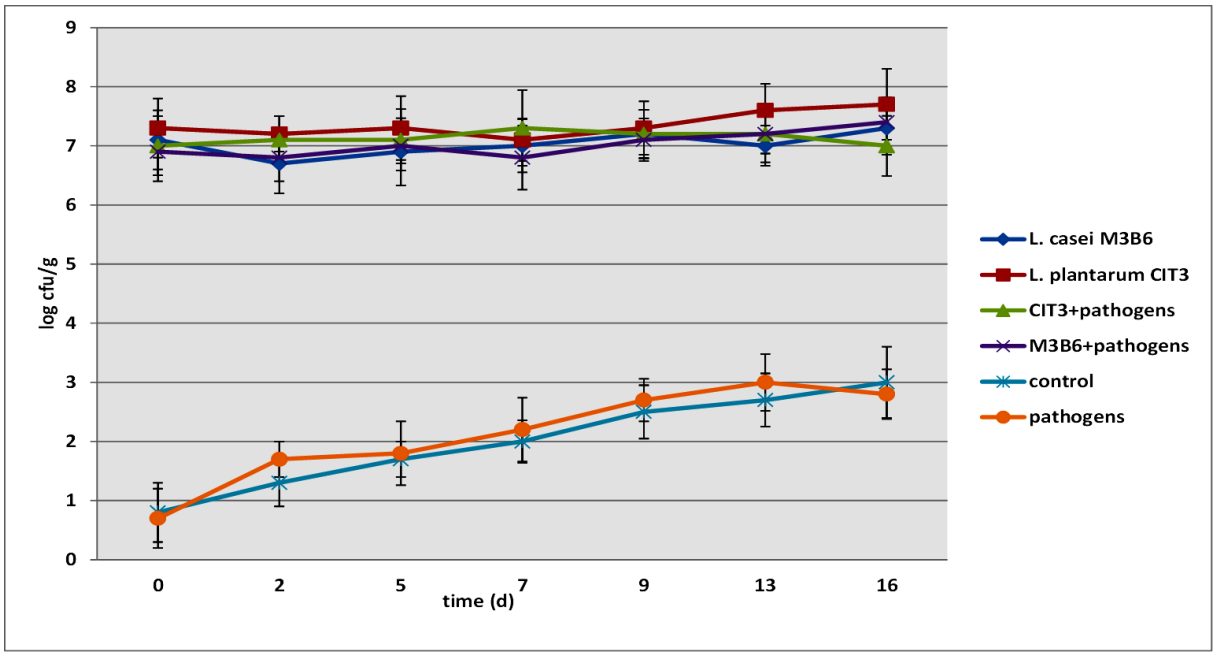


Figure 5.1- *Evolution of the lactic acid bacteria in sliced apples in relation to the addition of pathogenic species and/or* Lb. plantarum *(CIT3) or* Lb. casei *(M3B6)*

Regarding to the evolution of yeasts, no significant differences, in relation to the presence of biocontrol agents, were evidenced with the exception of the strain of *Lactobacillus paracasei* (M3B6). In fact, the latter allowed to keep the yeast cell load after 16 days of storage at 6 ° C at levels of about 1 logarithmic cycle lower compared to the other conditions. In these conditions, the yeast loads remained below the threshold of spoilage, that is considered for sliced apples 6 log cfu/g, even after 16 days of storage (data not shown).

The *Lactobacillus plantarum* CIT3 showed a significant inhibition against *Escherichia coli* and *Listeria monocytogenes* (Figures 5.2 and 5.3). In fact, this biocontrol agent accelerated significantly the kinetics of death of *Escherichia coli*, that, after 7 days, was below the limit of detection in products inoculated with *Lactobacillus plantarum* CIT3. Furthermore, this strain allowed to inhibit the growth of *Listeria monocytogenes* until the end of storage time. Also the strain *Lactobacillus paracasei* (M3B6) accelerated, although in a more limited extent, the death kinetics of *Escherichia coli* and exerted an inhibitory effect on *Listeria monocytogenes* in the first 9 days of storage at 6 °C. However, also the presence of biocontrol agents cannot guarantee the complete inactivation of *Listeria monocytogenes* when present at levels higher than the initial 1.5 log cfu/g.

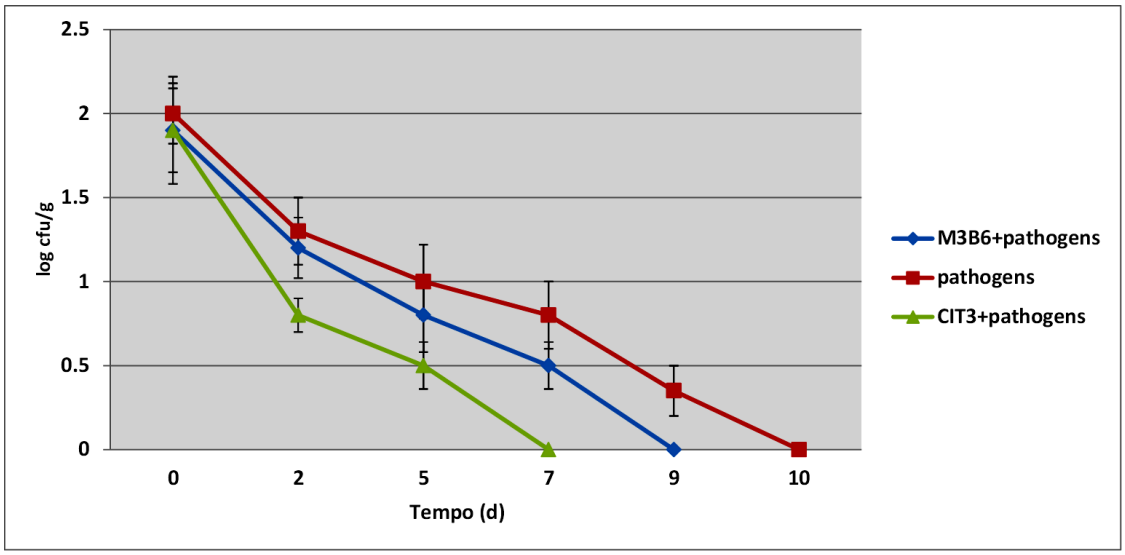


Figure 5.2- *Evolution of* Escherichia coli *(log cfu/g) inoculated in sliced apples in relation to the washing conditions and lactic strain adjunct*

These levels are quite unusual in real conditions of processing and real products. However, it is well known that *Listeria monocytogenes* is a psychrotrophic microorganism and it is able to grow, in the absence of other obstacles, also at refrigeration temperatures although with very low growth rate. This rate can increase dramatically if the product undergoes to thermal abuse (Beuchat, 2002). Therefore, biocontrol agents considered, and especially *Lb. plantarum* CIT3, seem to be, at least up to 16 days of refrigerated storage, an effective hurdle to the multiplication of *L. monocytogenes*. Moreover, the two biocontrol agents considered seem to increase the safety against *E. coli*, another pathogen frequently associated with fresh-cut products .

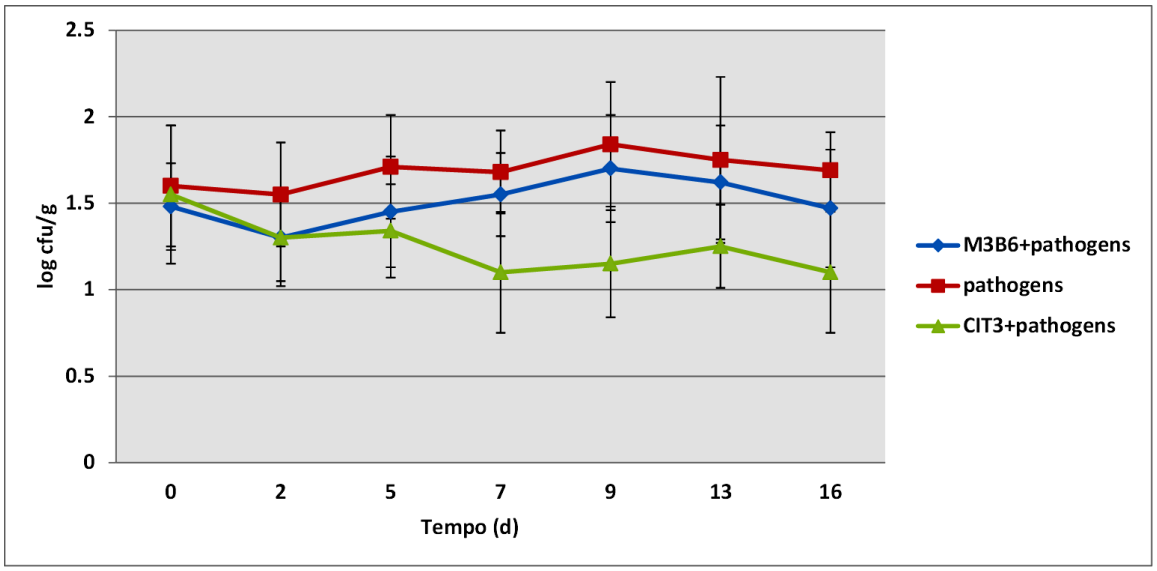
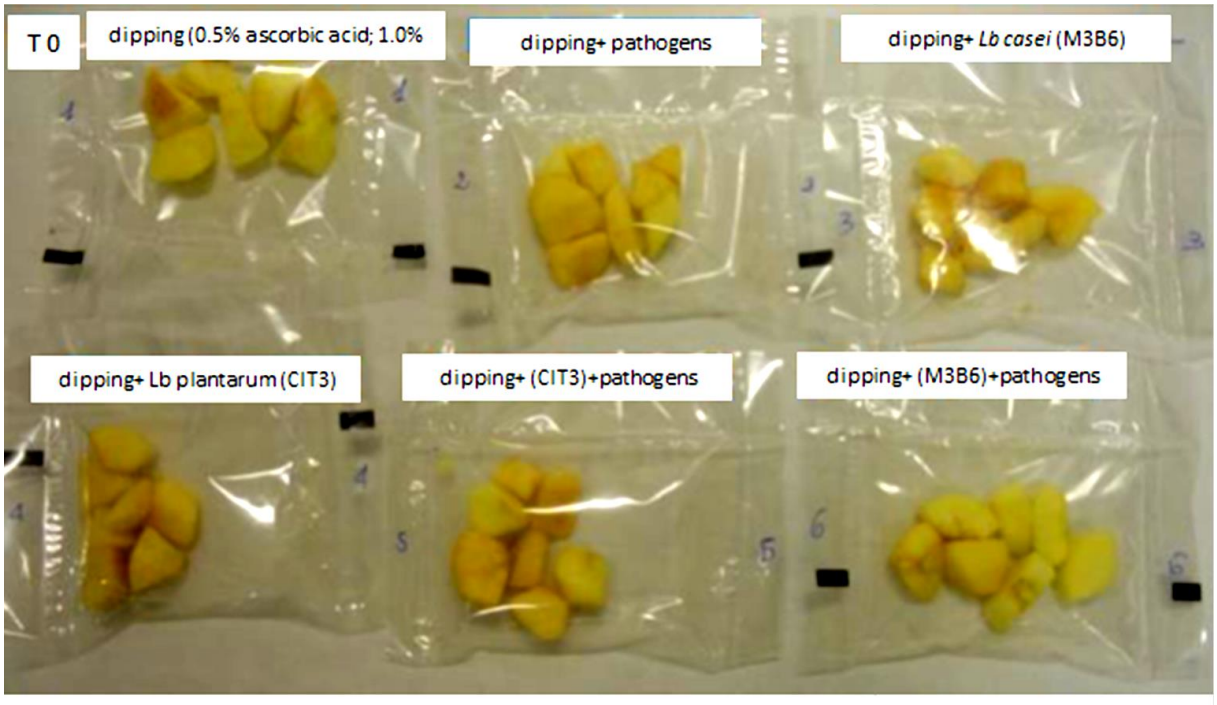


Figure 5.3- *Evolution of* Listeria monocytogenes *(log cfu/g) inoculated in sliced apples in relation to the washing conditions and lactic strain adjunct*

Although the considered strain of *E. coli* was not able to grow even in the control samples, where it showed a slow loss of viability, the addition of biocontrol agents fastened the death kinetics of the target microorganism. The data obtained are in agreement with those of the literature. In fact, similar effects on *Escherichia coli* and *Listeria monocytogenes* were obtained under laboratory conditions with selected strains of *Pseudomonas syringae*, *Pseudomonas graminis*, *Gluconobacter asai*, *Candida* spp and *Metschnikowia pulcherrima* inoculated as biocontrol agents in fresh-cut fruits (Harris et al., 2003; Beuchat, 2002; Trias et al., 2008a; Leverentz et al., 2006). However, the literature data do not exhaustively explain the effects of biocontrol agents on the spoilage microflora and more generally on the shelf-life of products. It is well known that the shelf life of fresh-cut fruits is mainly determined by the changes in color and texture of the product (Soliva-Fortuny & Martin-Belloso, 2003). The data obtained showed that the biocontrol agents considered (and especially *the Lactobacillus plantarum* CIT3) were able to inhibit significantly the growth of yeasts, but may negatively affect the sensory characteristics of the produce as evidenced by the pictures of apples taken during the storage (Figure 5.4). The presence of biocontrol agents leads to a more premature browning of products. However, the color also in the samples inoculated with the biocontrol agents remained acceptable up to 7 days of storage at 6 ° C.



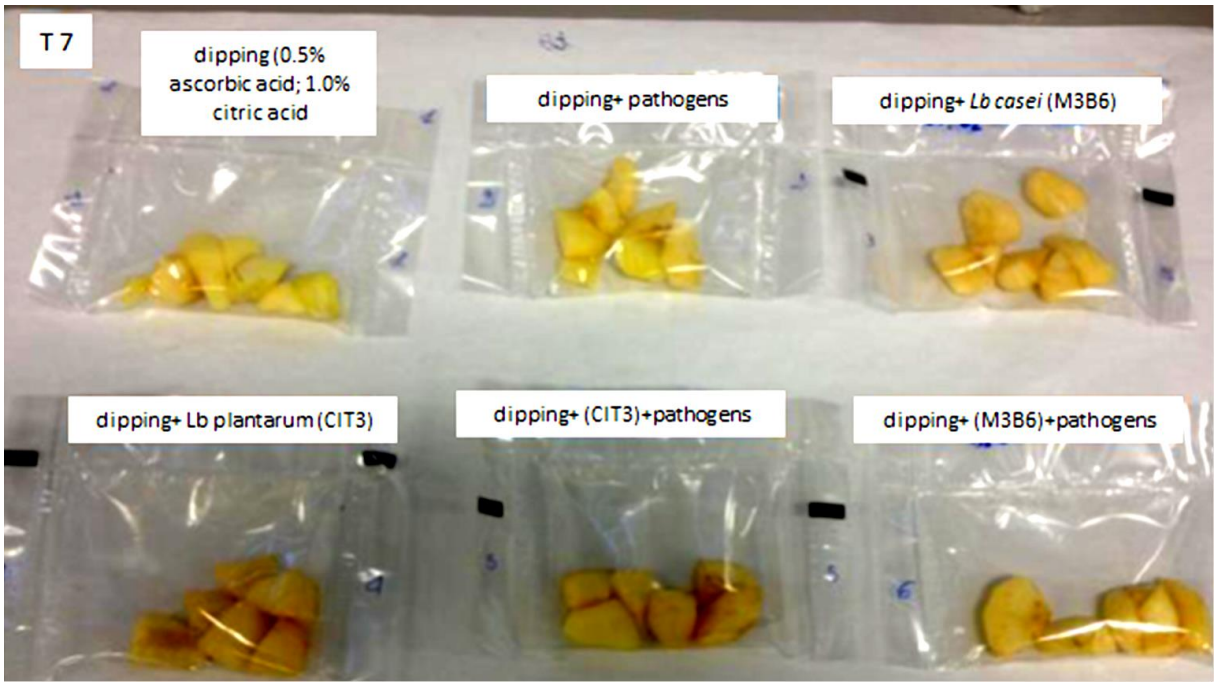


Figure 5.4- *Images of apples subjected to different washing conditions and added with pathogens and/or* Lb. plantarum *(CIT3) or* Lb. casei *(M3B6) immediately and after 7 days of storage*

Lb. casei (V4B4) and *Lb. plantarum* (V7B3) showed excellent adaptability to the stringent conditions of the minimally processed lamb's lettuce. In fact, in all the conditions considered lactic acid bacteria maintained cell loads similar to the inoculation levels for the whole storage period (Figure 5.5).

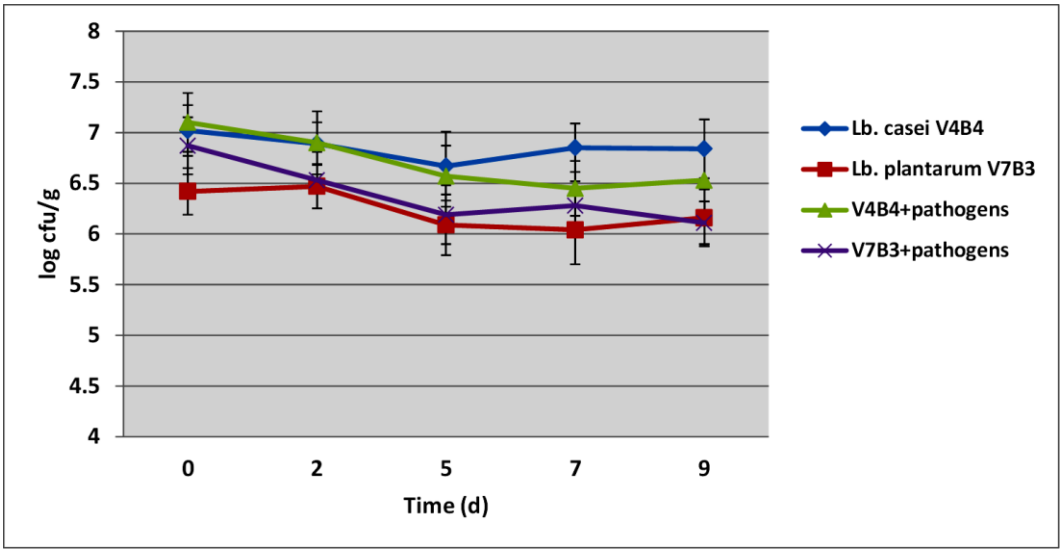


Figure 5.5- *Evolution of the lactic acid bacteria, in minimally processed lamb's lettuce in relation to the addition of pathogenic species and/or* Lb. plantarum *(V7B3) or* Lb. casei *(V4B4). The samples added only with pathogens, chlorine and chlorine+pathogens showed a load of LAB below the limit of detection (1 log cfu/g)*

Regarding the effect on the spoilage microflora, the two biocontrol agents caused a reduction of the levels of the total aerobic mesophilic similar to that of chlorine in the samples not inoculated with pathogens. The strain of *Lactobacillus plantarum* V7B3 determined a significant reduction of the mesophilic bacteria also in the samples inoculated with the pathogenic microorganisms considered (of about 1 logarithmic cycle). In contrast, the other samples inoculated with the considered pathogenic microorganisms showed a higher level of the total aerobic mesophilic bacteria. The differences highlighted immediately after washing, in relation to the agent of biocontrol considered and the presence or absence of pathogenic microorganisms inoculated, were attenuated during the storage period. After 9 days of storage in all samples the total aerobic mesophilic cell loads ranged between 6.5 and 7.5 log cfu/g of product. The lowest values were recorded in the presence of the biocontrol agent V7B3 that showed an effectiveness against the spoilage microflora similar to that exercised by traditional disinfectants (data not shown).

The strain V7B3 showed great potential also for the control of the considered pathogenic microorganisms (Figure 5.6 and 5.7). In fact, the presence of the strain V7B3 increased the death kinetics of *Escherichia coli* and induced a viability loss also in *Listeria monocytogenes*. *L. monocytogenes* was able to grow although very slowly in the samples washed only with water without the addition of chlorine or the biocontrol agent V7B3, and in the presence of biocontrol agent V4B4. In fact, *Listeria monocytogenes* increased its load of about 0.8-0.9 logarithmic cycles also in the control samples washed with chlorine inoculated with the target microorganisms. The inoculated strain of *Escherichia coli* lost viability under all conditions adopted even if with different kinetics. However, its cell load, even after 9 days of storage, was not lower than 2 log cfu/g. Even in this case, the inoculum levels of the pathogenic microorganisms considered were above those encountered in real conditions. Therefore, the results obtained on lamb's lettuce clearly indicate the good potential of the strains of lactic acid bacteria selected and in particular the strain V7B3, to control the spoilage microflora and to inhibit pathogenic microorganisms. In addition, the appearance and the color of the products were not affected by the addition of the lactic acid bacteria. In fact, after 9 days of storage the samples treated with the biocontrol agents had the same turgidity of the controls treated with chlorine in the washing step (Figure 5.8).

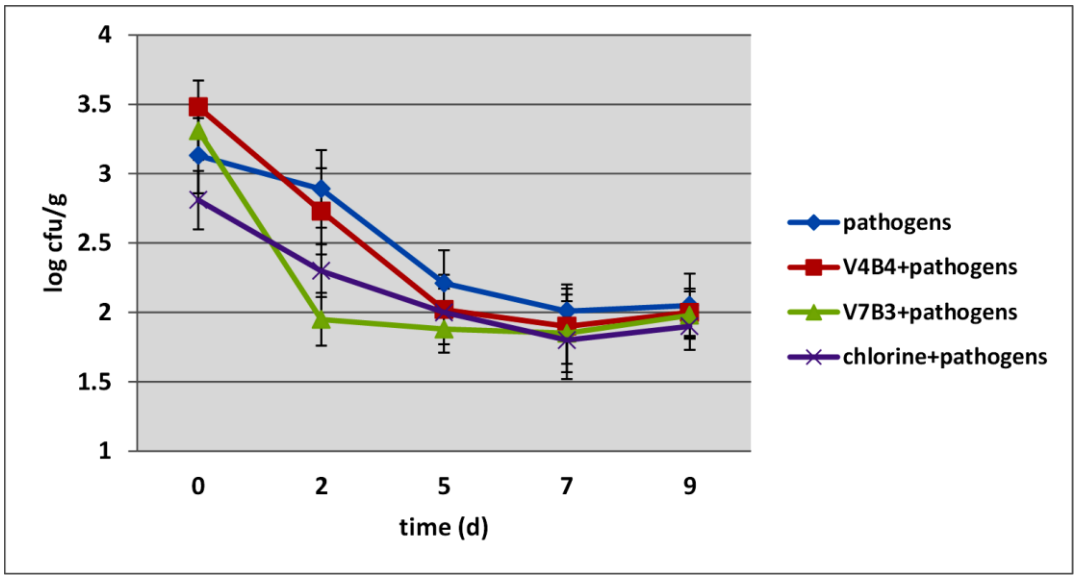


Figure 5.6- *Evolution of* Escherichia coli *(log cfu/g) inoculated in lamb's lettuce in relation to the washing conditions and lactic strain adjunct*

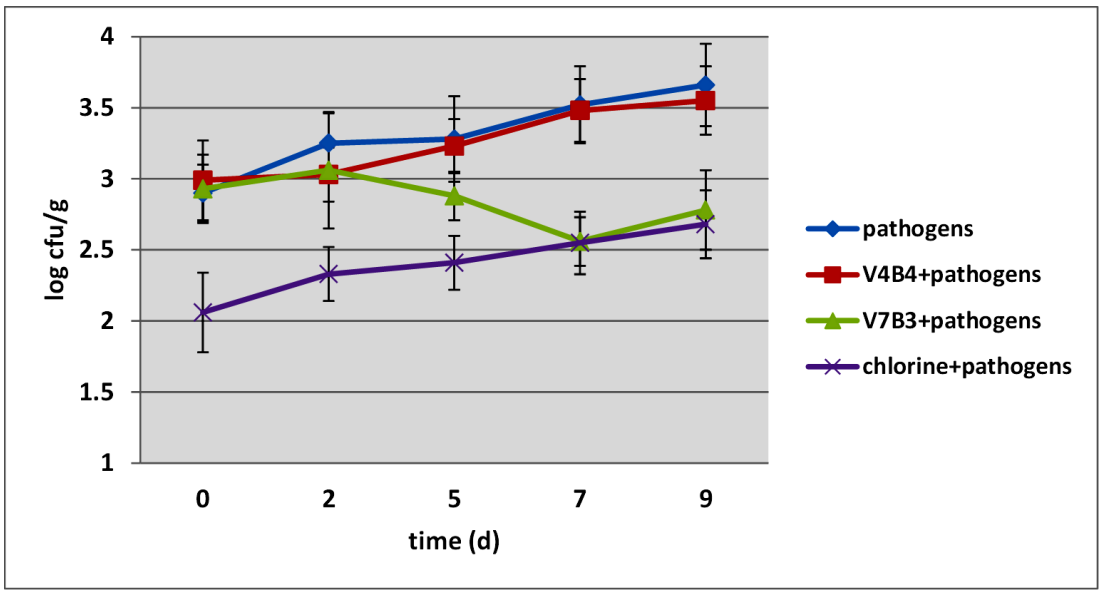


Figure 5.7-. *Evolution of* Listeria monocytogenes *(log cfu/g) inoculated in lamb's lettuce in relation to the washing conditions and lactic strain adjunct*

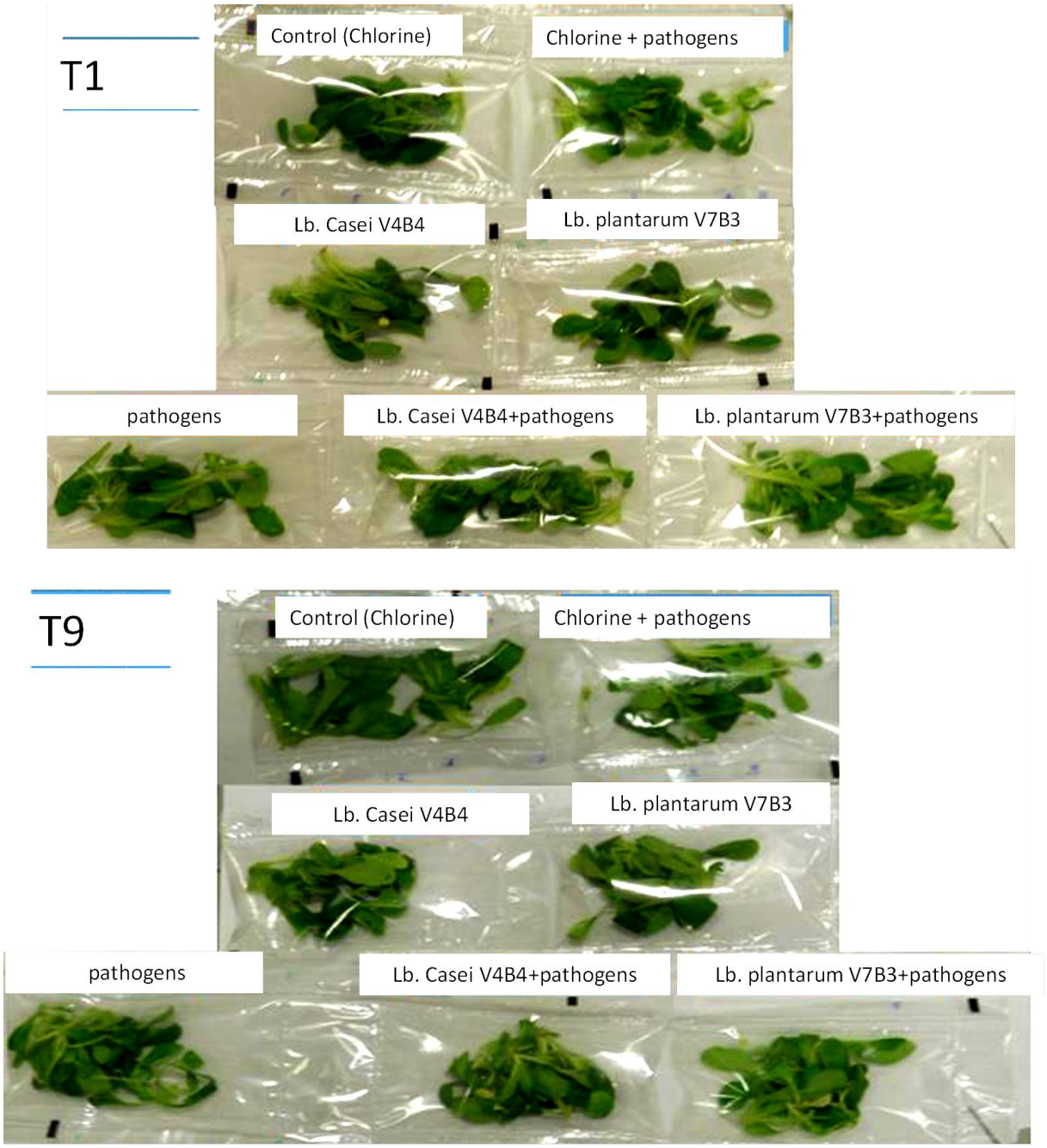


Figure 5.8- *Images of lamb's lettuce subjected to different washing conditions and added with pathogens and/or* Lb. plantarum *(V7B3) or* Lb. casei *(V4B4) after 1 and 7 days of storage*

Although the data obtained are comparable with those of the literature, regarding to the control of pathogens, the efficacy obtained in maintaining the quality parameters and the control of spoilage microorganisms indicate the possibility to transfer at industrial level the conditions developed in this work. In fact, other Authors have focused their attention on the ability of biocontrol cultures to inhibit pathogenic microorganisms frequently associated with fresh-cut products leaving out the effects on the shelf life of the product. Regarding to minimally processed vegetables, literature data indicate the good potential of selected strains of lactic acid bacteria and in particular of specific strains of *Lactobacillus casei* for the control

of *Aeromonas hydrophila*, *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes* (Palmai and Buchanan, 2002; Torriani et al.,1997). Furthermore, Allende et al. (2007) and Trias et al. (2008a) have proposed the use of bacteriocin producer strains belonging to the species *Lactobacillus plantarum*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Weissella cibaria* and *Pediococcus acidilactici* to increase the safety of the products and in particular the inhibition of *Listeria monocytogenes*.

3.2 Effects of selected LAB, in combination with natural antimicrobials, on microbiological quality of minimally processed apples and lamb's lettuce

In order to assess the effects of the selected biocontrol agents *Lb. plantarum* CIT3 and V7B3, in combination with the natural antimicrobials selected in the first part of experimentation, they were added simultaneously in the dipping and washing solution respectively on apples and lamb's lettuce. More specifically, mixtures of hexanal/E-(2)-hexenal, and E-(2)-hexenal/citral (at a concentration of 125 ppm for each compound) in combination with *Lb. plantarum* CIT3 were added in the dipping solution of sliced golden delicious apples, because it resulted the most suitable condition for this type of product. Regarding lamb's lettuce, thyme essential oil was used at a concentration of 250 ppm, during the washing process in combination with the biocontrol agent *Lb. plantarum* V7B3. Moreover, in order to evaluate the effectiveness of antimicrobial substances and of biocontrol culture chosen, challenge tests were performed by inoculating pathogenic microorganisms such as *Escherichia coli* and *Listeria. monocytogenes* directly in dipping or washing solutions of apples and lettuce, respectively.

As evidenced in Figure 5.9, the *Lactobacillus plantarum* CIT3 was absolutely well adapted to the considered system and it was not affected by the presence of the natural antimicrobials used in the dipping step. In fact, the load of lactic acid bacteria was constant and similar to the inoculation level during the first 10 days of storage in all the samples inoculated with the biocontrol agent. After 10 days, in the samples added with the mixture hexanal/E-(2)-hexenal, an increase of the LAB cell load of about one logarithmic cycle, independently on the addition of pathogenic species, was detected. By contrast, in the samples treated with E-(2)-hexenal/citral, the load of lactic acid bacteria remained almost constant during the storage at 6°C. In samples not treated with the biocontrol agent, the load of lactic acid bacteria was less than 3 log cfu/g during the whole period of storage (data not shown).

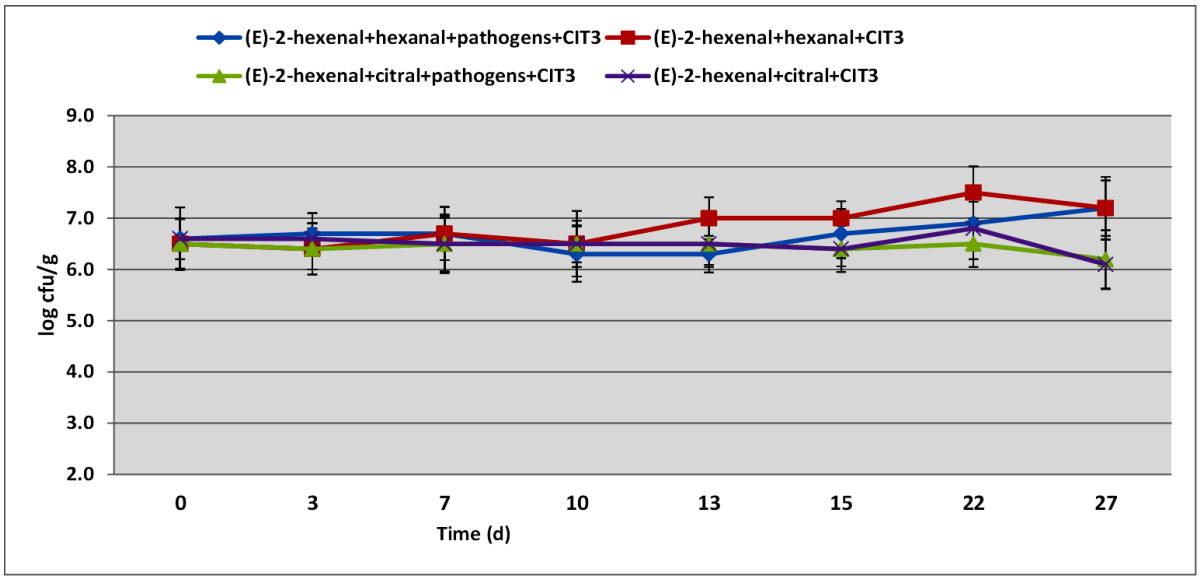


Figure 5.9- *Evolution of* Lb. plantarum *CIT3 (log cfu/g) inoculated in sliced apples in relation to the washing conditions and pathogenic species presence. In the samples not added with the biocontrol agent the load of LAB was below 3 log cfu/g during all the storage period*

As evidenced by the Figure 5.10, *E. coli* was not able to grow during the storage at 6°C. In fact, a loss of vitality was detected in all types of products considered, but with death kinetics more or less accelerated, in relation to the conditions adopted in the dipping step. In fact, the mere presence of natural antimicrobials contributes significantly to accelerate the death kinetics of the target microorganisms. In the presence of mixtures of natural antimicrobials, *E. coli* cell loads decreased below the detection limit with 24h in advance compared to controls subjected to traditional dipping. The addition of the biocontrol agent further increased the safety of the product.In fact, the cell loads of *E. coli* decreased below the determination limit already after 7 days in the samples treated with natural antimicrobials in combination with the strain of *Lactobacillus plantarum* CIT3.

In Figure 5.11, the evolution of *L. monocytogenes* is reported. This pathogenic species maintained a quite constant cell load during storage at 6°C; on the other hand it is a psychrotrophic microorganism, and therefore able to survive and multiply with a low growth rate at refrigeration temperatures. All conditions tested were not very effective for the inactivation of this microorganism. However, the use of the mixture 2-(E)-hexenal/citral, and especially the combined use of this mixture with the biocontrol agent, resulted in a reduction of the cell load of *L. monocytoges* during the first 10 days of storage, approximately of a logarithmic cycle.

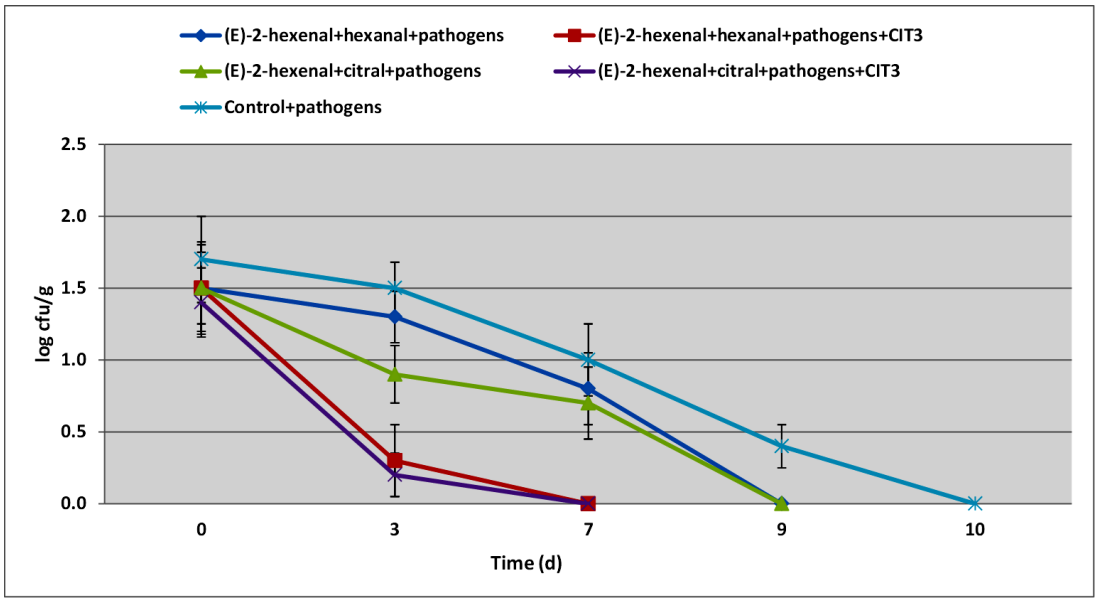


Figure 5.10- *Evolution of* Escherichia coli *(log cfu/g) inoculated in sliced apples in relation to the washing conditions and lactic strain adjunct*

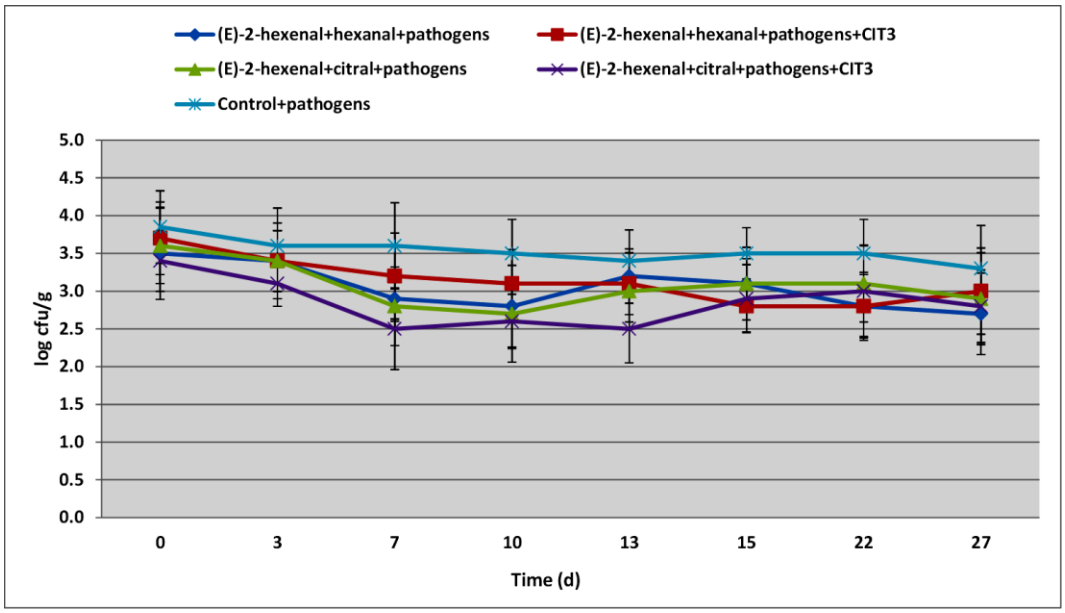


Figure 5.11- *Evolution of* Listeria monocytogenes *(log cfu/g) inoculated in sliced apples in relation to the washing conditions and lactic strain adjunct*

Yeast cell loads did not show significant differences in relation to the type of dipping in the first 15 days of storage. However, the presence of citral in the dipping solution was able to prevent the attainment of the spoilage threshold of yeasts (6 log cfu/g), even after 27 days of storage, with the exception of the samples added with pathogenic microorganisms. In all the other samples, the levels of yeasts overcame the spoilage threshold after 27 days of storage, while controls and controls added with pathogens after 22 days. The selected biocontrol

agent, although very effective in the control the inoculated pathogens, did not show any inhibitory effect against yeasts. However, it is known that in matrices with high C/N ratio, characterized by low pH, high sugar content the yeasts are clearly more competitive with respect to lactic acid bacteria (Patrignani et al., 2013).

In any case, the yeast spoilage threshold was reached when the product was already degraded in terms of color and texture (data not shown). Regarding to the visual quality, the conditions adopted allowed to maintain a good appearance of the product up to 15 days of storage; after this period, a marked decline in the quality of control samples and the samples treated with citral was evident. On the other hand, it is well known that the citral can exert cytotoxic effects on the cells of plant tissues (Belletti et al., 2008).

Regarding to the combined use of biocontrol agent *Lb. plantarum* V7B3 and thyme EO, the strain V7B3 showed excellent adaptability to the stringent conditions of the system considered, also in the presence of thyme EO (Figure 5.12). In fact, the loads of the added lactic acid bacteria were almost constant throughout the period of storage at 6°C, and similar to the inoculation levels. In the control samples and in the samples not supplemented with the biocontrol agent, LAB cell loads were always below the detection limit (1 log cfu/g).

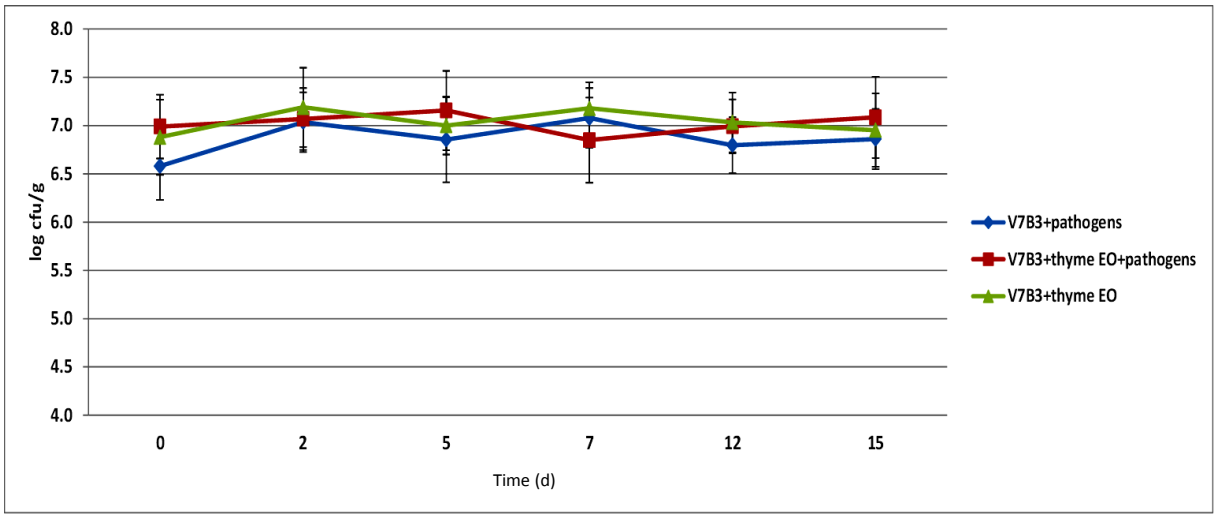


Figure 5.12- *Evolution of* Lb. plantarum *V7B3 (log cfu/g) inoculated in lamb's lettuce in relation to the washing conditions and pathogenic species presence. In the samples not added with the biocontrol agent the LAB loads were below the detection limit of 1 log cfu/g during all the storage period.*

As evidenced by the Figure 5.13, *E. coli* was not able to grow during storage of the products at 6°C; In fact a loss of viability was clear in all types of product, although with a lower death kinetics with respect to that observed in apples. In fact, in the control samples subjected to

washing with water, after 15 days, the level of *Escherichia coli* was decreased of 1 logarithmic cycle. The addition of chlorine or thyme essential oil, in combination with the biocontrol agent, accelerated the death kinetics of the microorganism considered. In particular, the combination of the biocontrol agent with thyme EO showed a marked effect on the inactivation of *E. coli* analogous to chlorine.

In Figure 5.14, the evolution of *Listeria monocytogenes* is reported. As it shown, the target microorganism maintained a constant load, during storage at 6°C, in the samples washed only with water. The addition of chlorine, thyme and/or biocontrol agent allowed a reduction of the initial *L. monocytogenes* loads of about one logarithmic cycle. The biocontrol agent and the thyme EO showed an effect comparable to that of chlorine, that represents the most commonly used disinfectant in minimally processed vegetables. It is well known that chlorine is able to reduce the microbial loads to levels never exceed 1-2 logarithmic cycles (Alegre et al., 2013). Furthermore, it is known that the antimicrobial efficacy of chlorine is strictly dependent on the organic matter content of the product: with increasing concentrations of the organic substance, the antimicrobial effectiveness of the chlorine decreases (Gil et al., 2009; Olmez & Kretzschmar, 2009). After 15 days the *L. monocytogenes* loads were similar, independently on washing solution adopted, with the exception of the samples treated with thyme and chlorine that showed a reduction of the cell loads, of about 1 log cycle, compared to the initial one.

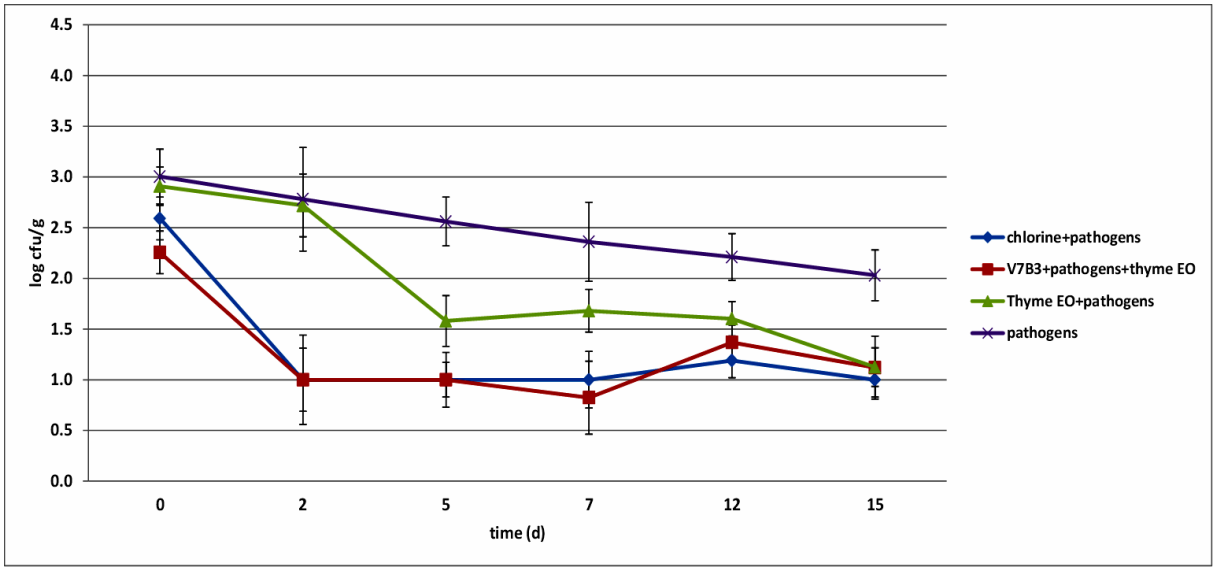


Figure 5.13- *Evolution of* Escherichia coli *(log cfu/g) inoculated in minimally processed lamb's lettuce in relation to the washing conditions and lactic strain adjunct*

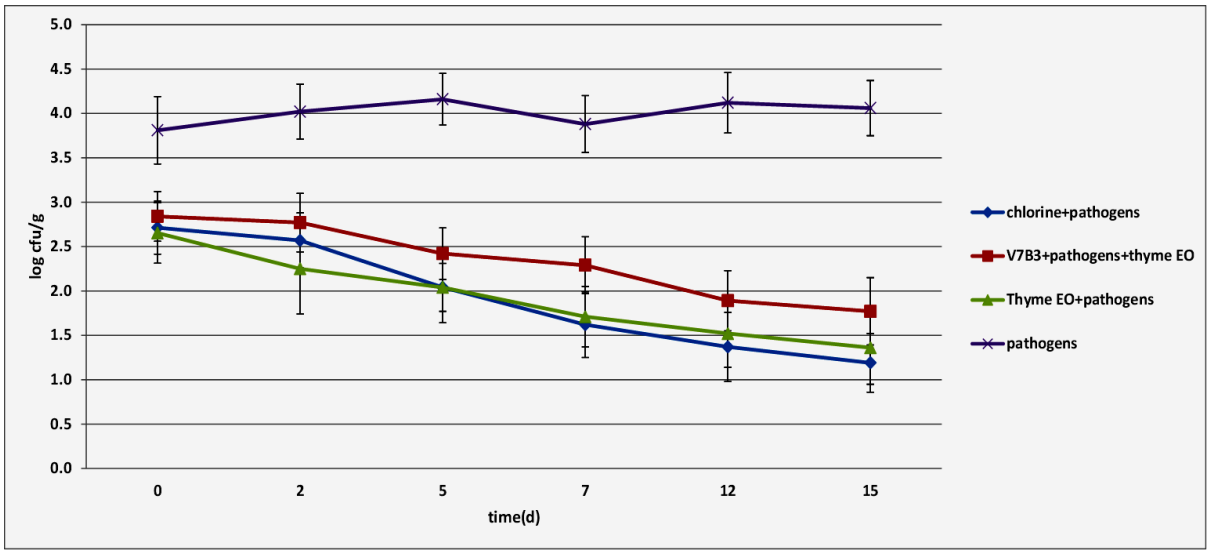


Figure 5.14. *Evolution of* Listeria monocytogenes *(log cfu/g) inoculated in minimally processed lamb's lettuce in relation to the washing conditions and lactic strain adjunct*

The total aerobic mesophilic bacteria, which can be considered as the main spoilage agents of this kind of product, exceeded the threshold of alteration (7 log cfu/g) after just 5 days of storage in the samples washed with water and inoculated with pathogens (Figure 5.15). In the samples treated with chlorine, total mesophiles reached the threshold of spoilage after 7 days of storage, independently on the presence or the absence of the deliberately inoculated pathogens. The replacement of chlorine with the thyme EO caused a positive effect on the shelf-life of the product that even after 15 days of storage showed a total mesophilic loads below the threshold of spoilage (7 log cfu/g), independently on the presence of pathogens in the washing water. The combination of thyme and biocontrol agent was more effective in prolonging the shelf-life than natural antimicrobials alone, presenting values of the total aerobic mesophilic significantly lower than the other samples for the whole period of storage. Moreover, the combination of thyme EO and the biocontrol agent was able to preserve the qualitative characteristics, color and texture of the products during the whole period of storage at 6°C (data not shown).

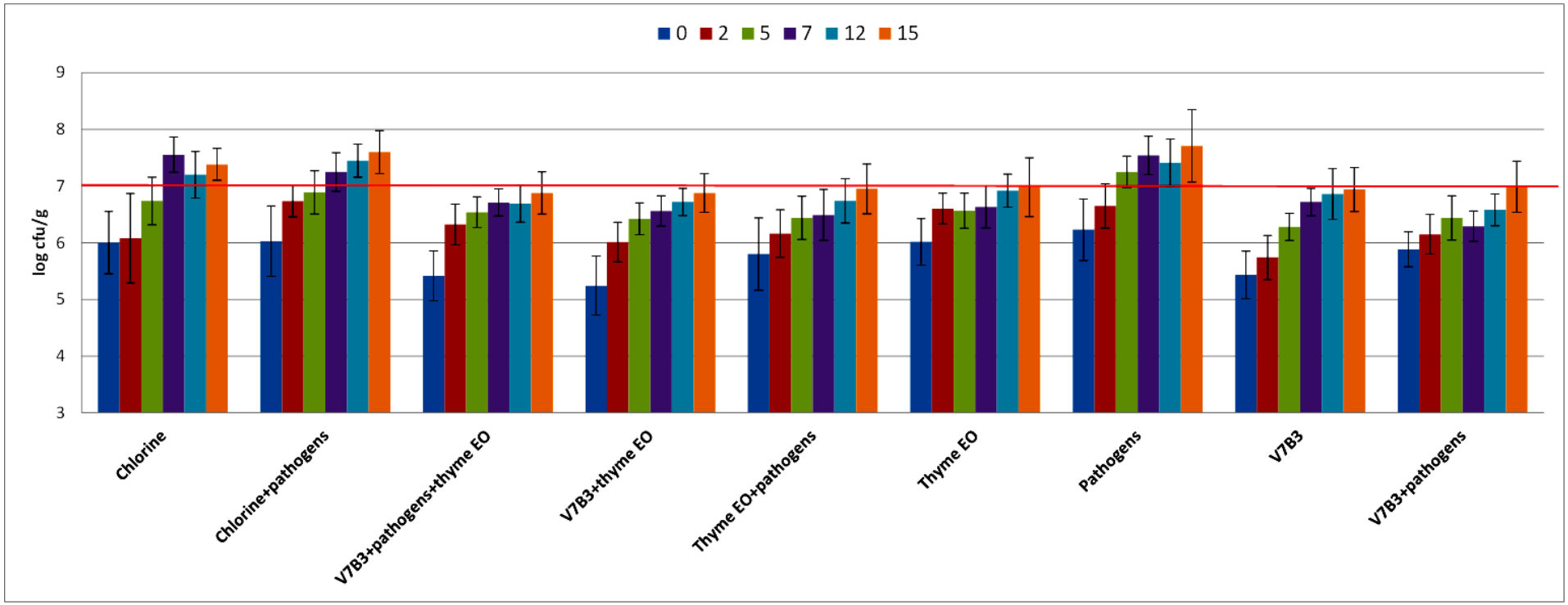


Figure 5.15. *Evolution of total aerobic mesophilic bacteria (log cfu/g) in minimally processed lamb's lettuce in relation to the washing conditions and lactic strain adjunct*

4. Conclusion

The use of the strains *Lactobacillus plantarum* CIT3 and V7B3 on apples and lettuce, respectively, provided encouraging results regarding the safety and shelf life of the minimally processed products considered. The obtained results are also more interesting because lactic acid bacteria are recognized as GRAS (Generally Recognized As Safe), and often they have a beneficial effect on consumer health . The results also highlighted the importance of isolation and selection of biocontrol agents from commercial products of the same type. In fact, the good performance of the strains used was not only against deliberately inoculated pathogens, but also against spoilage microorganisms. These abilities have to be attributed to the capability of the strains to colonize the product and survive under stringent conditions of refrigerated storage. Also the ability of biocontrol agents to not affect the quality indexes of the product is important. Regarding to the strain used in lamb's lettuce, it did not cause a reduction of the product quality, also when used in combination with thyme EO. We have to take into account the pressing for finding alternatives to the use of chlorine (the most common sanitizer used for leafy vegetables), and this research showed the good potential of the *L. plantarum* V7B3 alone or in combination with thyme EO as alternative to chlorine.

Also the strain CIT3, despite its higher potential degradation of the product compared to V7B3, was able to preserve the quality of fresh-cut apples treated up to 9 days when used alone and up to 16 days when used in combination with natural antimicrobials. However, further analyses should be performed to confirm that the application of these strains, as potential biocontrol agents, do not alter the sensory properties.

Acknowledgment

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CHAPTER 6

Use of a nisin-producing *Lactococcus lactis* strain, combined with natural antimicrobials, to improve the safety and shelf-life of minimally processed sliced apples and lamb's lettuce

Abstract

The demand of minimally processed fruits and vegetables has increased in the last years. However, their intrinsic characteristics may favor the growth of pathogens and spoilage microbiota. The negative effects on human health reported for some traditional chemical sanitizers have justified the search for substitutes to guarantee food safety and quality. In this context, the use of lactic acid bacteria (LAB) as biocontrol agents represents a good alternative, and numerous LAB have been identified as bioprotective agents, also due to their ability to produce bacteriocins. Furthermore the usage of natural antimicrobials has been proposed as alternative to the traditional sanitization methods.

In this experimentation, several *Lactococcus lactis* strains isolated from different sources were screened for their ability to produce nisin. Among the nisin-producing strains, the *Lactococcus lactis* CBM21 was chosen due to its capability to produce nisin Z. This strain was technologically characterized in order to verify its ability to growth at low pH and temperature and in the presence of sucrose 20%. Thus, the purpose of this study was to evaluate the potential application of the nisin-producing *Lactococcus lactis* CBM21 on the safety and shelf-life of sliced apples and minimally processed lamb's lettuce combined or not with natural antimicrobials such as hexanal, 2-(E)-hexenal, citral and thyme EO. To assess the effects on products safety, challenge tests in the presence of *Listeria monocytogenes* and *Escherichia coli* were also performed. The biocontrol agent, the natural antimicrobials, and pathogenic bacteria were added in the dipping or washing solution for apples and lamb's lettuce, respectively. The products were subsequently packed in modified atmosphere and stored at 6°C. During storage, microbiological analyses were performed during the storage of the products. In case of apples also color analyses and a panel test after 1 and 4 days of storage were performed.

The data highlighted the good performance of strain CBM21, combined or not with the antimicrobials employed, to inhibit both the inoculated pathogenic species *Listeria monocytogenes* and *Escherichia coli*, the naturally occurring yeasts and the total mesophilics. The addition of the biocontrol agent did not affect significantly the quality parameters of lamb's lettuce, while, the addition of the biocontrol agent affected the apples color parameters after 14 days of storage, but this negative effect was balanced by the presence of hexanal and 2-(E)-hexenal. The panel test showed that the consumer was not able to find differences between apples

added or not with the biocontrol agent. These results suggest that the considered alternative "hurdles" can represent a new strategy to ensure the safety and quality of this kind of products.

1. Introduction

The demand of minimally processed fruits and vegetables has incessantly increased in the last years reflecting the interest of consumers for fresh and healthy products with an easy way of preparation. The intrinsic characteristics of ready-to-eat vegetables and fruits, such as the low acidity and high humidity, together with the high number of cut surfaces, may favor the microbial growth, as well as foodborne pathogens and spoilage microorganisms (Ongeng et al., 2006). These products have been implicated in outbreaks of foodborne infections provoked by human pathogens like *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Viswanathan & Kaur, 2001; Francis et al., 1999; Tian et al., 2012; Salleh et al., 2003; Beuchat, 1996, 2002). Consequently, the use of raw materials of good quality and correct decontamination procedures are critical steps to ensure the safety of ready-to-eat fresh fruits and vegetables (Silva et al., 2007; Legani & Leoni, 2004). The negative effects on human health reported for some chemical compounds such as chlorine (Gil et al., 2009; Selma et al. 2008; Sao Josè a& Vanetti, 2012; López-Gálvez et al., 2010; Tomás-Callejas et al., 2012), and the development of resistant strains of pathogenic microorganisms, have justified the search for substitutes to guarantee food safety and quality. In this context, the use of biocontrol agents fits well with this new trend, and numerous microorganisms have been identified as bioprotective agents (Vermeiren et al., 2004). Numerous studies have shown the great potential of several microorganisms to inhibit the growth of foodborne pathogens in minimally processed fruits and vegetables (Vescovo et al. 1996; Bennik et al. 1999; Leroy et al. 2003; Palmai & Buchanan 2002). The use of biocontrol agents such as *Candida* sp., *Gluconobacter* sp., *Discosphaerina* sp. and *Metschnikowia* sp., have been reported to inhibit the growth of *L. monocytogenes*, *E. coli* and *S. enterica* in fresh-cut apples but negative effects such as browning of fruits were observed (Leverentz et al., 2006). Also Torriani et al. (1997) and Scolari and Vescovo (2004) showed the potential of a strain of *Lactobacillus casei* to increase the safety of minimally processed vegetables due to the inhibition of *Aeromonas hydrophila*, *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes*. However, literature data do not exhaustively explain the effects of biocontrol agents on the spoilage microflora and more

generally on the shelf-life of products. For this reason, there is still a need for new bioprotective microorganisms that fulfill desired characteristics such as biosafety and limitation of non-target effects (Trias et al., 2008a,b).

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS) by the USA Food and Drug Administration (FDA), and their use to preserve (trough fermentation) meat and dairy products and to bioprotect fermented vegetables is well documented (Ruiz-Barba et al., 1994; Stiles & Holzapfel, 1997). Moreover, the capability of LAB to produce bacteriocins and other antimicrobial molecules such as organic acids, diacetyl, acetoin, reuterin, reutericyclin, peroxidase, etc., and their general acceptability in foods, make them interesting to be used as alternatives to chemicals in food preservation. In particular, the antimicrobial effects of bacteriocins, an heterogeneous group of antibacterial peptides with different molecular weight and composition, classified into different groups and produced by bacteria to compete against bacteria of the same species or other genera, is well documented (Yang et al., 2012; Alakomi et al., 2000; Cleveland et al., 2001; Cotter et al., 2005). Moreover, bacteriocins have been consumed unconsciously by humans for thousands years like natural ingredients of fermented foods.

Several authors have reported the great potential of bacteriocins to inhibit Gram-positive bacteria both in model and in real food systems such as cheese, meat and ready-to-eat vegetables (Cai & Farber, 1997; Molinos et al., 2005; Buyong et al., 1998; Ennahar et al., 1998; Loessner et al., 2003; McAuliffe et al., 1999; Jamuna et al., 2005).

Among bacteriocins, nisin is produced by *L.lactis* and it was the first characterized bacteriocin .

It is generally recognised as safe (GRAS) and, consequently, permitted as preservative in food.

(FDA, 1988; Delves-Broughton et al., 1996; Jones et al., 2005). Recent works have shown synergistic effect of nisin when used in combination with other food additives such as chlorine, sodium lactate, citric acid, phytic acid, potassium sorbate, H_2O_2 in fresh cut lettuce and various kinds of minimally processed fruits and vegetables (Allende et al., 2007; Bari et al., 2005; Leverentz et al., 2003; Ukuku et al., 2005).

Several possible strategies for the application of bacteriocins in the preservation of foods have been proposed: i) addition of the purified or semipurified bacteriocin as a food preservative; ii) use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing or iii) inoculation of the food with LAB (starter or protective cultures) able to produce the bacteriocin in the product (Schillinger et al., 1996; Deegan et al., 2006; Settanni & Corsetti,

2008). The success of the latter application is related to the ability of the bacteriocin-producing LAB to grow and to produce the bacteriocin in the food system under real production and storage conditions.

In this context, the purpose of this experimental phase was to identify and characterize nisin-producing *Lactococcus lactis* strains,, since this nisin has the most potential applications in food sector. In fact, nisin, and in particular the Z type, is commonly used in various food products, due to its high solubility and stability in foods, in order to increase the microbiological safety (de Arauz et al., 2009). It is well-known the activity of nisin against Gram-positive bacteria, and particularly *Listeria monocytogenes*, both under laboratory conditions as well in foodstuffs, as well the synergistic effect of nisin combined with food additives which can increase the range of antimicrobial activity also against Gram-negative bacteria, yeasts and molds. In this context the second objective of this research was to evaluate the potential application of selected nisin-producing strain of *Lactococcus lactis* in minimally processed apples and lamb's lettuce, combined or not with other "hurdles" to microbial growth. More specifically, the strain *Lactococcus lactis* CBM 21 was added in the dipping or washing solution, respectively, for apples and lettuce, in combination with hexanal, trans-2-hexenal and citral for sliced apples, and thyme EO for lamb's lettuce. To assess the effects on product safety, also challenge tests with *Listeria monocytogenes* and *Escherichia coli*, added in the washing step, were performed. Apples and lamb's lettuce were subsequently packed in modified atmosphere (0% CO_2 and 7% O_2) or artificial ordinary atmosphere, respectively, and stored at 6°C. The conditions of dipping, washing and the composition of the atmosphere were chosen on the basis of previous experiments, reported in chapter 3 and 4, which had proven their ability to significantly increase the shelf-life of minimally processed apples and lettuce.

2. Material and methods

2.1 *Screening for the detection of nisin-producing Lactococcus lactis strains*

Thirty-one strains of *Lactococcus lactis* isolated from different food sources, previously identified and belonging to the Department of Biotechnology of Verona University, were screened for the capability to produce nisin. An agar spot test was used to verify the antimicrobial activity against an indicator strain such as *Lactobacillus plantarum* ATCC 14917^T, which was

shown to be sensitive to nisin (Rossi et al., 2008). More specifically, 5 µl of overnight cultures of each tested strain were spotted onto M17 (Oxoid Ltd., Basingstoke, England) agar plates. The spotted agar plates were then incubated at 30°C for 24h. Subsequently, spots were covered with 10 mL of MRS (Oxoid Ltd., Basingstoke, England) soft agar (0.75%) inoculated with 100 µl of an overnight culture of *Lactobacillus plantarum* ATCC 14917^T (indicator strain). These plates were then incubated at 37°C for 24h. Positive cultures were then considered in the following steps.

2.2 *Search for nisin encoding gene through PCR*

Total genomic DNA was extracted from microbial cells and purified, using the Wizard^R Genomic Purification Kit (Promega corporation, Madison, WI, USA) following the manufacturer's recommendations.

Primers reported by de Vos et al. (1993) (forward: 5'–CGCGAGCATAATAAACGGCT-3'; reverse:5'-GGATAGTATCCATGTCTGAAC-3'), were employed for the amplification of the nisin-encoding gene. These sequences are complementary to 80bp upstream and 29bp downstream the coding region of the *nis*A and *nis*Z genes, respectively (Mulders et al., 1991). The PCR mixture (50 µl) was composed by 2 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of deoxyribonucleotide triphosphates (dNTPs), 0.02 U/µl *Taq* polymerase, 1× PCR buffer and approximately 20 µg of genomic DNA. Thermocycling conditions were preliminary denaturation at 94°C for 5 min; 30 cycles of 93°C for 2 min, 54°C for 1 min, 72°C for 1.5 min, then a final extension at 72°C for 10 min. PCR products were visualized on 1.5% agarose gel. PCR products were purified following an internal protocol and sequenced at BMR Genomics sequencing center (Padua, Italy). sequences were then compared with those available in GeneBank database retrieved thorugh BLASTn searches and then aligned using the GeneDoc 2.7 software.

2.2 Phenotypic characterization and evaluation of antagonistic activity of the strain Lactococcus lactis CBM21

The nisin Z-producing *Lactococcus lactis* CBM21 was characterized on the basis of the capability to grow in different environmental conditions. The selected conditions were different temperatures (4, 8, 15 and 30°C), different levels of sodium chloride (2, 4 and 6%), high

concentrations of sucrose (20%) and low pH values (3.5, 4.0 and 4.5). The protocol used is the same reported in paragraph 2.2 of chapter 5.

The evaluation of the ability of CBM21 to antagonize typical spoilage and pathogenic microorganisms was performed on the following target strains: *Lactobacillus casei* V4B4, *Lactobacillus plantarum* CIT3 and V7B3, *Listeria monocytogenes* Scott A and OSP4, *Salmonella enteritidis* E5, *Escherichia coli* 555, *Bacillus cereus* SV90, *Staphylococcus aureus* F1, *Enterococcus faecalis* 29212, *Saccharomyces cerevisiae* spa, *Lactococcus lactis* S1, *Lactobacillus rhamnosus* C 111 2, *Lactobacillus sakei* S8 and *Lactobacillus brevis* IOEB 9809. The antimicrobial activity was evaluated through an agar spot assay following the method reported by Schillinger and Lucke (1989).

The capability of *L. lactis* CBM21 to survive in the presence of the natural antimicrobials, previously used on minimally processed apples and lamb's lettuce, was evaluated by the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the selected natural antimicrobials against the strain CBM21. In particular, the MIC and MBC values of thyme essential oil, citral, 2-(E)-hexenal and hexanal against the *L. lactis* CBM21, were determined by following the method reported in paragraph 2.3 of chapter 3.

2.3 Preparation of minimally processed apples and lamb's lettuce added of Lactococcus lactis CBM21 alone or in combination with natural antimicrobials

The effect of *Lactococcus lactis* CBM21, alone or in combination with natural antimicrobials, on the shelf-life and safety of minimally processed apples and lamb's lettuce was tested. Minimally processed apples and lamb's lettuce were prepared by following the protocols reported in Figure 3.2 and 4.2 of chapter 3 and 4, respectively, for sliced apples and lettuce. As antimicrobials, the mixtures citral/2-(E)-hexenal and hexanal/2-(E)-hexenal were employed for apples, at a concentration of 125 ppm for each compound, while thyme EO was used for lettuce at a concentration of 250 ppm. Challenge tests with *Listeria monocytogenes* and *Escherichia coli* were performed in order to evaluate the effects of the added biocontrol agent and natural antimicrobials on the safety of the products. The supplementation of the biocontrol agent (7-8 log cfu/mL) and/or natural antimicrobials and/or pathogens (3-4 log cfu/mL) occurred in the dipping

or in the washing solution for apples and lamb's lettuce, respectively. All the conditions employed in this experimental phase both for apples and lettuce are reported in Table 6.1. After the treatments, apples were dried with paper, packaged in active modified atmosphere with 7% O_2 and 0% CO_2 and stored at 6°C until the end of shelf-life. Regarding lamb's lettuce, after the treatments, lettuce was spin dried and packaged in artificial ordinary atmosphere, and then stored at 6°C until the end of shelf-life.

Table 6.1- *Conditions employed on apples and lamb's lettuce; the inoculation of* L. lactis *CBM21 at a level between 7-8 log cfu/mL; the pathogenic microorganisms were inoculated at a level ranged between 3 and 4 log cfu/mL. Thyme EO was employed at a concentration of 250ppm while the two combinations 2-(E)-hexenal/hexanal and citral/2-(E)-hexenal were used at 125ppm for each compound. The addition of* L. lactis *CBM21 and/or antimicrobial compounds and/or pathogens was in the dipping and washing solution for apples and lamb's lettuce, respectively.*

Tested Conditions	
Apples	**Lamb's lettuce**
1. dipping (0.5% ascorbic acid; 1.0% citric acid	1. washing water+ pathogens (*L. monocytogenes, E. coli*)
2. dipping+2-(E)-hexenal/hexanal	2. washing water+Chlorine 120ppm
3. dipping+2-(E)-hexenal/hexanal+pathogens (*L. monocytogenes, E. coli*)	3. washing water+Chlorine 120ppm+pathogens (*L. monocytogenes, E. coli*)
4. dipping+2-(E)-hexenal/hexanal+pathogens+*Lc. lactis* CBM21	4. washing water+thyme EO
5. dipping+2-(E)-hexenal/hexanal+*Lc. lactis CBM21*	5. washing water+thyme EO+pathogens (*L. monocytogenes, E. coli*)
6. dipping+2-(E)-hexenal/citral	6. washing water+*Lc. lactis* CBM21
7. dipping+2-(E)-hexenal/citral+pathogens (*L. monocytogenes, E. coli*)	7. washing water+thyme EO+*Lc. lactis* CBM21
8. dipping+2-(E)-hexenal/citral+pathogens +*Lc. lactis CBM21*	8. washing water+ pathogens (*L. monocytogenes, E. coli*)+*Lc. lactis* CBM21
9. dipping+2-(E)-hexenal/citral+*Lc. lactis CBM21*)	9. washing water+thyme EO+pathogens+*Lc. Lactis* CBM21
10. dipping+ pathogens (*L. monocytogenes, E. coli*)	
11. dipping+*Lc. lactis* CBM21	
12. dipping+pathogens (*L. monocytogenes, E. coli*)+*Lc. lactis* CBM21	

2.4 Microbiological analyses

During storage, the evolution over time of LAB, yeasts and mesophylic aerobic bacteria was evaluated by plate counting respectively on de Man Rogosa and Sharpe Agar (MRS, Oxoid Ltd. Basingstoke, United Kingdom) with cycloheximide (0.05%) (Sigma-Aldrich), Sabouraud Dextrose Agar (SAB, Oxoid Ltd.), with chloramphenicol (Sigma-Aldrich) and Plate Count Agar (PCA, Oxoid Ltd., Basingstoke, United Kingdom). After homogenization, samples were serially diluted in physiological solution (10 g of sample diluted into 90 mL of physiological water (0.9% (w/v) NaCl). Lactic acid bacteria were incubated at 37°C for 48h, while yeasts and mesophylic aerobic bacteria were incubated at 30°C for 48h. The detection of the inoculated pathogens *Listeria monocytogenes* and *Escherichia coli* was evaluated by plate counting on Listeria Selective Agar Base (LSO, Oxoid) with selective listeria supplement (Oxoid) and violet red bile

agar (Oxoid) with 4-methylumbelliferyl-β-D-glucuronide (Oxoid), respectively. Plates were incubated at 37°C for 24h. Microbiological analyses were performed immediately after treatments and after 2, 5, 7, 9, 12, 14, 16, 19, 21, 23, 26 and 28 days of storage in case of apples, while for lamb's lettuce immediately after treatments and after 2, 5, 7, 12 and 15 days of storage.

2.4 Color analyses and panel test

Surface color of sliced apples was measured using a color-spectrophotometer mod. Colorflex (Hunterlab, USA). Color was measured using the CIELab scale and Illuminant D65. The instrument was calibrated with a white tile (L*98.03, a* - 0.23, b* 2,05) before the measurements. Results were expressed as L* (luminosity), a* (red index) and b* (yellow index).
At each storage time, 21 readings were obtained for each sample from the seven packages, measuring three slices for each package.
A panel test was performed for sliced apples after 1 and 4 days of storage. The panel was composed by 30 untrained consumers, and the quality parameters evaluated were flavor, taste, browning, firmness, crispiness, sweetness, bitterness, acidity, flower, juiciness and overall impression. Four different conditions were evaluated by consumers: control apples, apples added with citral/2-(E)-hexanal and apples added with hexanal/2-(E)-hexanal and apples added with the biocontrol agent.

3. Results

3.1 *Selection of a nisin Z-producing Lactococcus lactis strain*

In this experimental phase thirty-one strains of lactic acid bacteria belonging to the species *Lactococcus lactis,* isolated mainly from dairy products, were screened for the capability to produce nisin by an agar spot test using the sensitive strain *Lactobacillus plantarum* ATCC14917^{T} as target. Table 6.2 shows the strains taken into consideration, their isolation source and the eventual inhibition of the indicator strain. Only four strains (CBM21, CBM44, Lc caciotta and DSM 20729) were able to inhibit the indicator strain. To prove that the bacteriocin produced by the positives *L. lactis* strains was nisin, the presence of the nisin-encoding gene was assayed by PCR. As reported in Figure 6.1, only strains CBM21 and DSM 20729^{T} were shown to harbor the nisin-encoding gene.

Table 6.2- *Screened* Lactococcus lactis *strains, source of isolation and eventual inhibition of the nisin-sensitive indicator strain*

Species	Strain	Source	Inhibition of indicator strain *Lb. plantarum* ATCC 14917^T
L.lactis sub. cremoris	CBM 2	cheese	-
L.lactis sub. Lactis	CBM 17	cheese	-
L.lactis sub. Lactis	CBM 18	cheese	-
L.lactis sub. Lactis	CBM 21	cheese	+
L.lactis sub. cremoris	CBM 26	cheese	-
L.lactis sub. Lactis	CBM 28	cheese	-
L.lactis sub. cremoris	CBM 34	cheese	-
L.lactis sub. Lactis	CBM 36	cheese	-
L.lactis sub. Lactis	CBM 38	cheese	-
L.lactis sub. Lactis	CBM 39	cheese	-
L.lactis sub. Lactis	CBM 41	cheese	-
L.lactis sub. Lactis	CBM 43	cheese	-
L.lactis sub. Lactis	CBM 44	cheese	+
L.lactis sub. Lactis	CBM 45	cheese	-
L.lactis sub. Lactis	CBM 58	cheese	-
L.lactis sub. Lactis	CBM 72	cheese	-
L.lactis sub. Lactis	CBM 77	cheese	-
L.lactis sub. Lactis	RAC 2410	cheese	-
L.lactis sub. Lactis	RAC 2412	cheese	-
L.lactis sub. Lactis	PBCF 56	cheese	-
L.lactis sub. Lactis	RAC 248	cheese	-
L.lactis sub. Lactis	RAL C5	cheese	-
L.lactis	Lc CACIOTTA	cheese	+
L.lactis sub. Lactis	6049	milk	-
L.lactis sub. Lactis	LMG 6880^T	milk	-
L.lactis sub. Lactis	LMG 7949^T	milk	-
L.lactis sub. Lactis	LMG 6890^T	milk	-
L.lactis sub. Lactis	DSM 20729^T	milk	+
L. lactis	Lc 2,12 G II p	cheese	-
L. lactis	Lc 2,12 G II g	cheese	-
L.lactis sub. Lactis	M10G19	apple	-

Legend: +: Inhibition observed; -: no inhibition observed

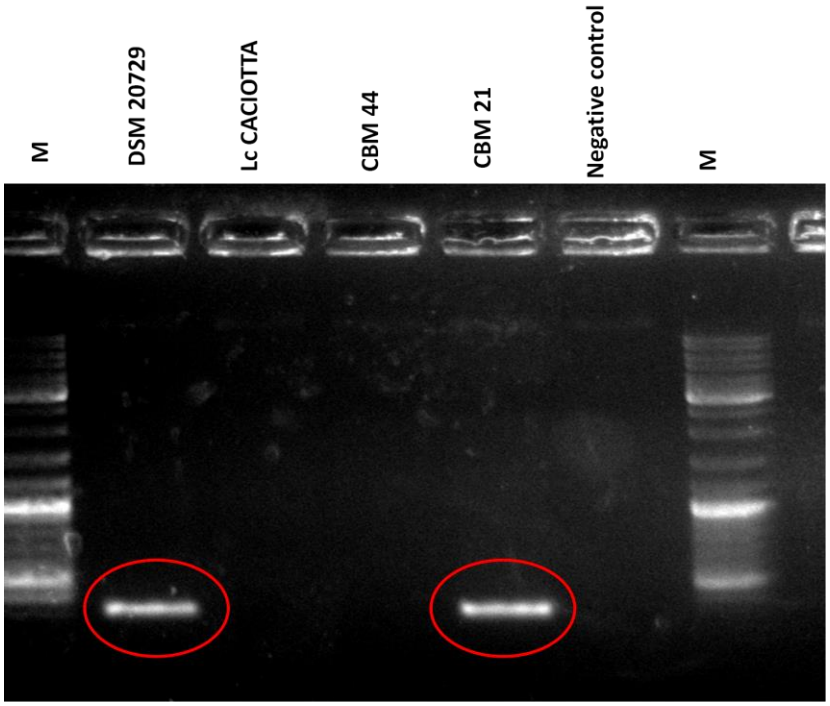


Figure 6.1- *Agarose gel electrophoresis of PCR products obtained with nisin gene specific primers from the* L. lactis *strains CBM21, CBM44, Lc caciotta and DSM 20729^T*

The amplified PCR products of *L. lactis* CBM21 and DSM 20729^T were subsequently sequenced as shown in Figure 6.2. Sequences of CBM21 and DSM 20729^T showed 100% similarity values with nisin Z and nisin A, respectively, indicating the production of nisin Z by CBM21 and nisin A by DSM 20729^T. Since nisin Z is characterized by a better solubility in food systems compared to the A type (de Arauz et al., 2009), strain CBM21 was selected to be used on food products.

```
                *       20        *       40        *       60        *       80        *      100        *
nis_A     : ATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTG : 117
DSM20729  : ATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTG : 117
nis_Z     : ATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTG : 117
NIS_CBM21 : ATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTG : 117
            ATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTG

            120       *       140       *       160
nis_A     : ATGGGTTGTAACATGAAAACAGCAACTTGTCATTGTAGTATTCACGTAAGC : 168
DSM20729  : ATGGGTTGTAACATGAAAACAGCAACTTGTCATTGTAGTATTCACGTAAGC : 168
nis_Z     : ATGGGTTGTAACATGAAAACAGCAACTTGTAATTGTAGTATTCACGTAAGC : 168
NIS_CBM21 : ATGGGTTGTAACATGAAAACAGCAACTTGTAATTGTAGTATTCACGTAAGC : 168
            ATGGGTTGTAACATGAAAACAGCAACTTGT ATTGTAGTATTCACGTAAGC
```

Figure 6.2- *Nucleotide sequences of the nisZ and nisA gene isolated respectively from* L. lactis *CBM21 and DSM 20729^T and aligned with the nisZ and nisA gene sequences found in GeneBank*

3.2 *Technological characterization and evaluation of the antimicrobial activity of L. lactis CBM21*

Strain CMB21 was characterized for technologically important features in order to assess the potential for its use in minimally processed apples and lettuce. The considered strain showed the ability to grow at low temperatures, at low pH values and in substrates with low water activity. In particular, the strain CMB21 was able to grow within 24 hours at 30°C and 15°C, with 2% and 4% NaCl , and with 20% sucrose. By contrast, at 4°C and 8°C a growth reached levels which were higher than 10^8 cfu/mL after seven days of incubation. Also the increase of NaCl concentration to 6% resulted in a significant decrease of the growth kinetics of the strain, which however, reached the stationary phase of growth after 5 days of incubation at 30°C. Regarding pH conditions, values of 4.5 and 4.0 allowed the strain to reach levels higher than 10^8 cfu/mL in 2 and 5 days, respectively. On the contrary, pH values of 3.5 did not allowed the microorganism to grow, at least in 15 days of incubation at 30°C. However, in the latter condition no significant decrease in the viability of the strain *L. lactis* CBM21 was detected. In addition, the assessment of the antagonistic activity was performed against several Gram-positive and Gram-negative microorganisms, and a strain of *Saccharomyces cerevisiae.* Target strains and results obtained are reported in Table 6.3. In particular *Lactococcus lactis* CBM21 showed a high antagonistic activity (diameter of inhibition higher than 3 mm) against *Listeria monocytogenes*, *Staphylococcus aureus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactococcus lactis* and the two *Lactobacillus plantarum* strains considered. In contrast, *L. lactis* CBM21 did not show antagonistic activity against both Gram-negative and the yeast considered. On the other hand, it is well-known the good activity of nisin against Gram-positive bacteria, while it is quite ineffective against Gram-negative bacteria and yeasts unless the outer membrane is compromise or damaged (Stevens et al., 1991; Helander & Sandholm, 2000).

Considering that the raw materials showed pH values of 4.1±0.2 and 5.6±0.3 for apples and lettuce, respectively, and the storage conditions of minimally processed fruits and vegetables rarely maintained the cold chain at 4°C, the selected strain showed good technological characteristics for the application in a real system.

Table 6.3- *Antagonistic activity of* L.lactis *CBM21 against several microorganisms and determined by agar spot test*

Antagonistic activity of L. lactis CBM21	
Indicator strains	**Inhibition**
L. plantarum CIT3	+++
L. monocytogenes OSP4	++
L. brevis IOEB9809	++
L. monocytogenes Scott A	+++
S. aureus F1	+++
L. casei V4B4	+++
E. coli 555	-
B. cereus SV90	+
L. plantarum V7B3	+++
L. rhamnosus C1112	+++
S. enteritidis E5	-
S. cerevisiae spa	-
L. sakei S8	+++
L. lactis S1	+++
E. faecalis 29212	++

Legend: - No inhibition zone; + small inhibition zone (0.5 to 1 mm); ++ medium inhibition zone (1 to 2 mm); +++ large inhibition zone (>2mm)

3.2 Effects of L. lactis CBM21, in combination with natural antimicrobials, on microbiological quality of minimally processed apples

Given the ineffectiveness against Gram-negative bacteria considered by *L. lactis* CBM21, as well as the frequent association of Gram-negative pathogens to fresh-cut products, the use of natural antimicrobials was combined with the bioprotective culture. In fact, the antimicrobial activity of essential oils (EO) and their main component against both Gram-positive and Gram-negative bacteria is well documented (Dorman & Deans 2000; Cosentino et al., 2003; Smith-Palmer et al., 1998; Kalemba and Kunicka 2003; Burt, 2004) and also confirmed by the experimental activities reported in the previous chapters of this thesis.

More specifically, biocontrol effects of *L. lactis* CBM21 in minimally processed products based on apples and lettuce were analysed in combination with natural antimicrobials. These compounds were sensory compatible with the food matrices considered and selected for their efficacy in real systems in the previous chapters. In fact, on the basis of previous results, mixtures hexanal/2-(E)-hexenal and citral/2-(E)-hexenal combined with the biocontrol agent were

employed on minimally processed apples. In order to verify the impact of the natural antimicrobials on the viability of the biocontrol agent, their MIC (Minimum Inhibitory Concentration) and MCB (Minimum Bactericidal Concentration) values after 24 and 48 hours of incubation at 30°C were determined.

Lactococcus lactis CBM 21 was shown to be extremely resistant against citral and hexanal (MIC and MCB higher than 700 ppm) when inoculated at a level of 4 log cfu/mL. On the contrary, it was more sensitive towards 2-(E)-hexenal, anyway the MIC values were always higher than 200 ppm. Generally, literature data indicate that significant increase in shelf-life can be obtained by using less than 50 ppm of 2-(E)-hexenal in products based on minimally processed fruits (Lanciotti et al., 2003, 2004). In addition, literature reports concerning minimally processed fruits show that citral and hexanal has a good antimicrobial potential associated with a good organoleptic compatibility at concentrations less than 200 ppm (Lanciotti et al., 1999; Belletti et al., 2008).

The biocontrol agent (7 log cfu/mL) and the natural antimicrobials (125 ppm for each compound) were added during the dipping of sliced apples while samples obtained with the same protocol but treated (in the process of dipping) with only ascorbic acid and citric acid were considered as controls. In the phase of dipping, pathogenic microorganisms such as *Listeria monocytogenes* Scott A and *Escherichia coli* 555 were inoculated also in order to assess the efficacy of biocontrol and/or of natural antimicrobials employed on the safety of the products. The pathogens were inoculated between 3 and 4 log cfu/mL. During storage at 6°C, the effects of dipping conditions tested on the microbiological quality of the product were evaluated.

The considered biocontrol agent was able to survive in the products considered independently on the presence or the absence of natural antimicrobials; in fact, in each condition adopted, *Lactococcus lactis* CBM21 showed a level of about 7 log cfu/g during the storage (28 days at 6°C; data not shown). In the control samples, lactic acid bacteria did not exceed 3 log cfu/g.

As shown in Figure 6.3, the biocontrol agent considered allows the significant increase of the safety of the products since it inhibits the growth of *Listeria monocytogenes* when used alone, but especially when used in combination with the proposed natural antimicrobials.

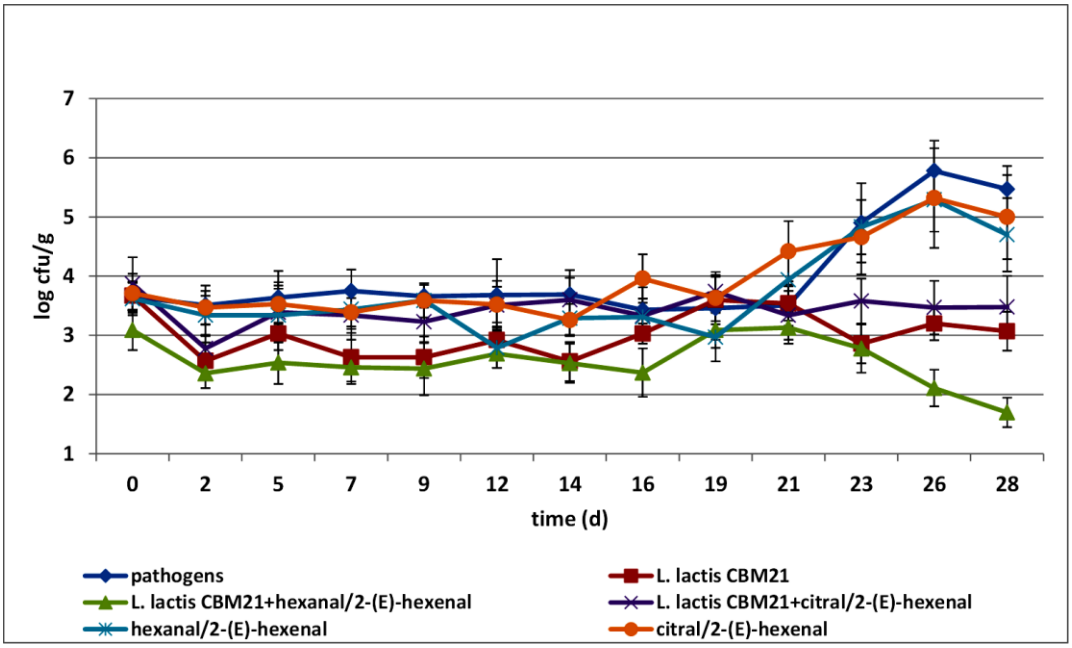


Figure 6.3- *Evolution of* Listeria monocytogenes *(log cfu/g) inoculated in sliced apples in relation to the washing conditions employed*

The higher effectiveness against *Listeria* monocytogenes was observed when strain CBM21 was used in combination with hexanal/2-(E)-hexenal. In fact, in these conditions, *Listeria monocytogenes* showed a loss of viability of more than 2 logs. By contrast, *L. monocytogenes* was able to grow, although very slowly, in the controls and in the samples added with hexanal/2-(E)-hexenal and citral/2-(E)-hexenal in the absence of the biocontrol agent. Regardind *E. coli*, the low storage temperatures did not allow its proliferation, independently on the conditions employed in the dipping processes. Furthermore, the use of natural antimicrobials slightly increased the kinetics of death of the target microorganism (Figure 6.4).

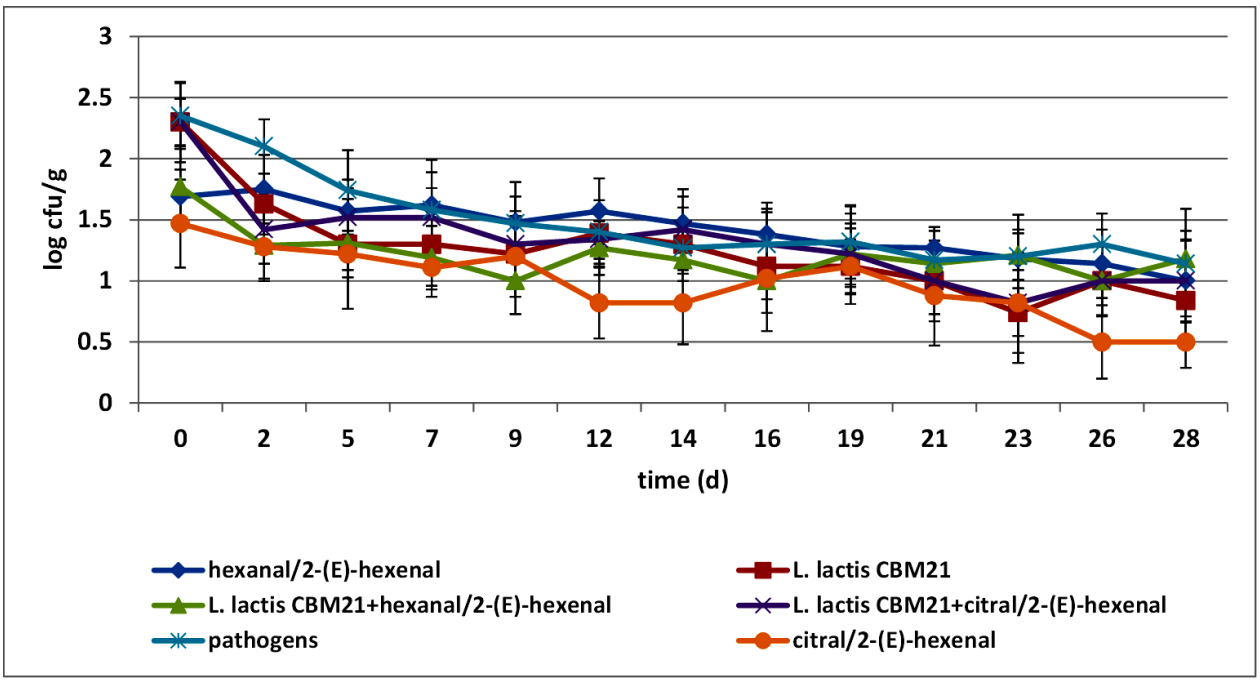


Figure 6.4- *Evolution of* Escherichia coli *(log cfu/g) inoculated in sliced apples in relation to the washing conditions employed*

Yeast loads during storage at 6°C in relation to the dipping conditions employed are reported in Figure 6.5. Obtained data clearly showed that yeasts were able to overcome the level of 6 log cfu/g, which is considered as the spoilage threshold for this type of product, only in control samples subjected to the traditional dipping and in control samples which were previously inoculated with the considered pathogens. The addition of the biocontrol agent and/or natural antimicrobials significantly delayed the growth of yeasts, allowing a significant increase of the product shelf-life. In fact, the yeast cell loads in all the samples supplemented with *Lactococcus lactis* CBM 21 and/or natural antimicrobials did not reach 6 log cfu/g also after 28 days of refrigerated storage.

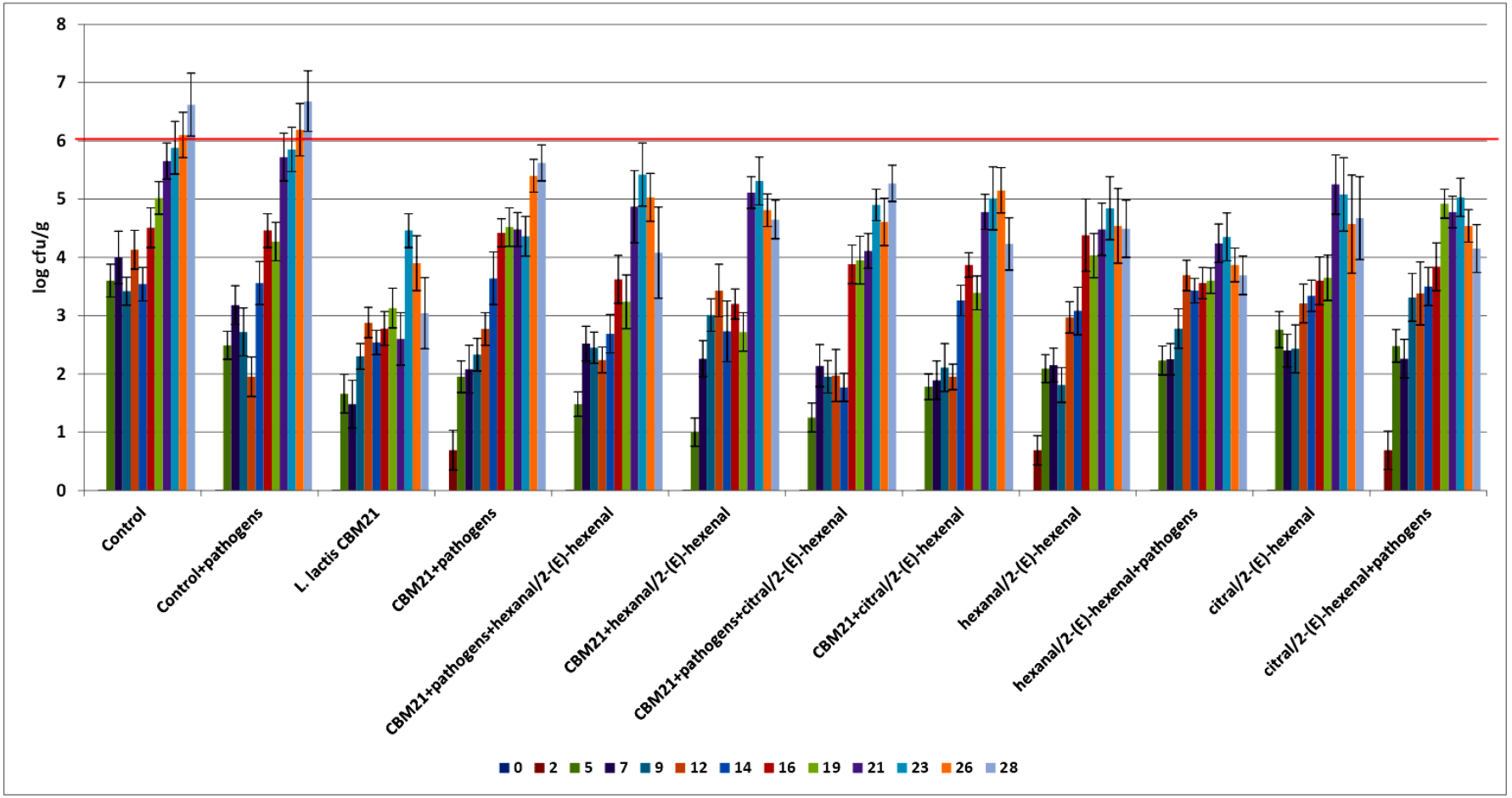


Figure 6.5- *Evolution of the load of yeasts in sliced apples in relation to the washing conditions employed*

Although *L. lactis* CBM21 showed good potential for the control of inoculated pathogens and spoilage agents naturally present in the products, color data showed a negative effect on the evolution of the colorimetric measurement indexes. In fact, samples inoculated with the biocontrol agent were characterized by a rapid worsening of L* (luminosity) and a* (red index) values. The evolution of luminosity L* is reported in Figure 6.6. The presence of natural antimicrobials mitigated the negative effects of the *Lactococcus lactis* strain selected. However, also in samples treated only with the biocontrol agent, the color parameters were acceptable up to 16-19 days. Photographs shown in Figure 6.7 confirmed the negative effects of the *Lactococcus lactis* strain from the sixteenth day of storage at 6 °C.

Panel test performed after 1 and 4 days of storage at 6°C on control samples, samples added with *L. lactis* CBM21, samples added with the mixture hexanal/2-(E)-hexenal and samples supplemented with citral/2-(E)-hexenal (Figure 6.8), showed that the addition of the biocontrol agent had a positive effect on the overall impression of the consumers after 1 day of storage. Otherwise, all the other quality parameters evaluated were similar or better than those detected in the controls and in the other treated samples. The browning of the samples added with *L. lactis* CBM21 after four days of storage was perceived by consumers as similar to the controls. The addition of citral/2-(E)-hexenal was perceived as negative for the flavor and the odor of the sliced apples by the consumers, both after 1 and 4 days, mainly due to the presence of citral that is characterized by a lemongrass flavor. On the contrary, the addition of citral/2-(E)-hexenal dramatically reduced the browning of the product after 4 days of storage. For the consumers, the addition of hexanal/2-(E)-hexenal resulted more compatible with apple flavor and all the other quality parameters. Moreover, samples added with this mixture of natural antimicrobials were perceived quite similar to the controls after 1 days of storage, while, after 4 days of storage, their quality parameters resulted better, in particular the flavor, the odor and the overall impression, than all the other samples.

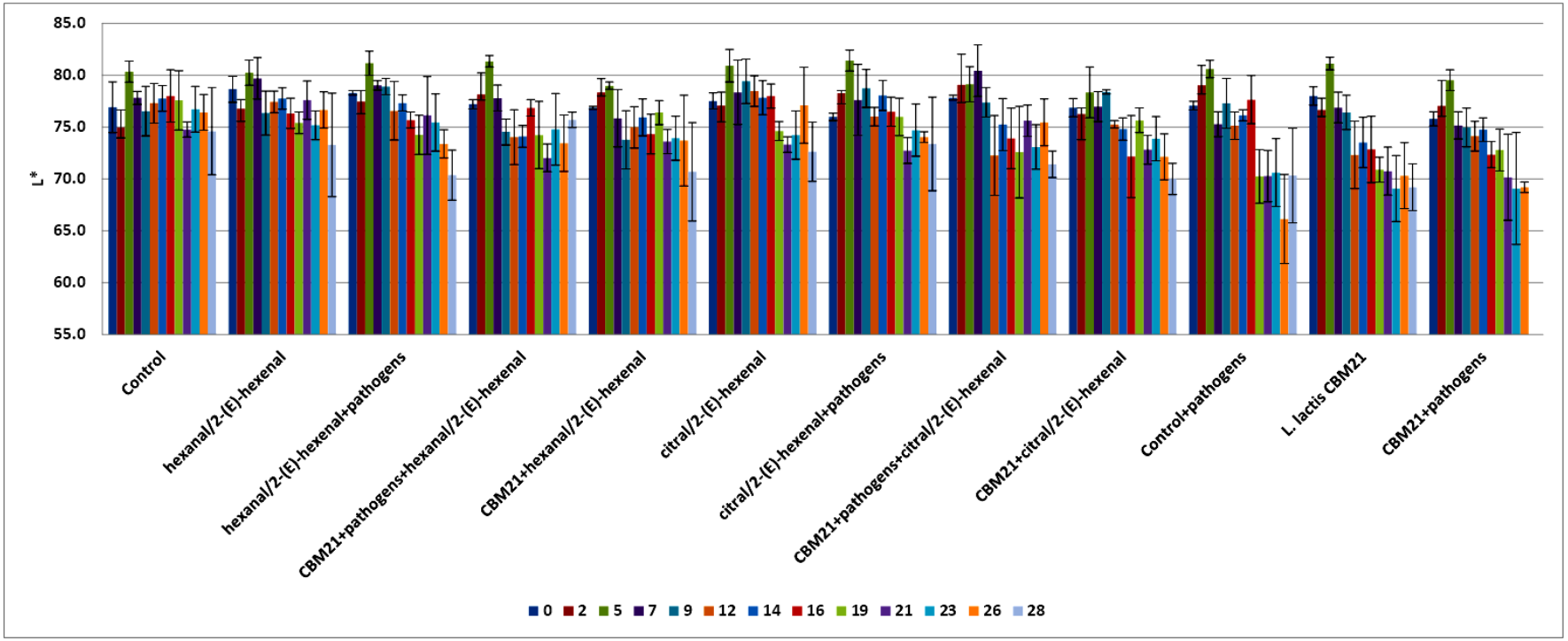


Figure 6.6- *Evolution of luminosity, L*, of apples in relation to the washing conditions employed during storage time.*

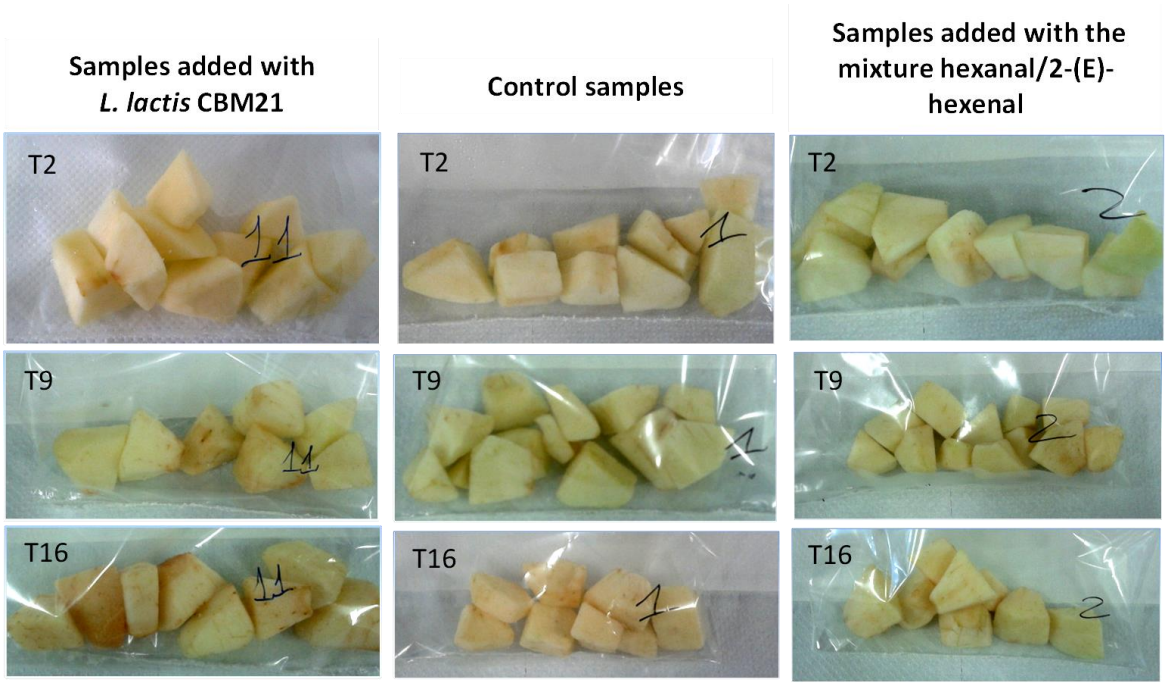
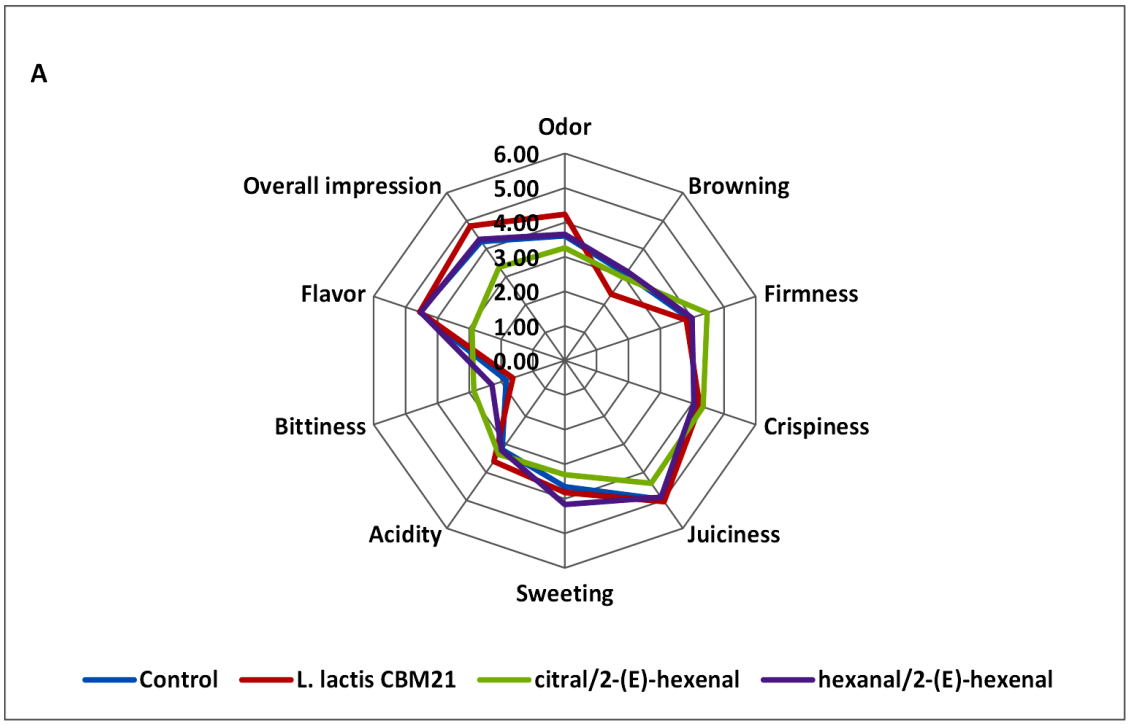


Figure 6.7. *Evolution during storage of the samples inoculated only with the* L. lactis *CBM 21, in comparison with the control samples and samples supplemented with the mixture hexanal/2-(E)-hexenal*



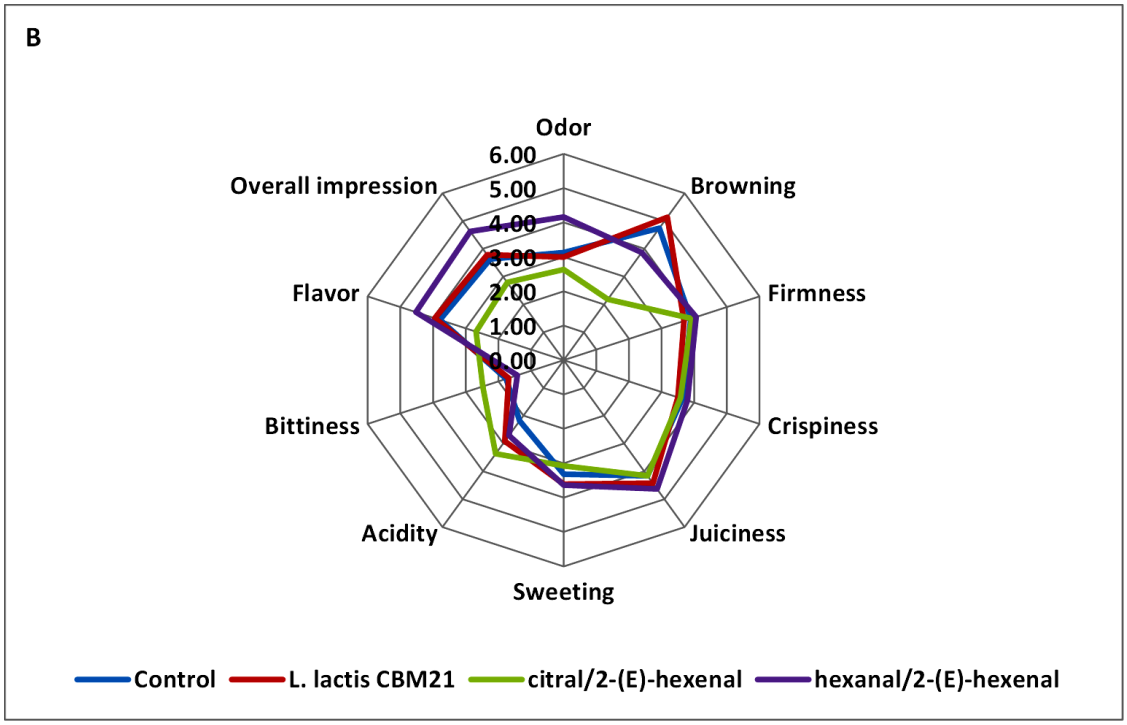


Figure 6.8- *Sensory data of sliced apples, in relation to the washing employed, after 1 day (A) and 4 days (B) of storage at 6°C*

3.3 Effects of L. lactis CBM21, in combination with natural antimicrobials, on microbiological quality of minimally processed lamb's lettuce

On the basis of previous results, thyme EO combined with the biocontrol agent were employed on minimally processed lamb's lettuce. Also in this case, in order to verify the impact of thyme EO on the viability of the biocontrol agent considered, the MIC and MBC values after 24 and 48 hours of incubation of thyme EO against *L. lactis* CBM21 were recorded.

Lactococcus lactis CBM21 proved to be resistant to thyme EO (MIC and MBC higher than 300 ppm) with cell concentrations of 4 log cfu/mL. On the contrary, it was more sensitive when inoculated at 2 log cfu/mL, anyway the MIC values were always higher than 250 ppm. On the other hand the effects of inoculation level on MIC and MBC values is well known (Lambert et al., 2001).

The biocontrol agent (7 log cfu/mL) and/or thyme EO (250 ppm) were added during the washing step, samples obtained with the same protocol but treated (in the washing process) with 120 ppm of chlorine were considered as controls. In the phase of washing, in some conditions, pathogenic microorganisms such as *Listeria monocytogenes* and *Escherichia coli* were also inoculated in order to assess the

efficacy of the biocontrol agent and/or thyme EO on the safety of the products. Pathogens were inoculated at levels ranging between 3 and 4 log cfu/mL. During storage at 6°C the effects of the tested washing conditions on the microbiological quality of the product were evaluated.

The considered *Lactococcus lactis* CBM21 was sensitive to the stringent conditions of the system independently on the presence of thyme EO. In fact, the population of *Lactococcus lactis* CBM21 decreased during storage at 6°C, both in the presence or the absence of thyme EO (data not shown). The latter, however, accelerates the death kinetics of the added strain which, after 15 days of storage showed a cell load of about 10^4 cfu/g compared to the initial loads that ranged between 6 and 7 log cfu/g.

The addition of the biocontrol agent in combination with thyme EO decreased the initial loads and the growth kinetics of the total aerobic mesophilic bacteria. In particular, the addition of *Lactococcus lactis* CBM21 induces the reduction of the initial total aerobic mesophilic loads of a logarithmic cycle, while the addition of the strain in combination with thyme EO reduced the load of the total mesophilic of about two logarithmic cycles. Samples which were washed separately with chlorine exceed the level of 7 log cfu/g of product after 7 days storage at 6°C, whereas all samples added with antimicrobials and/or protective agent did not exceed the level of 7 log cfu/g even after 15 days storage at 6°C.

Concerning the antimicrobial activity against *Escherichia coli*, Figure 6.9 shows the loss of viability of this microorganism during storage at 6°C, independently on the presence of biocontrol agent and antimicrobials. However, the presence of these compounds increased the inactivation kinetics of *E. coli*. In particular, similarly to chlorine, the addition of thyme EO showed the major effect on the viability reduction of *E. coli* and, consequently, the highest effectiveness in increasing the safety of the product.

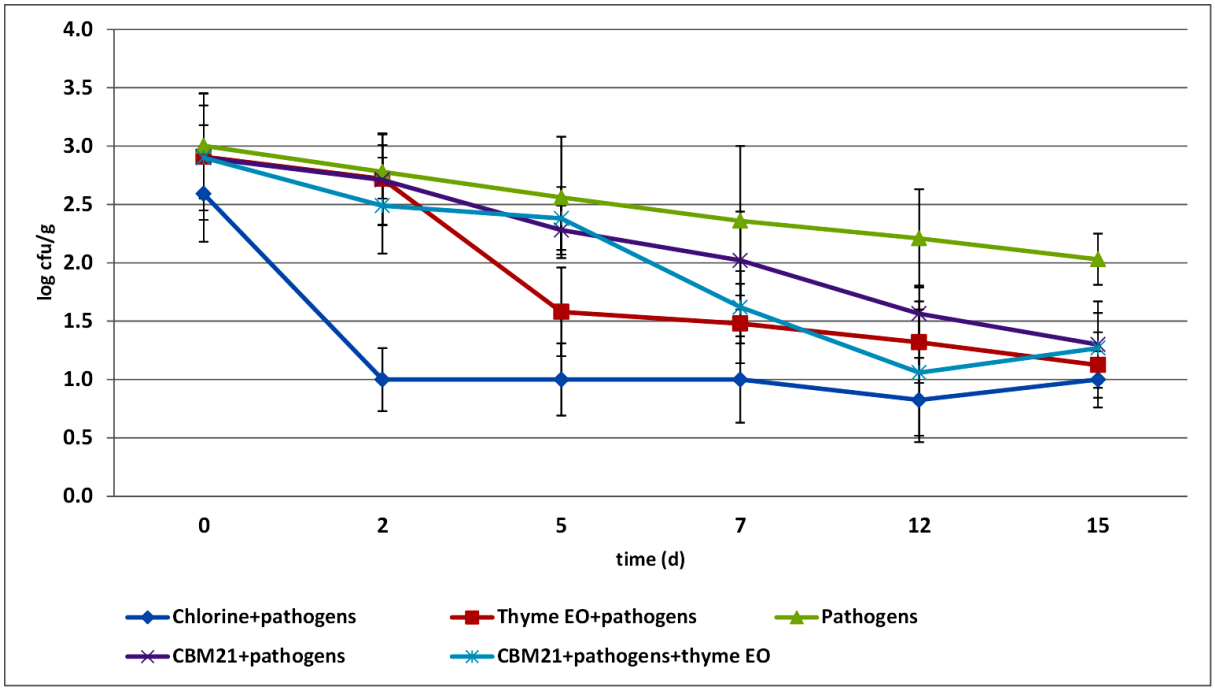


Figure 6.9- *Evolution of* Escherichia coli *(log cfu/g) inoculated in minimally processed lamb's lettuce in relation to the washing conditions employed*

Listeria monocytogenes remained viable without showing growth in the control samples without chlorine and in the samples added with the biocontrol agent. The stringent characteristics of the raw materials and the competition with the endogenous microflora presumably prevented its proliferation. *L. lactis* CBM21 seems to be less effective against *L. monocytogenes* in lamb's lettuce than apples. This is probably due to the lower production of nisin in these conditions. In fact, it is well documented in literature that nisin production increases only in the late exponential phase of growth (De Vuyst & Vandamme, 1992). Based on this, *L. lactis* CBM21 was not affected by the presence of competitive microflora when grown on apples; consequently, strain CBM21 remained viable or also increased its load during the refrigerated storage. Contrarily, on lamb's lettuce, the presence of competitive microflora caused a reduction of the biocontrol agent cell load during storage.

However, the presence of thyme EO in combination or not with the biocontrol agent showed a significant efficacy (comparable to that of chlorine) in reducing the levels of *L. monocytogenes* during storage at 6°C (Figure 6.10).

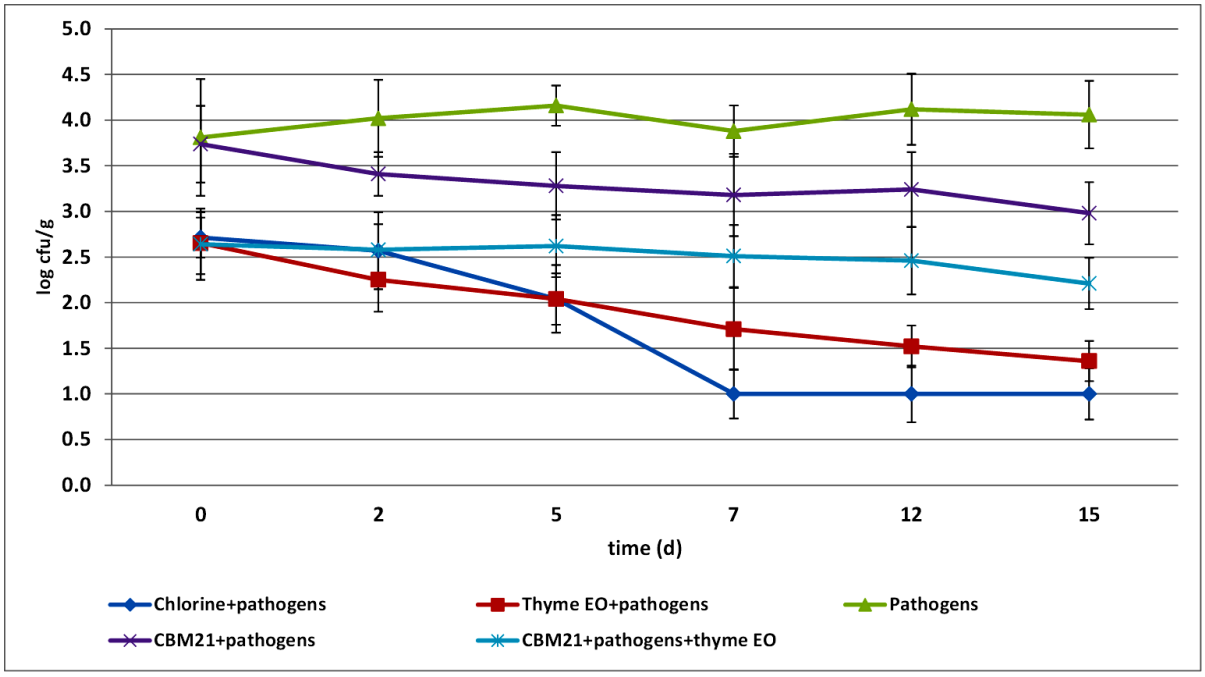


Figure 6.10- *Evolution of* Listeria monocytogenes *(log cfu/g) inoculated in minimally processed lamb's lettuce in relation to the washing conditions employed*

As regard to the effect of *L. lactis* CBM21 and natural antimicrobials on the color and texture of lamb's lettuce, not significant differences were evident among the different treatments compared to the samples added with chlorine during the storage at 6°C.

4. Conclusions

The results obtained showed that *L. lactis* CBM21, selected on the basis of the ability to produce nisin Z as well as the physiological characteristics (ability to grow at low pH, low temperature and in the presence of high concentrations of sugars), significantly increased the safety and shelf-life of sliced apples and minimally processed lamb's lettuce. In particular, the considered biocontrol agent was able to inhibit both the pathogenic microorganisms inoculated as well as yeasts (apples) or mesophilic aerobic bacteria (lettuce), that represent the main spoilage agents of this type of product. The effectiveness against *Listera monocytogenes* observed on sliced apples seems to be attributed to the production of nisin Z more than to the competition for space and nutrients. The effects obtained against *Listeria monocytogenes* was absolutely relevant and comparable to that observed for other biocontrol agents used on fresh-cut apples. For example, Alegre et al. (2013) observed a reduction of 2.5 log cfu/g

of *Listeria monocytogenes* in Golden Delicious apples inoculated with *Pseudomonas graminis* CPA-7 packaged in modified atmosphere and stored for 7 days at 10°C.

The selected strain, in particular when combined with the natural antimicrobials employed, showed a good inhibition also against microorganisms not specifically sensitive to nisin Z such as the deliberately inoculated *Escherichia coli* and the naturally occurred yeasts. On the other hand the inoculated strain of *Escherichia coli* was not able to grow at the conditions adopted, but the presence of the biocontrol agent increased the death kinetics. Also Abadias et al (2009) obtained similar results using *Candida sake* CPA-1 as a biocontrol agent.. In this case the inhibition was attributed to the competition for space and for nutrients. However, it is possible that the selected strain was able to produce other molecules with antimicrobial activity; in fact it is well known that lactic acid bacteria can produce a broad spectrum of antimicrobial substances (volatile ketoacids, furanones, diacetyl, lactic acid, hydrogen peroxide) (Schillinger et al., 1996). Moreover, the selected strain showed interesting inhibition also against the spoilage agents in both the food system considered. Since literature data concerning minimally processed fruits and vegetables are mostly focused on the effect of biocontrol agents on pathogen microorganisms, without any reference to the shelf-life of the product, these results can be useful to better understand the effects of biocontrol agents on spoilage microorganisms. The use of *Lactococcus lactis* CBM21 allowed to limit the growth of yeasts on apples below 5 log cfu/g during all the time of storage considered, while on lamb's lettuce allowed to not exceed the level of 7 log cfu/g in the population of total meshopilic bacteria, also after 15 days storage at 6°C. The addition of *Lactococcus lactis* CBM21 affected the quality parameters of apples such as color after 14 days of storage at 6°C. However, the recognized average life of this category of products is generally less than this preservation period. Moreover, the negative effects due to the inclusion of biocontrol agent after 14 days of storage was balanced by the presence of the natural antimicrobials, particularly hexanal. On the other hand, the positive effect of hexanal on the maintenance of the color of minimally processed apples is reported in literature (Lanciotti et al., 1999; Corbo et al. 2000). The consumers were not able to recognized the presence of the biocontrol agent on sliced apples after 1 and 4 days of storage. Moreover the panel test showed that the addition of the biocontrol agent positively affected quality parameters such as the flavor and odor, which were preferred by the consumer compared to control samples.

Therefore, the selected biocontrol agent, and in particular its combination with natural antimicrobials, may represent a good strategy to increase the safety and the shelf-life of minimally processed fruits and

vegetables. Furthermore, since important health properties have been attributed to lactic acid bacteria, their use in this kind of products could also contribute to confer specific healthy properties to these products. However, the introduction of biocontrol agent can be further optimized, focusing on the level and mode of inoculation and to limit the negative effects observed on the color parameters.

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CHAPTER 7

Effect of sub-lethal concentrations of thyme and oregano essential oils, carvacrol, thymol, citral and 2-(E)-hexenal on membrane fatty acid composition and volatile compounds of *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enteritidis*

Abstract

In recent years, the interest of the food industry and consumers in natural antimicrobials, alternative to traditional chemical additives, to prevent the growth of spoilage and pathogenic microorganisms is increased significantly. Thyme and oregano essential oils are two of the most effective as antimicrobials. Their antimicrobial activity is well documented both in *in vitro* screening and in food model media, and carvacrol and thymol are present as major and effective components of thyme and oregano essential oils. Also aldehydes, such as citral and 2-(*E*)-hexenal, which are component of the aroma of many fruits and vegetables are endowed with strong antimicrobial activity both in model and real system. Although the antimicrobial properties of essential oils and their major components are well known, their mechanisms of action have not been fully understood. Because the usage of these compounds as antimicrobials in foods has to be supported by the comprehension of their action mechanisms, the aim of the research was to investigate the modifications of cell membrane fatty acid composition and volatile molecule profiles of pathogenic microorganisms, such as *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli,* during the growth in the presence of different sublethal concentrations of thyme and oregano essential oils as well as carvacrol, thymol, 2-(E)-hexenal and citral. The results obtained evidenced that the tested molecules induced noticeable modifications of membrane fatty acid profiles and volatile compounds produced during the growth. Although specific differences in relation to the species considered were identified, the tested compounds induced a marked increase of some membrane associated fatty acids, particularly unsaturated fatty acids, trans-isomers, and specific released free fatty acids.

1. Introduction

Essential oils (EOs), can be defined as complex mixture of volatile compounds, characterized by a strong sensorial impact and produced by many plants as secondary metabolites. In recent years, the interest of the food industry and consumers in natural antimicrobials, alternative to traditional chemical additives, to prevent the growth of spoilage and pathogenic microorganisms, is increased significantly. For this reason the number of publications regarding the antimicrobial activity and the potential application of EOs and their main components as natural preservatives in different food matrices such as meat, dairy products, minimally processed fruits and vegetables is greatly augmented (Holley & Patel 2005; Espina, et al, 2011; Gutierrez et al, 2009a; Smith-

Palmer et al, 2001; Burt, 2004; Karabagias et al., 2011). Is well documented that these volatile molecules play a key role in defence of fresh vegetables against microbiological decay (Ben-Yehoshua et al., 1998). The antimicrobial properties of EOs and their components have been tested in the past (Dorman & Deans 2000; Cosentino et al., 2003; Smith-Palmer et al., 1998; Kalemba & Kunicka, 2003). In addition, these molecules are widely used as flavouring in foodstuffs and are generally recognized as safe (GRAS).

Normally EOs contain about 20–60 components at different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts (Bakkali et al. 2008). Among EOs, thyme and oregano EO are two of the most effective as antimicrobials. Their antimicrobial activity is well documented both in *in vitro* screening (Cosentino et al., 1999; Ivanovic et al., 2012; Gutierrez et al., 2008a) and in food model media (Gutierrez et al., 2009b; Chouliara et al., 2007; Nowak et al., 2012). Carvacrol and thymol are presents as major and effective components of thyme and oregano EOs (Burt, 2004). These two phenolic monoterpenes have shown a strong *in vitro* antimicrobial activity against a wide range of pathogenic microorganisms (Bagamboula et al., 2004; Zhou et al., 2007, Oussalah et al., 2007), fungi and insects (Kordali et al., 2008). Other volatile compounds are aldehydes, such as hexanal and 2-(*E*)-hexenal, which are component of the aroma of many fruits and vegetables and endowed with strong antimicrobial activity both in model and real system (Gardini et al., 2001; Lanciotti et al., 2004). Moreover, citral (3,7-dimethyl-2-7-octadienal), a component of several citrus EOs, is a mixture of two isomeric acyclic monoterpene aldehydes: geranial and neral, characterized by a wide spectrum antimicrobial activity both in model system and foodstuffs (Hayes & Markovic, 2002; Wuryatmo et al., 2003 and Belda-Galbis et al., 2013).

Although the antimicrobial properties of EOs and their major components is well known, their mechanisms of action have not been fully understood (Hyldgaard et al., 2012). In general, the hydrophobicity of EOs and their components, allow them to be distributed in the lipids of the cell membrane. The presence of the hydroxyl group, in thymol and carvacrol, is connected to the inactivation of the microbial enzymes. Probably, this group interacts with the cell membrane causing leakage of cellular components, change in fatty acids (FAs) and phospholipids composition, and disruption of the proton motive force, electron flow, active transport, coagulation of cell contents and influencing genetic material synthesis (Burt, 2004).

2-(E)-hexenal and citral, have mechanisms of action on microbial cells, similar to that of other aldehydes. These molecules are able to permeate the plasma membrane through passive diffusion. Inside the cells α, β-unsaturated aldehydes can reacts with biologically important nucleophilic groups (Kubo & Fujita, 2001). Moreover, aldehydes may cross link amino groups in the cell wall and cytoplasm and increase in its permeability and inhibit enzymes with a thiol group at the cytoplasmic membrane (Aiemsaard et al., 2011). At high concentrations, aldehydes may also cause coagulation and precipitation of cytoplasmic constituents (Denyer, 1995).

It is well known that when microbial cells are exposed to a sub-lethal stress, the cell membrane is able to change the fluidity in order to deal with the new environment (Russell et al., 1995). This is fundamental in maintaining membrane integrity and functionality against external stresses (Russell et al., 1995). To reduce the effects of environmental condition changes on cell membrane, the cell can regulate the fluidity by modifying the unsaturation level, the fatty acid length and the presence of branched chains or hydroxylic groups (Guerzoni et al., 1997; 2001).

In this perspective, the aim of the research was to investigate the modifications in cell membrane fatty acid composition and volatile molecule profiles of pathogenic microorganisms, such as *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli,* during the growth in the presence of different sublethal concentrations of thyme EO, oregano EO, carvacrol, thymol, 2-(E)-hexenal and citral.

2. Materials and Methods

2.1 Natural antimicrobials and microbial strains

Oregano and thyme EO were obtained from Flora s.r.l. (Pisa, Italy), the others compounds (2-(E)-hexenal, Citral, Carvacrol and thymol) were purchased from Sigma-Aldrich (Milano, Italy).

The strains used in this study, *L. monocytogenes* Scott A, *E. coli* 555, *S. enteritidis* E5, belonging to the Department of Agricultural and Food Sciences. The strains were maintained at -80°C and cultured in brain heart infusion (BHI) broth (Oxoid, Basingstoke, Humpshire, UK) for 24 h at 37°C. Before experiments, the strains were sub-cultured, on BHI broth for 24h at 37 °C.

2.2 Determination of the growth kinetics of L. monocytogenes, E. coli and S. enteridis in presence of EO

The growth curves of the tested microorganisms, were performed by using the most effective EOs and their components, selected on the basis of MIC results (reported in chapter 3 and 4). Thyme EO, oregano EO, carvacrol and thymol were used on all the selected pathogens. Citral was tested only on *L. monocytogens*, whereas 2-(*E*)-hexenal was used on *E. coli*. Each EOs or components was used at three different concentrations (approximately 1/2, 1/3, 1/5 of the MIC value), the EOs or components and the relative concentrations used on *L. monocytogenes*, *S. enteritidis* and *E. coli* are reported in Table 7.1. The tested strains were inoculated in 100 mL flasks, containing 50 mL of BHI broth at a level of about 2.5 log cfu/mL. Immediately after the inoculum, the samples were supplemented with the selected concentrations of each compound. The EOs or their components used were conveyed through 1% of ethanol (0.5 mL:50 mL). Three flasks for each condition were considered in different days. Inoculated samples added just with 1% of ethanol and samples without any addition were considered as controls. The samples were incubated at 37°C, and the growth evaluated on the basis of the optical density at a 600nm (OD_{600}) using a spectrophotometer UV-1204 (Shimadzu, Kyoto, Japan), and periodically (every hour during exponential growth phase) a plate count was performed. The OD growth kinetics were modelled by using Gompertz equation with the statistica 8 software.

Table 7.1- *Molecules and relative concentrations (ppm) used respectively on* E. coli, L. monocytegenes *and* S. enteritidis *for the determination of the growth kinetics and subsequently for study the modifications in fatty acid composition of the cell membranes*

	Oregano EO (ppm)	Thyme EO (ppm)	Thymol (ppm)	Carvacrol (ppm)	Citral (ppm)	2-(*E*)-hexenal (ppm)
E. Coli	50	70	40	40		100
	80	120	70	70		170
	120	170	100	100		250
L. monocytogenes	30	40	40	20	50	
	40	70	70	35	85	
	50	100	100	50	125	
S. enteritidis	60	50	40	40		
	100	90	70	70		
	150	135	100	100		

2.3 Cell treatments

The same molecules and strains used in the determination of the growth kinetics and reported in Table 7.1, were used in the next step.

The tested strains, grown overnight, were inoculated in 1.0 L flasks, containing 500 mL of BHI broth at a level of about 2.5 log cfu/mL. Immediately after the inoculum, the samples were supplemented with the selected concentrations of each compound. The EOs or their components used were conveyed through 1% of ethanol (5 mL : 500 mL). Three flasks for each condition were considered. Inoculated samples added just with 1% of ethanol and samples without any addition were considered as controls.

The incubation was performed at 37°C, until was reached the stationary growth phase. The times of incubation for each condition were previously defined through the determination of the growth kinetics.

2.4 Fatty acid analyses

Late exponential phase cells were collected by centrifugation (8000 *g* × 15 min) and washed in physiological water. Lipid extraction and membrane fatty acid analyses were performed according to Suutari et al. (1990). The fatty acid composition analyses was executed through gas chromatography combined to mass spectometry (GC/MS) technique. For peak detection, an Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett–Packard, Geneva, Switzerland) and a fused silica capillary column coated with a 0.2 μm film of Carbowax (Supelco) as stationary phase was used. The injector and detector temperatures were respectively 125 and 225 °C. Helium was used as carrier gas at a flow-rate of 3 mL/min; the splitting ratio was 1:20 (v/v). The oven temperature program was 120 °C for 5 min, then heated to 215 °C at 3 °C/min, this temperature was maintained for 0 min, then heated to 225 °C at 0.5 °C/min, this temperature was maintained for 2 min. Compounds were identified by comparing their retention times with those of a standard mix, BAME (Sigma-Aldrich, Milan, Italy) and by the use of the Agilent Hewlett–Packard NIST 98 mass spectral database. The results were the average of three different biological replicates.

2.5 Volatile molecule profile analyses through GC/MS/Solid Phase Microextraction (SPME)

Late exponential phase cells, for each strain and added antimicrobial, were collected by centrifugation and then 5 mL of the supernatant were sterilely taken and placed in 10 mL vial sealed by PTFE/silicon septa. Three replicates for each condition were analysed and the samples were stored at -40 °C until analyses.

Before analysis the samples were thawed and then conditioned for 5 min at 50 ° C. An SPME fiber covered by 50 μm divinylbenzene-carboxen-poly(dimethylsiloxane) (DVB/CARBOXEN/PDMS StableFlex) (Supelco, Steiheim, Germany) was exposed to each sample at 50 °C for 40 min, and finally, the adsorbed molecules were desorbed in the GC for 10 min. Regarding fibre and gas-chromatographic conditions, the method reported by Patrignani, et al. (2008) was used. Compounds were identified by the use of the Agilent Hewlett–Packard NIST 98 mass spectral database.

2.6 Statistical analyses

The growth curves of the tested microorganisms were modelled according to the Gompertz equation as modified by Zwietering et al. (1990). Three replicates for each strain and each pH condition were performed.

The relative percentages of the FAs (as peak areas of the methyl esters) are means of 3 replicate determinations. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 2 and 5%.

The unsaturation degree (Δ/mol) in the lipid fraction was calculated as:

$$\Delta/\text{mol} = [\%\text{monoenes}+2\ (\%\text{dienes})]/100$$

The mean fatty acid chain length was expressed as:

$$\text{Mean chain length} = \Sigma(\text{FAP} \times \text{C})/100$$

where FAP is the percentage of fatty acid and C the number of carbon atoms.

Principal component analysis (PCA) were performed using Statistica software (version 8.0; StatSoft., Tulsa, Oklahoma, USA) to obtain a visual overview of FA composition of cell membranes and of the volatile molecule profile.

The volatile molecule compounds detected for the tested microorganisms grown in presence of the selected compounds are expressed as peak area x 10^5. The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 5 and 10%

3. Results

3.1 Effects of the selected compounds on the growth kinetics of Listeria monocytogenes, Escherichia coli and Salmonella enteritidis

In Table 7.2a the Gompertz parameters of *E. coli*, grown in the presence of different antimicrobials such as carvacrol, 2-(E)-hexenal, oregano, thymol and thyme are reported. As shown by Table 7.2a, the growth in the presence of the tested antimicrobials caused a reduction of A value (maximum increase of the population in stationary phase), associated to a reduction of μ max (growth rate in exponential phase) and an increase of λ (duration of lag phase), respect to the control and the control added with 1% of ethanol. Clearly, this trend resulted more accentuated increasing the concentrations used for each antimicrobial. In particular, the addition of 2-(E)-hexenal at different concentrations and 100 ppm of oregano increased significantly the durations of lag phase respect to the control, and all the other treated samples. 2-(E)-hexenal, at all the concentrations used, carvacrol and oregano, at the highest concentration (100ppm), were the most effective to reduce the growth rate in exponential phase.

Table 7.2b reports the Gompertz parameters of *S. enteritidis*, supplemented with different concentrations of carvacrol, oregano, thymol and thyme. Also for *Salmonella*, a decrease of A and μ max parameters, associated to an increase of λ was detected in the treated samples respect to the control. The effectiveness to delay the growth kinetics were dependent on the antimicrobials used and their concentrations. Oregano and thyme, when used at the higher concentrations, carvacrol at 100 ppm were the most effective in slowing the growth of *Salmonella*.

In Table 7.2c the Gompertz parameters of *L. monocytogenes*, grown in presence of different antimicrobials such as carvacrol, citral, oregano, thymol and thyme are reported. The employed strain of *Listeria* monocytogenes had a lower growth rate than the other tested microorganisms and the highest sensitiveness to antimicrobials used. In particular, the addition of thyme and

oregano, independently on the concentration used, caused a marked increase of the lag phase, while thyme and thymol were the most effective to reduce μ max.

Table 7.2 a,b and c. *Gompertz parameters of* E. coli *(a)* S. enteritidis *(b) and* L. monocytogenes *(c), grown in the presence of different concentrations of carvacrol, 2-(E)-hexenal, citral, oregano, thymol and thyme*

2a	Control	Ethanol 1%	Carvacrol			Oregano EO			Thymol			Thyme EO			2-(E)-hexenal		
			40 (ppm)	70 (ppm)	100 (ppm)	40 (ppm)	80 (ppm)	100 (ppm)	40 (ppm)	70 (ppm)	100 (ppm)	70 (ppm)	120 (ppm)	170 (ppm)	100 (ppm)	170 (ppm)	250 (ppm)
A[a]	0.84	0.81	0.86	0.82	0.71	0.76	0.75	0.70	0.78	0.77	0.74	0.71	0.73	0.71	0.91	0.99	0.75
μ max[b]	0.35	0.32	0.30	0.31	0.26	0.32	0.30	0.26	0.32	0.31	0.30	0.30	0.29	0.28	0.25	0.15	0.16
λ[c]	7.21	7.43	7.82	7.85	7.68	7.18	7.29	11.20	7.55	7.70	7.53	7.62	7.72	8.96	9.51	15.56	40.73

2b	Control	Ethanol 1%	Carvacrol			Oregano EO			Thymol			Thyme EO		
			40 (ppm)	70 (ppm)	100 (ppm)	60 (ppm)	100 (ppm)	150 (ppm)	40 (ppm)	70 (ppm)	100 (ppm)	50 (ppm)	90 (ppm)	135 (ppm)
A[a]	0.75	0.72	0.70	0.68	0.72	0.69	0.73	0.69	0.74	0.71	0.69	0.68	0.70	0.72
μ max[b]	0.37	0.36	0.33	0.29	0.25	0.31	0.26	0.29	0.32	0.31	0.31	0.28	0.28	0.25
λ[c]	6.27	6.53	6.61	6.71	6.69	7.04	7.51	9.86	6.61	6.54	6.79	6.79	7.13	7.87

2c	Control	Ethanol 1%	Carvacrol			Oregano EO			Thymol			Thyme EO			Citral		
			20 (ppm)	35 (ppm)	50 (ppm)	30 (ppm)	40 (ppm)	50 (ppm)	40 (ppm)	70 (ppm)	100 (ppm)	40 (ppm)	70 (ppm)	100 (ppm)	50 (ppm)	85 (ppm)	125 (ppm)
A[a]	0.84	0.85	0.72	0.74	0.73	0.71	0.69	0.71	0.82	0.68	0.65	0.66	0.70	0.73	0.79	0.74	0.73
μ max[b]	0.15	0.13	0.17	0.16	0.15	0.15	0.14	0.09	0.12	0.09	0.10	0.08	0.08	0.10	0.15	0.12	0.13
λ[c]	17.62	18.33	18.60	18.77	19.28	25.31	32.30	41.63	18.46	21.16	24.02	31.96	43.50	49.74	19.69	21.54	26.23

Gompertz parameters calculated according to Zwietering et al. (1990) for *E. coli* (2a), S. enteritidis (2b) and L. monocytogenes (2c) grown in the presence of different concentrations of natural antimicrobials. The reported values are average of three different replicates. The coefficients of variability, expressed as the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.

[a] Maximum growth extent. It corresponds to the asymptotic optical density attained as time increases indefinitely (final optical density).

[b] The maximun specific growth rate as variation of O.D. 600 nm/h.

[c] Lag Time in hours

3.2 Cell fatty acid changes induced by sublethal concentrations of 2-(E)-hexenal, citral, carvacrol, thymol, thyme EO and oregano EO

The presence of the tested substances affected both membrane associated and released fatty acids (FAs) of the tested strains. Table 7.3 reports the FAs composition of late exponential phase cells of *L. monocytogenes* Scott A in relation to the stress condition applied during the growth. The main FAs detected in the control cells were C15ante, C15iso, C17ante, C16:0 and C18:0. The addition of ethanol did not modify the FAs profile of cells. On the contrary, the addition of carvacrol modified the FAs profiles in relation to the concentrations used. In particular independently to the concentration employed, carvacrol induced the decrease of C17iso and

C17ante, associated to the increase of C14:0, C16:0, C16iso, and particularly of C18:1z9. The marked increase of C18:1z9 accounted for the increase of the unsaturation level (UL).

The growth in the presence of citral (50, 85 and 125 ppm) did not affect significantly the percentage of C15iso, C15ante and C18:0, whereas a small increase of C16:0 and C16iso and a decrease of C17iso and C17 ante, compared to the control were observed. The UL increased with the increase of the concentration of citral in the growth medium. This result was due to the increment of the relative percentage of C14:1, C16:1, C18:2 and C18:1. The presence of oregano EO (30, 40 and 50 ppm) strongly affected FAs composition compared to the control. In fact, the unsaturated fatty acids (UFAs) % was at least two times higher than the control and all the detected UFA showed higher relative percentage with respect to the control cells. Moreover an increase of C16:0 and C18:0 was detected associated to and augmentation of the CL. Thymol (40, 70 and 100 ppm) had a similar effect of oregano on the membrane FAs composition of the *L. monocytogenes* cells. The UL and the relative percentage of UFAs were similar to those of the control, while a marked decrease of C18:0 and an increase of C15ante were evident. An increase of the UL was detected for samples treated with 40 and 100 ppm of thyme EO, while in all the samples added with thyme, an increase of the CL was detected. This trend was mainly due to the increase of relative percentage of C18:0, in particular for the samples treated with 40 ppm of thyme, and of C17ante for the samples treated with 70 and 100 ppm of thyme EO.

In Table 7.4 the relative percentages of free fatty acids (FFAs) in relation to the chemical stress conditions applied to *L. monocytogenes* are shown. The addition of ethanol did not affect UL compared to the control. Instead, an increase of C15iso, C15ante, C16iso and C17ante, associated to a decrease of C16:0 and C18:0 was observed. The growth in the presence of different concentrations of carvacrol increased the relative percentages of medium chain FAs (C10:0 and C12:0) and of C16iso. These changes were associated to the reduction of the CL. In case of the samples treated with the higher concentrations of carvacrol (35 and 50 ppm), also an increase of C18:2 and C18:1 and, consequently, of the UL, respect to the control, were noticed. Citral, independently on the concentration employed, caused an increase of the relative percentages of medium chain FAs. In samples treated with citral, respect to the control, an increase of C18:0, C18:1 and, in case of the treatment with 50 ppm, also of C18:2 were detected. Opposite trends were evident for C15ante, C17iso, C17ante and also for the CL. Oregano EO was the substance that mainly affected the FFAs composition. In fact, a marked increase of the relative percentages

of medium chain FAs, UFAs (C14:1, C16:1, C18:1 and C18:2) and C15iso, associated to a decrease of the saturated FAs C16:0, C18:0, C15ante and C17ante, was detected.

As consequence, in all the samples treated with oregano EO, the UL was at least three times higher than that of the control, while the CL was significantly reduced. The samples treated with thymol showed different FAs composition in relation to the concentration added. An increase of the relative percentages of C10:0, C12:0 and C14:0 was observed with the increase of the concentration of thymol. The relative percentages of these FAs were higher, with few exceptions, with respect to the control. An opposite trend was observed for C15iso, C15ante, C16iso and C17ante. These FAs showed higher percentages, with the exception of C17ante, with respect to the control cells. In addition a decrease of their relative percentages was observed increasing the thymol concentration. The samples treated with 40 ppm of thymol showed a decrease of the UFAs and consequently of the UL. The other samples supplemented with thymol, showed a slight increase of the UL and of UFAs percentages. Moreover, in all the cells treated with thymol a decrease of C16:0 and C18:0, and, consequently, of CL was observed. The cell membrane FAs profiles showed an increase of C10:0, C12:0, C14:1, C15iso, C16iso, C16:1, C17iso, C17ante, C18:2 and C18:1, accompanied by a marked decrease of C16:0 and C18:0 respect to the control, independently on thyme concentration.

Table 7.3- *Membrane fatty acid composition of* Listeria monocytogenes *Scott A in relation to the stress condition applied*

	Total Fatty acids (%)														UL[a]	CL[b]
	C 10:0	C 12:0	C 14:1 Z11	C 14:0	C 15 iso	C 15 anteiso	C16iso	C 16:1 Z9	C 16:0	C 17 iso	C 17 anteiso	C 18:2 (ZZ)	C 18:1 Z9	C 18:0		
Control	0.1	1.2	0.0	2.5	16.2	28.6	4.3	0.4	10.1	7.3	16.6	0.6	3.9	5.8	0.06	1587.0
Ethanol 1%	0.1	0.7	0.2	3.2	17.7	28.6	9.1	0.5	9.6	5.6	14.7	0.5	3.0	2.2	0.05	1568.6
Carvacrol 20ppm	0.0	1.3	0.5	4.5	16.0	22.2	5.4	0.5	16.7	4.8	9.1	0.5	9.7	4.3	0.12	1584.8
Carvacrol 35ppm	0.1	1.3	0.4	3.9	13.3	19.7	3.3	1.8	19.8	3.3	6.6	0.6	13.0	8.4	0.18	1603.2
Carvacrol 50ppm	0.0	0.3	0.7	2.8	14.2	20.3	4.5	1.1	18.4	6.2	8.2	2.4	11.3	5.8	0.18	1605.0
citral 50ppm	0.0	1.6	0.4	2.6	17.1	25.5	7.0	0.5	11.1	6.0	15.4	0.7	4.3	4.2	0.07	1578.6
citral 85ppm	0.1	0.7	0.3	2.5	14.8	24.1	7.2	0.9	15.3	5.4	11.6	1.1	6.0	5.7	0.10	1591.1
citral 125ppm	0.1	1.0	0.4	3.6	16.4	23.7	4.8	0.6	15.0	4.8	10.2	3.7	6.5	5.4	0.16	1589.5
oregano 30ppm	0.1	0.5	0.6	3.2	8.3	11.4	3.0	4.1	23.8	3.5	5.9	1.7	13.8	14.0	0.24	1638.1
oregano 40ppm	1.0	1.1	2.0	2.9	6.6	9.5	2.5	2.5	22.4	2.4	7.9	1.8	16.3	17.9	0.25	1645.1
oregano 50ppm	0.0	1.1	0.2	2.9	9.0	13.7	6.4	2.4	18.9	5.2	10.0	0.5	10.7	15.8	0.15	1632.7
thymol 40ppm	0.1	1.3	0.1	3.4	17.4	33.6	8.1	0.1	7.8	5.8	15.4	0.2	2.8	1.2	0.04	1561.3
thymol 70ppm	0.0	0.4	0.1	2.5	16.8	29.7	8.8	0.3	9.2	6.3	15.4	0.6	3.2	2.5	0.06	1577.4
thymol 100ppm	0.1	0.4	0.2	2.6	15.1	30.1	4.3	0.2	9.7	8.8	17.6	0.8	3.9	2.1	0.06	1581.3
Thyme 40ppm	0.6	0.2	0.7	2.6	5.3	18.9	5.0	0.6	16.2	4.7	16.4	0.7	6.1	17.8	0.11	1639.7
Thyme 70ppm	0.0	0.2	0.1	0.6	10.9	26.7	6.5	0.1	10.3	7.9	21.9	0.1	4.1	8.3	0.05	1611.2
Thyme 100ppm	0.5	0.3	0.7	0.6	7.8	23.1	6.1	0.2	11.1	7.1	26.0	0.5	4.8	7.9	0.08	1623.5

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.

[a] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100

[b]Mean chain length calculated as (FAP*C) (where FAP is the percentage of fatty acid and C the number of carbon atoms).

Table 7.4- *Free fatty acid of* Listeria monocytogenes *Scott A in relation to the stress condition applied*

	Total Fatty acids (%)														UL[a]	CL[b]
	C 10:0	C 12:0	C 14:1 Z11	C 14:0	C 15 iso	C 15 anteiso	C16iso	C 16:1 Z9	C 16:0	C 17 iso	C 17 anteiso	C 18:2 (ZZ)	C 18:1 Z9	C 18:0		
Control	0.3	3.9	0.4	6.0	7.6	11.8	0.6	0.7	28.4	3.8	9.2	0.9	1.5	21.8	0.05	1612.3
Ethanol 1%	0.4	4.8	0.2	5.8	10.9	20.8	5.0	0.2	17.8	4.2	11.1	0.6	1.7	11.9	0.04	1570.8
Carvacrol 20ppm	8.4	7.2	0.2	14.8	6.6	7.1	2.7	0.2	18.8	4.6	3.2	1.0	1.3	17.1	0.05	1515.5
Carvacrol 35ppm	5.0	20.6	1.1	5.7	1.7	6.0	4.3	1.0	22.3	0.5	1.1	1.6	3.4	21.6	0.10	1512.8
Carvacrol 50ppm	7.1	7.7	0.6	7.3	4.4	4.7	2.9	0.5	25.6	2.4	4.3	2.1	3.3	20.6	0.11	1558.3
citral 50ppm	4.0	4.5	0.6	5.4	7.2	6.2	3.5	0.4	25.3	2.9	5.2	2.1	3.4	23.9	0.10	1590.4
citral 85ppm	1.1	9.8	0.4	4.1	6.7	5.1	3.5	0.3	27.7	2.6	5.5	0.6	2.8	25.7	0.05	1599.2
citral 125ppm	3.9	7.2	0.4	5.6	2.5	3.7	0.9	0.3	33.4	0.9	1.8	0.8	1.7	32.0	0.05	1600.0
oregano 30ppm	7.7	6.1	6.3	6.6	10.4	6.8	0.9	3.6	18.0	3.3	1.9	2.5	5.9	12.9	0.23	1522.5
oregano 40ppm	6.6	15.2	4.8	6.3	13.0	7.7	1.9	3.0	12.9	6.4	5.3	1.8	3.7	8.3	0.15	1489.6
oregano 50ppm	9.0	7.7	5.8	3.8	9.3	4.4	0.3	1.3	25.5	2.6	1.6	1.5	4.8	18.8	0.16	1521.6
thymol 40ppm	1.1	6.3	0.4	5.9	15.4	25.2	6.2	0.1	13.2	4.6	9.2	0.1	0.4	7.4	0.01	1535.8
thymol 70ppm	1.8	12.0	0.9	9.8	11.7	16.6	4.2	0.3	14.0	5.4	8.1	1.1	1.7	5.7	0.07	1517.9
thymol 100ppm	2.0	17.9	2.5	16.4	7.3	10.2	1.5	0.3	17.3	4.0	5.2	1.0	0.8	8.9	0.06	1482.6
Thyme 40ppm	1.5	5.5	1.0	8.3	10.4	7.7	4.3	1.5	17.3	8.3	12.0	1.5	2.0	14.1	0.08	1569.4
Thyme 70ppm	1.4	8.6	2.0	4.8	14.8	13.6	5.4	0.9	13.9	8.8	14.3	1.4	1.3	6.3	0.08	1567.0
Thyme 100ppm	0.5	6.0	2.1	6.2	9.1	8.4	5.7	1.7	16.0	8.0	19.0	1.2	3.1	8.4	0.12	1593.4

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.
[a] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100
[b]Mean chain length calculated as (FAP*C) (where FAP is the percentage of fatty acid and C the number of carbon atoms).

The fatty acids composition of *E. coli* 555 is shown in Table 7.5. The main FAs detected in the control cells were C12:0, C14:0, C12cyc, C16:0, C17cyc, C18:2, C18:1z9, C18:1e9, C18:0 and C19cyc. In general, the growth in the presence of the tested antimicrobials (ethanol, 2-(*E*)-hexenal, carvacrol, thymol, thyme EO and oregano EO) caused an increase of the UL, with exception of the samples added with 40 ppm of thymol. In particular, the samples treated with carvacrol, thyme EO and oregano EO showed an UL at least two time higher than control samples. Moreover, in all the treated samples, there was a marked decrease of the FAs C12:0 and C14:0, compared to the control. The decrease of medium chain FAs resulted in the increase of the CL in the treated samples. The samples supplemented with oregano showed also a marked increase of C12cyc and the UFAs (C16:1, C18:1z9, C18:1e9 and C18:2) associated to the decrease of the other branched FAs (C17cyc and C19cyc). In the samples added with thyme EO, an increase of the UFAs, with exception of C18:2 for samples added with 70 ppm of thyme, was detected. The same trend was evident for C16:0 and C18:0, except C18:0 for the samples added with 170 ppm of thyme. In addition a decrease of all the branched FAs (C12cyc, C17cyc and C19cyc) was observed. The samples added with carvacrol showed a marked increase of UFAs and C18:0 relative percentages as well as a slight diminution of C16:0, C12cyc and C19cyc. On

the contrary, the relative percentages of C17cyc detected in the cell membranes of cell treated with carvacrol were similar to those of the control. The addition of different concentrations of thymol provoked a different FAs profiles in relation to the amount added. In fact, at the lowest concentration of thymol (40 ppm) the UL was quite similar to the controls; this was due to the diminution of the relative percentage of C16:1, C18:2 and C18:1z9 associated to an increase of C18:1e9. At the highest concentrations of thymol (70 and 100 ppm) a raise of the UL respect to the control was observed. Even if a slight diminution of C18:2 was detected, an increase of the relative percentage of C16:1 and C18:1e9 were found. All the samples treated with thymol, independently of the concentration added, showed the increase of C16:0 and C18:0 respect to the controls. In addition, a general decrease of C19cyc, respect to the control, was observed, while the relative percentage of C12cyc and C17cyc were quite similar to the control. The only exception was C12cyc in samples treated with 40 ppm of thymol which showed a marked decrease.

Table 7.5- *Membrane fatty acid composition of* Escherichia coli *555 in relation to the stress condition applied*

	Total Fatty acids (%)												UL[a]	CL[b]
	C 12:0	C 14:0	C12 cyc	C 16:1 Z9	C 16:0	C17 cyc	C 18:2 ZZ	C 18:1 Z9	C 18:1 E9	C 18:0	C19 cyc			
Control	8.01	8.01	7.44	0.51	37.23	12.16	1.02	4.34	1.92	5.91	9.99		0.10	1576.71
Ethanol 1%	0.22	5.22	5.77	0.98	45.86	17.01	1.26	4.99	4.22	2.84	10.30		0.13	1639.33
oregano 50ppm	0.51	4.33	11.72	2.16	40.84	7.56	1.58	10.71	6.15	5.64	3.80		0.24	1606.23
oregano 80ppm	0.22	3.90	10.91	5.27	42.10	6.45	1.48	11.80	8.61	5.33	2.09		0.30	1614.65
oregano 120ppm	1.23	4.07	16.45	1.97	31.96	1.84	2.82	19.27	6.09	11.45	1.64		0.33	1605.66
2-(E)-hexenal 100ppm	0.20	3.33	5.02	1.69	52.91	11.50	1.17	5.87	7.04	4.47	4.88		0.18	1634.91
2-(E)-hexenal 170ppm	0.20	2.49	6.76	0.97	53.89	7.43	1.36	6.05	3.40	11.20	4.17		0.14	1629.95
2-(E)-hexenal 250ppm	0.40	3.30	6.49	3.42	46.65	13.81	0.64	3.50	9.37	4.58	5.87		0.19	1632.88
thymol 40ppm	0.12	4.40	1.23	0.36	50.64	13.41	0.34	2.99	4.71	9.68	8.34		0.09	1611.29
thymol 70ppm	0.92	6.60	7.66	1.71	46.60	10.88	0.65	2.68	4.41	9.68	7.50		0.11	1636.47
thymol 100ppm	1.50	3.08	8.07	0.69	39.23	15.87	0.91	6.60	5.16	12.15	5.90		0.16	1646.81
Thyme 70ppm	0.09	3.28	6.19	2.76	44.72	10.22	1.01	9.73	7.35	10.57	2.57		0.22	1626.83
Thyme 120ppm	3.69	4.82	4.86	0.56	42.00	5.00	1.58	10.67	8.71	15.77	0.00		0.24	1644.57
Thyme 170ppm	0.39	3.65	4.52	7.36	48.49	6.17	1.29	5.52	13.89	4.84	2.23		0.30	1635.81
Carvacrol 40ppm	1.23	1.69	3.46	0.72	34.12	11.50	2.63	17.51	4.48	12.93	5.84		0.29	1666.22
Carvacrol 70ppm	0.86	3.15	1.76	2.30	35.62	12.84	1.50	15.60	6.89	10.68	6.45		0.30	1687.06
Carvacrol 100ppm	0.33	2.75	6.69	5.01	31.41	10.64	2.81	11.16	13.58	9.39	4.48		0.36	1663.68

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.

[a] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100

[b]Mean chain length calculated as (FAP*C) (where FAP is the percentage of fatty acid and C the number of carbon atoms).

The addition of 2-(E)-hexenal caused a marked and general increase of C16:1 and C18:1e9. A slight increase of C18:2 and C18:1z9 was observed only in the samples supplemented with the lowest concentrations of 2-(E)-hexenal (100 and 170 ppm); at the highest concentration of 2-(E)-

hexenal (250 ppm) the trend was opposite. The relative percentage of C16:0 showed an evident rise compared to the controls in all the samples added with 2-(E)-hexenal. The same increase was detected for C18:0 in the samples added with 170 ppm of 2-(E)-hexenal, while no significantly differences respect to the control were found for the other concentrations used. A slight decrease or not significantly differences of the relative percentages of branched FAs, respect to the control, were detected in all the samples treated with 2-(E)-hexenal.

In Table 7.6 the relative percentages of free fatty acids (FFAs) in relation to the chemical stress conditions applied to *E. coli* are shown. In general, the growth in presence of the tested molecules (ethanol, 2-(*E*)-hexenal, carvacrol, thymol, thyme EO and oregano EO) caused an increase of the UL, with exception of the samples treated with 50 ppm of oregano, and the lowest concentrations of 2-(E)-hexenal (100 and 170 ppm) employed. Only the samples supplemented with ethanol, thymol or 2-(E)-hexenal showed an increase of the CL respect to the controls. The samples added with oregano at the lowest concentrations used (50 and 80 ppm) had a lower CL, while the others did not show significantly differences. The relative percentages of medium length saturated FAs such as C12:0 and C14:0 in the treated samples did not show differences compared to the controls; the only exceptions were the samples added with oregano, 2-(E)-hexenal and thyme. In case of oregano a slight increase of C14:0 was detected, while an opposite trend was found when 2-(E)-hexenal and thyme were added, independently on the concentration used. Regarding to C16:0, it represented the most present FA in FFAs profiles. In general, a raise in C16:0 relative percentages respect to the control, with the exception of the samples supplemented with thymol, and carvacrol at the lowest concentration, was detected. On the contrary, the relative percentages of C18:0 of the treated samples showed a decrease, in some cases remarkable. The only exception was represented by the samples added with thymol. In this sample the relative percentages of C18:0 were similar or higher than those of control cells. In all the treated samples, the UFAs C16:1 and C18:1e9 showed an increase; this was particularly evident for C16:1 in the sample supplemented with the highest concentrations of thyme and oregano. Regarding to C18:2 and C18:1z9, the relative percentages of the treated samples were lower or quite similar to those of the controls. About the branched FAs detected, the stresses applied caused an increase of C17cyc with the exception of the samples supplemented with ethanol or carvacrol, that showed relative percentages similar to the control cells. Contrarily, C19cyc in the treated samples decreased or was similar respect to the control. Only the samples added with thymol or 2-(E)-hexenal at 170 ppm showed a slight increase of this FA.

Table 7.7 reports the FAs composition of late exponential phase cells of *S. enteritidis* in relation to the stress condition applied during the growth. The growth in the presence of sublethal concentrations of the tested molecules caused a modulation of the FAs composition. In particular, in the treated samples, a remarkable increase of UL respect to the controls was detected. The same trend, respect to the control, was noted for the CL, with exception of samples added with oregano; in these samples a decrease of the CL was found. Regarding to the UFAs, C16:1z9 and C18:1e9 showed a remarkable increase of the relative percentages in the presence of the added molecules. Contrarily, C18:1z9 in treated samples decreased or was similar to the control, with the exception of samples added with thyme at 50 ppm. Not significant differences were observed for medium length saturated FAs (C12:0 and C14:0). In fact, only the samples added with 70 ppm of carvacrol, 100 ppm of thymol and oregano showed a marked increase of both C12:0 and C14:0. C16:0 represented the most present FA, and independently on the added antimicrobial, the relative percentages of the treated samples were similar or lower than the control.

Table 7.6- *Free fatty acid of* Escherichia coli *555 in relation to the stress condition applied*

	Total Fatty acids (%)											UL[a]	CL[b]
	C 12:0	C 14:0	C12 cyc	C 16:1 Z9	C 16:0	C17 cyc	C 18:2 ZZ	C 18:1 Z9	C 18:1 E9	C 18:0	C19 cyc		
Control	2.94	6.11	0.24	0.97	43.09	4.14	1.49	3.11	10.50	15.39	4.52	0.20	1635.60
Ethanol 1%	1.57	6.76	0.12	1.86	45.78	5.14	0.48	1.20	15.83	11.97	4.70	0.23	1651.05
oregano 50ppm	5.77	8.25	0.00	3.00	49.03	7.00	0.35	1.85	12.90	6.09	2.00	0.20	1612.37
oregano 80ppm	3.22	9.20	0.16	5.25	43.82	9.23	0.91	1.02	15.96	3.76	3.07	0.27	1622.11
oregano 120ppm	2.27	7.48	0.04	11.82	47.45	7.96	0.54	1.08	13.61	5.16	1.83	0.29	1654.39
2-(E)-hexenal 100ppm	1.10	4.21	0.32	2.30	53.99	6.67	0.29	0.59	12.10	11.07	3.35	0.18	1645.87
2-(E)-hexenal 170ppm	1.60	4.13	0.00	1.41	49.16	12.37	0.95	1.93	11.76	6.95	6.19	0.19	1653.33
2-(E)-hexenal 250ppm	1.03	3.59	0.20	4.11	48.22	15.81	0.18	0.52	14.43	3.86	3.95	0.22	1650.34
thymol 40ppm	2.98	6.28	0.00	1.28	37.28	10.24	1.75	3.18	11.82	15.34	5.67	0.22	1658.18
thymol 70ppm	1.97	7.16	0.22	3.34	39.26	8.92	0.68	2.95	11.79	14.13	5.44	0.22	1655.80
thymol 100ppm	2.26	4.63	0.00	1.38	36.01	8.64	0.94	4.82	10.95	18.75	5.36	0.25	1692.81
Thyme 70ppm	1.68	3.81	0.00	4.00	53.40	7.70	0.38	1.98	10.40	11.33	0.00	0.20	1627.20
Thyme 120ppm	3.35	3.70	0.06	6.76	45.91	10.61	1.66	2.22	12.15	7.14	2.49	0.27	1634.35
Thyme 170ppm	2.73	3.61	0.06	10.54	50.72	9.97	0.31	0.57	14.53	3.16	1.52	0.28	1629.40
Carvacrol 40ppm	3.38	6.26	0.00	2.33	42.30	5.04	2.27	2.15	13.23	12.43	3.95	0.27	1638.46
Carvacrol 70ppm	1.88	6.54	0.00	4.47	53.07	4.08	1.29	1.66	11.07	10.70	1.59	0.22	1630.60
Carvacrol 100ppm	1.99	6.04	0.07	5.95	52.02	4.27	0.35	1.39	14.10	8.15	0.78	0.25	1630.05

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.

[a] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100

[b]Mean chain length calculated as (FAP*C) (where FAP is the percentage of fatty acid and C the number of carbon atoms).

The same trend was detected for C18:0, with the exceptions of the samples supplemented with 60 ppm of thymol and 70 ppm of carvacrol. A different trend on the basis of the added molecules

was observed for the branched FAs. The addition of ethanol and thyme, independently on the concentration employed, caused a remarkable decrease of C12cyc associated to an increase of C17cyc and C19cyc. A decrease of the branched FAs was observed with the addition of carvacrol, independently on the concentration. The supplementation of thymol at different concentrations provoked a decrease of C12cyc and C17cyc, while an increase of C19cyc was detected in the samples supplemented with 40 and 70 ppm of thymol. In contrast to the other treatments, the addition of oregano, independently on the concentration, caused a marked increase of C12cyc associated to decrease of C17cyc and C19cyc.

Table 7.7- *Membrane fatty acid composition of* Salmonella enteritidis *E5 in relation to the stress condition applied*

	Total Fatty acids (%)										UL[a]	CL[b]
	C 12:0	C 14:0	C12 cyc	C 16:1 Z9	C 16:0	C17 cyc	C 18:1 Z9	C 18:1 E9	C 18:0	C19 cyc		
Control	0.6	4.3	5.9	0.8	51.8	13.9	5.3	2.1	6.4	6.8	0.11	1630.23
Ethanol 1%	0.87	5.52	0.04	1.68	47.88	20.67	2.48	5.21	1.75	13.05	0.10	1663.81
Carvacrol 40ppm	0.24	6.40	1.41	2.96	52.19	7.30	2.43	13.87	7.17	4.22	0.21	1648.70
Carvacrol 70ppm	3.46	10.21	0.52	2.44	34.60	5.21	0.00	18.12	8.16	3.70	0.32	1638.74
Carvacrol 100ppm	0.26	2.89	1.21	6.79	49.47	6.06	4.01	18.81	4.95	3.71	0.32	1661.78
Thyme 50ppm	0.90	4.42	0.16	2.72	34.14	20.28	6.92	9.87	3.89	13.57	0.23	1690.79
Thyme 90ppm	0.26	4.25	0.05	3.22	47.87	14.42	1.86	11.97	3.17	8.68	0.19	1665.39
Thyme 135ppm	0.50	3.09	0.36	5.08	43.13	15.88	4.37	12.04	2.56	9.34	0.28	1677.29
thymol 40ppm	0.39	5.64	0.17	2.56	51.30	11.77	3.04	11.47	2.95	8.07	0.19	1641.17
thymol 70ppm	0.71	4.07	0.48	2.88	46.26	10.84	3.91	14.66	4.87	9.36	0.23	1672.89
thymol 100ppm	1.25	5.56	0.32	3.62	49.53	7.25	3.57	16.00	5.20	4.96	0.26	1655.96
oregano 60ppm	0.00	3.61	14.52	1.20	48.83	6.21	4.50	4.38	11.67	4.02	0.12	1595.15
oregano 100ppm	0.70	6.99	18.85	2.02	44.13	8.17	3.63	5.07	5.20	2.37	0.14	1550.35
oregano 150ppm	0.18	2.29	13.89	6.73	43.78	7.97	1.51	10.60	7.41	2.14	0.20	1560.45

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.

[a] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100

[b]Mean chain length calculated as (FAP*C) (where FAP is the percentage of fatty acid and C the number of carbon atoms).

In Table 7.8 the relative percentages of FFAs in relation to the chemical stress conditions applied to *S.enteritidis* are shown. Also in this case the increase of UL of treated samples respect to the control, with the exception of samples added with ethanol and the lower concentrations of carvacrol (40 and 70 ppm), was evident. This change was linked to the increase, in the treated samples, of the relative percentages of the two main UFAs, C16:1z9 and C18:1e9. The only exceptions were the samples added with carvacrol at 40 and 70 ppm, that showed a similar percentages of C18:1e9 with respect to the control. The addition of carvacrol, independently on the concentration, provoked a decrease of C14:0, while the other treatments did not change

significantly the relative percentage of C14:0. Also the percentages of C12:0 in treated samples were not different to the ones of the control with the exception of the samples added with the lower concentrations of oregano (60 and 100 ppm), that showed a slight increase of this FA. With the addition of ethanol no changes in C16:0 percentages were detected, while a slight decrease of this FA was observed when oregano, thymol and thyme were added. On the contrary, the supplementation with carvacrol caused a remarkable increase of C16:0 relative percentages, compared to the control. The addition of carvacrol and oregano provoked a marked increase of the relative percentage of C18:0. Moreover the addition of carvacrol and oregano caused a remarkable decrease of the branched FAs (C17cyc and C19cyc) with respect to the control. Regarding the growth in the presence of thyme the relative percentages of branched FAs were quite similar or slightly lower compared to the control. When thymol was added at the lowest concentration (40ppm) an increase of branched FAs was detected, but increasing the thymol concentration a reduction of the relative percentages of these FAs was observed.

Table 7.8- *Free fatty acid of* Salmonella enteritidis *E5 in relation to the stress condition applied*

	Total Fatty acids (%)										UL[a]	CL[b]
	C 12:0	C 14:0	C12 cyc	C 16:1 Z9	C 16:0	C17 cyc	C 18:1 Z9	C 18:1 E9	C 18:0	C19 cyc		
Control	0.9	6.2	0.2	1.2	47.7	20.7	1.6	5.2	4.1	8.4	0.11	1644.48
Ethanol 1%	1.38	6.24	0.08	1.49	46.13	22.91	1.40	3.99	5.41	8.23	0.09	1647.93
Carvacrol 40ppm	0.30	2.88	0.00	2.29	68.89	6.64	0.46	5.75	8.81	2.25	0.09	1620.13
Carvacrol 70ppm	1.21	1.32	0.00	2.18	65.31	4.17	0.35	5.93	17.68	0.37	0.09	1644.19
Carvacrol 100ppm	1.28	1.94	0.05	3.01	65.24	5.52	1.94	6.59	8.55	1.65	0.14	1635.18
Thyme 50ppm	0.74	5.26	0.00	3.12	43.96	20.17	1.93	9.90	3.49	9.25	0.17	1662.70
Thyme 90ppm	1.40	4.73	0.33	2.24	43.29	15.70	3.79	10.56	3.38	5.39	0.21	1647.95
Thyme 135ppm	1.36	5.22	0.00	3.27	42.54	17.91	6.92	10.27	2.34	5.75	0.25	1642.84
thymol 40ppm	0.65	5.41	0.00	2.35	40.37	25.16	2.19	8.67	2.42	10.45	0.15	1668.26
thymol 70ppm	0.74	5.47	0.00	2.25	42.48	19.22	2.48	10.47	3.07	11.74	0.17	1670.45
thymol 100ppm	1.31	6.51	0.14	1.34	45.71	13.06	1.17	9.60	4.20	8.51	0.15	1604.08
oregano 60ppm	2.11	6.42	0.09	3.15	42.77	7.95	0.99	8.88	19.36	2.91	0.16	1633.04
oregano 100ppm	3.30	8.60	0.12	4.50	46.74	7.01	0.73	11.40	11.17	2.08	0.20	1622.99
oregano 150ppm	0.62	5.89	0.02	6.89	44.08	12.74	1.19	12.84	11.94	1.59	0.23	1653.59

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.

[a] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100

[b]Mean chain length calculated as (FAP*C) (where FAP is the percentage of fatty acid and C the number of carbon atoms).

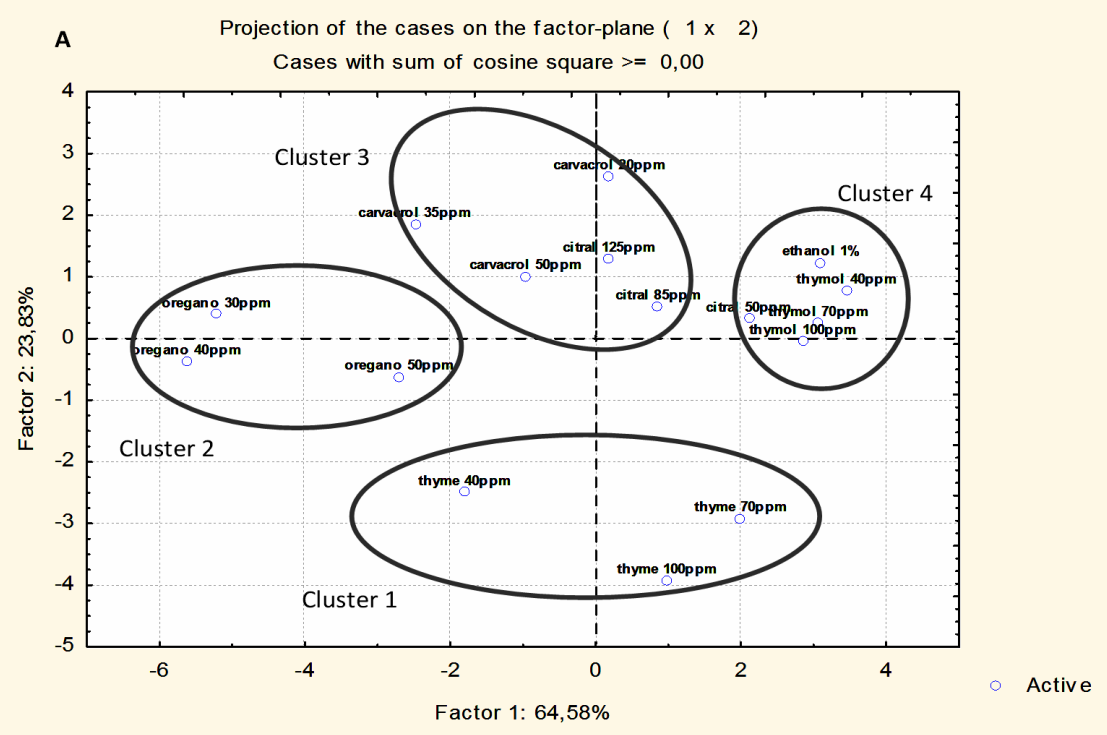
3.3 PCA analyses of cell fatty acid changes

To better show the relationships between membrane FAs composition and the EOs treatments, a principal component analysis (PCA) was carried out with the FA percentages detected in the

controls and treated strains. In fact, the PCA is a very powerful technique, able to emphasize sample clusters in a two-dimensional space (Cruz et al. 2013; Tabanelli et al. 2013).

In Figure 7.1a are reported the PCA loading plot of FAs composition of late exponential phase cells of *L. monocytogenes* in relation to the stress condition applied during the growth. All the samples were mapped in the space spanned by the first two principal components PC1 versus PC2. PC1 accounted for 64.58% of the variability, and PC2 for 23.83%. The samples were grouped mainly on the basis of the added molecules independently on the concentrations added. Four clusters were evident: cluster 1 included all the samples added with thyme EO; in cluster 2 were present the samples treated with oregano EO independently on its concentration; cluster 3 grouped all the samples added with carvacrol and the sample added with 85 and 125 ppm of citral. Cluster 4 grouped the samples added with 1% ethanol, 50 ppm of citral and all the samples supplemented with thymol.

Figure 7.1b reports the variable factor coordinates for the first two factors. Factor 1 was highly positively related with the FAs C15iso and C15ante, and highly negatively related with C18:1 z9 and C16:1e9. Regarding factor 2, the main negative effects were determined by C17ante, C17iso and C18:0, while the main positive effects were related with C14:0, C14iso and C15iso.



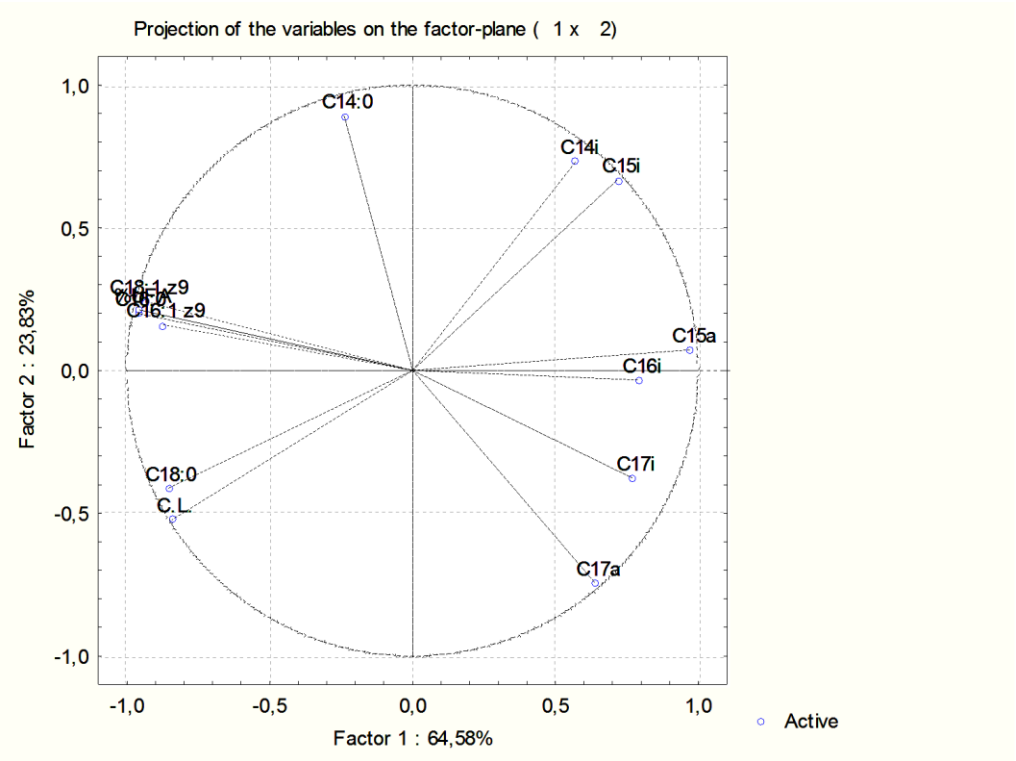
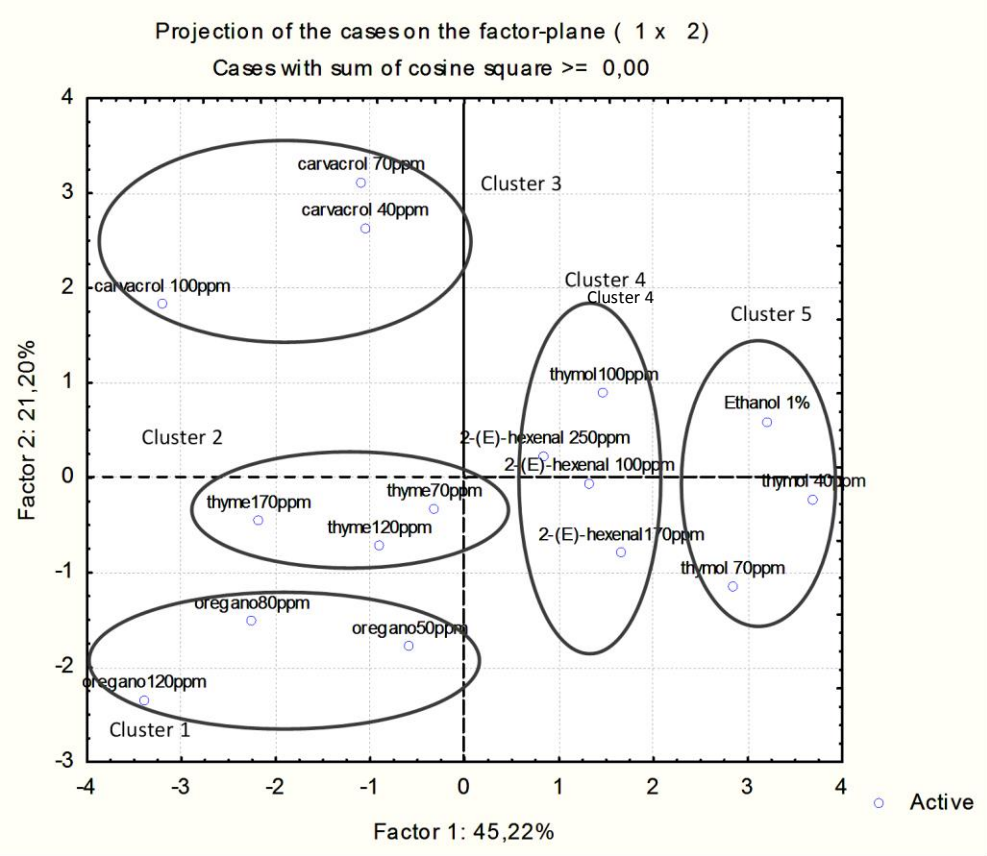


Figure 7.1a,b *Principal component analysis (PCA) loading plots of the two-first factors relative to the fatty acid membrane composition of* Listeria monocytogenes *Scott A in relation to the stress condition added (a) and variable factor coordinates for the two-first factors (b);*

In Figure 7.2a are reported the PCA loading plot of FAs composition of late exponential phase cells of *E. coli* in relation to the stress condition applied during the growth. In this case, PC1 accounted for 45.22% of the variability, and PC2 for 21.20%. Five clusters were evident: cluster 1 included all the samples treated with oregano EO; in cluster 2 the samples added with thyme EO, independently on the concentration, were present; cluster 3 grouped the samples added with carvacrol. In cluster 4 all the samples added with 2-(E)-hexenal and the sample supplemented with the highest concentration of thymol (100ppm) were present. Finally cluster 5 was characterized by the presence of samples added with the lower concentrations of thymol (40 and 70 ppm) and the sample supplemented with 1% ethanol. However, the clusters 4 and 5 showed marked scatterings among the samples along the PC2 that explained the 21.2% of the variance.

Figure 7.2b reports the variable factor coordinates for the first two factors. Factor 1 was highly positively related with C16:0, C14:0, C17cyc and C19cyc, and negatively related with the UFAs C16:1z9, C18:1z9 and C18:1e9. Regarding factor 2, the main negative effects were determined

by C14:0, C16:0 and C12cyc, while the main positive effects were related with C17cyc, C19cyc and the chain length (CL).



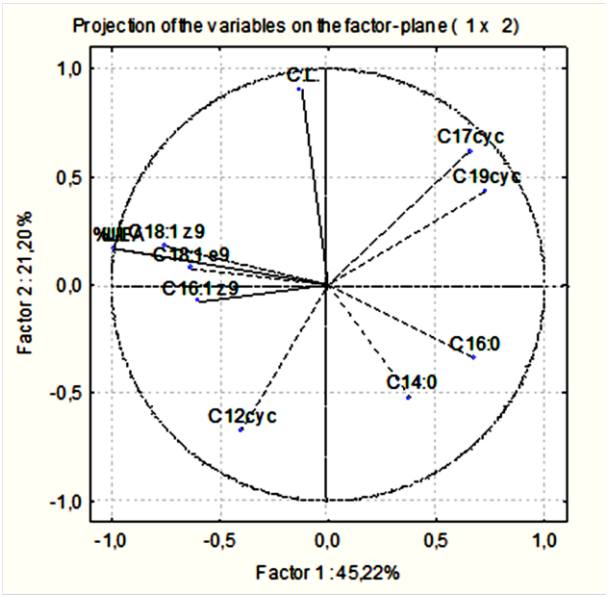
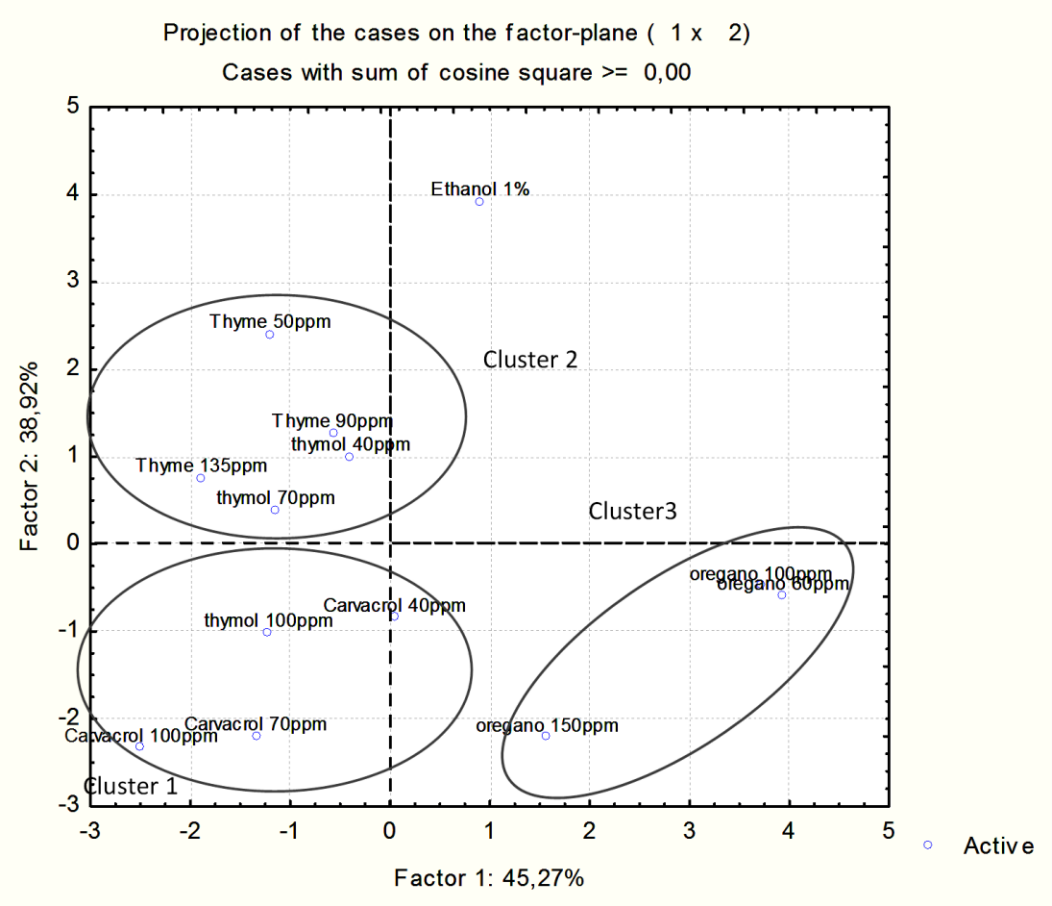


Figure 7.2a,b- *Principal component analysis (PCA) loading plots of the two-first factors relative to the fatty acid membrane composition of* Escherichia coli *555 in relation to the stress condition added (a) and variable factor coordinates for the two-first factors (b);*

The PCA loading plot of FAs composition of late exponential phase cells of *S. enteritidis* in relation to the stress condition applied during the growth are reported in Figure 7.3a. PC1 accounted for 45.27% of the variability, and PC2 for 38.92%. The samples were grouped mainly on the basis of the supplemented compounds independently on the concentrations added. Three clusters were evident: cluster 1 included all the samples added with carvacrol and the sample treated with the highest concentration of thymol (100ppm); in cluster 2 the samples treated with thyme EO independently on the concentration and with the lower concentrations of thymol (40 and 70ppm) were included; while cluster 3 was composed by the samples added with oregano EO. The sample added with 1% of ethanol did not grouped with any other sample.

Figure 7.3b reports the variable factor coordinates for the first two factors. Factor 1 was positively related with C12cyc and C18:0, and negatively related with the UFAs C16:1z9, C18:1e9. Regarding factor 2, the main negative effects were determined by C18:0, C12cyc, C16:1z9 and C18:1e9, while the main positive effects were related with C17cyc, C19cyc and the CL.



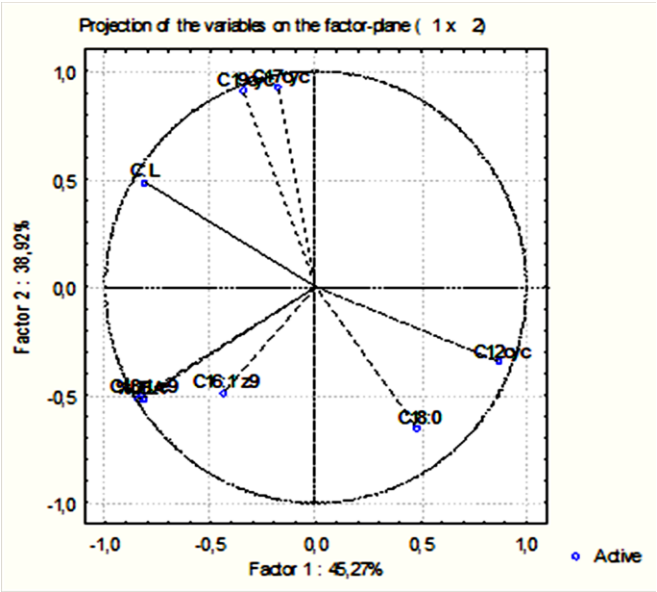


Figure 7.3a,b- *Principal component analysis (PCA) loading plots of the two-first factors relative to the fatty acid membrane composition of* Salmonella enteritidis *E5 in relation to the stress condition added (a) and variable factor coordinates for the two-first factors (b);*

3.4 Volatile molecule profile changes induced by sublethal concentrations of 2-(E)-hexenal, citral, carvacrol, thymol, thyme EO and oregano EO

The supplementation of the growth media with the considered natural antimicrobials provoked marked modifications of the GC–MS-SPME profiles of the tested strains. As expected, the volatile profiles of the strains differed according to the species. With respect to the controls and the cultures added with ethanol, which were characterized by GC profiles with a lower number of metabolites, the exposure to the tested compounds resulted in the release of enhanced levels of several molecules. In particular, aldehydes, hydrocarbons, pyrazines and alcohols were the principal families of metabolites. As shown by Tables 7.9, 7.10 and 7.11, the supplementation of the growth medium with the tested compounds significantly affected the volatile molecule profiles of cells, In fact, the volatile molecule profiles of treated cells showed the presence of the supplemented antimicrobials and their detoxification compounds. The occurrence of the 2-(*E*)-hexenal detoxification products, such as 2-(*E*)-hexen-1-ol, 2-(Z)-hexen-1-ol, 2 hexenoic acid, ethyl ester and butane, 1,1-diethoxy characterized the SPME-GC profile of *E. coli* subjected to the unsaturated aldehyde exposure; while the presence of citral detoxification molecules such as nerol, geraniol, β-citronellol, 5-hepten-2-one, 6-methyl and 1-pentene, 2,3-dimethyl characterized the volatile molecule profile of *L. monocytogenes* subjected to citral exposure.

Table 7.9 *Major volatile compounds (expressed as peak area × 10^5 detected for* Listeria monocytogenes *Scott a in relation to the different stress conditions*

	Control	Ethanol 1%	Citral			Carvacrol			Thymol			Thyme EO			Oregano EO		
			50 ppm	85 ppm	125 ppm	20 ppm	35 ppm	50 ppm	40 ppm	70 ppm	100 ppm	40 ppm	70 ppm	100 ppm	30 ppm	40 ppm	50 ppm
Ethyl alcohol	121.1	641.7	520.7	2569.4	3759.8	5502.7	533.2	502.4	510.2	507.6	533.1	2550.9	517.8	492.2	2937.6	1476.3	3290.2
1-Propanol, 2,2-dimethyl	7.4	21.4	-	-	-	-	17.0	10.2	2.3	2.6	-	-	-	-	-	-	-
2-heptanol, 2-methyl	5.6	16.1	1.1	15.9	34.6	-	4.6	1.4	-	-	-	-	4.0	-	-	14.8	-
γ-terpinene	-	-	-	-	-	-	-	-	-	-	-	770.9	23.9	24.3	240.5	138.1	260.8
p-cymene	-	-	-	-	-	-	-	-	-	-	-	1224.3	111.8	137.0	516.1	1179.0	490.0
caryophillene	-	-	-	-	-	-	-	-	-	-	-	227.8	39.3	81.1	37.4	132.6	84.2
Pyrazine, methyl-	24.7	29.0	13.3	59.7	-	-	5.9	11.9	19.2	14.4	10.3	-	-	-	-	-	9.8
Pyrazine, 2,6-dimethyl-	128.6	173.2	90.3	198.2	119.9	108.1	57.5	65.0	116.9	92.3	24.7	268.3	82.4	73.0	283.9	260.1	256.9
Pyrazine, ethyl-	10.4	15.4	-	-	-	-	18.9	16.3	32.9	16.0	52.7	-	-	-	6.7	27.6	3.8
5-Hepten-2-one, 6-methyl-	-	-	1189.8	2382.6	2080.8	9.5	-	-	-	-	4.1	-	7.6	15.1	21.3	-	12.4
1-Pentene, 2,3-dimethyl-	-	-	101.1	195.7	158.6	-	-	-	-	-	-	-	16.6	21.5	-	-	-
Nonanal	9.8	22.4	23.9	51.6	29.3	35.9	85.3	96.0	10.0	9.2	16.8	-	59.9	15.3	16.9	225.4	27.1
Pyrazine, trimethyl-	12.4	15.8	13.9	23.0	-	11.2	11.4	8.2	12.6	6.7	6.7	-	8.3	8.3	12.4	22.5	11.2
1-Octen-3-ol	-	-	9.4	21.6	58.1	15.5	9.1	-	-	-	-	846.6	137.7	154.0	435.3	457.3	427.2
Pyrazine, 3-ethyl-2,5-dimethyl-	24.8	41.1	100.6	18.2	19.8	7.8	57.8	42.2	43.3	22.5	25.9	34.1	23.4	21.9	18.5	92.5	67.7
Linalool	-	-	-	-	-	-	-	-	-	-	-	5038.6	1163.5	1209.3	52.6	292.5	-
Benzaldehyde	27.4	47.6	10.6	1266.3	772.1	753.9	6.0	6.2	41.7	28.7	17.6	-	-	-	1767.2	238.6	3197.2
1-Terpinen-4-ol	-	-	-	-	-	-	-	-	-	-	-	1088.3	285.2	282.6	956.6	954.1	1102.7
Cyclohexene, 1-methyl-3-(1-methylethenyl)	7.3	31.7	6.6	-	-	-	7.7	23.3	58.3	33.4	-	-	25.7	10.6	-	-	-
Thiophene-2-acetic acid, dodec-9-ynyl ester	47.1	85.8	29.4	-	-	414.3	20.4	68.8	180.2	106.6	68.5	621.1	67.0	28.7	449.5	171.1	227.1
Neral	-	-	167.1	7952.1	6999.3	-	-	-	-	-	-	-	-	-	-	-	-
1,2,4-Triazol-4-amine, N-(2-thienylmethyl)-	13.6	15.9	42.8	-	-	131.7	16.2	22.9	57.4	25.0	26.1	238.1	18.8	13.0	279.0	93.6	13.7
borneol	-	-	-	-	-	-	-	-	-	-	-	586.4	187.5	206.3	167.5	153.7	171.0
Geranial	-	-	253.6	10290.3	9105.6	-	-	-	-	-	-	-	-	-	-	-	-
beta citronellool	-	-	140.9	540.2	508.4	-	-	-	-	-	-	-	-	-	-	-	-
nerol	-	-	135.3	1690.4	1451.8	-	-	-	-	-	-	-	-	-	-	-	-
geraniol	-	-	433.2	2626.8	2321.8	-	-	-	-	-	-	-	-	-	-	-	-
thymol	-	-	-	-	-	115.6	2.9	14.6	2400.1	2597.6	2855.1	5913.2	1915.8	1928.3	2763.1	1724.3	669.6
Carvacrol	-	-	-	-	-	8106.3	2386.5	2720.4	3.4	5.8	14.1	7009.5	2239.7	2145.9	7998.4	4826.7	9073.8
Phenol, 4-(1,1,3,3-tetramethylbutyl)-	-	19.0	-	217.5	83.2	154.4	6.6	19.0	24.9	19.9	27.0	160.8	18.3	10.4	151.2	116.9	97.1

The results are means of three independent experiments. The coefficients of variability, expressed as percentage ratios between the standard deviations and the mean values, ranged between 5 and 10%.
- Undetectable level

Table 7.10- *Major volatile compounds (expressed as peak area × 10^5 detected for* Escherichia coli *555 in relation to the different stress conditions*

	Control	Ethanol 1%	Carvacrol			Thyme EO			Thymol			Oregano EO			2-(E)-hexenal		
			40 ppm	70 ppm	100 ppm	70 ppm	120 ppm	170 ppm	40 ppm	70 ppm	100 ppm	50 ppm	80 ppm	120 ppm	100 ppm	170 ppm	250 ppm
Ethyl alcohol	236.2	11592.4	9190.4	10111.2	3031.5	5041.1	2632.7	469.9	10442.8	4789.5	3122.3	3357.0	3555.3	2544.3	2725.7	2146.4	2658.2
Ethyl acetate	748.7	418.6	97.1	53.6	10.6	26.4	42.1	-	167.2	-	18.2	26.4	19.3	14.5	222.6	313.0	268.5
2-butanone	979.1	568.8	162.5	76.4	12.3	35.8	-	-	270.9	-	26.9	46.6	15.5	24.3	-	-	-
2-Hexenal, (E)-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1998.3	1932.8	2586.0
γ-terpinene	-	-	-	-	-	11.6	184.0	612.5	-	-	-	706.9	433.0	1148.4	-	-	-
p-cymene	-	-	-	-	-	23.7	1083.3	2312.2	-	-	-	1990.5	954.5	2494.0	-	-	-
Pyrazine, 2,5-dimethyl-	1859.0	2452.6	1919.2	967.1	323.7	201.2	73.9	20.2	1674.4	94.0	147.4	494.8	278.1	363.5	134.8	84.1	86.7
2-Hexenoic acid, ethyl ester	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2437.0	3017.8	2463.4
2-Hexen-1-ol, (E)-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3680.0	2811.2	3788.7
2-Hexen-1-ol, (Z)-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	475.5	286.2	377.4
Butane, 1,1-diethoxy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1802.5	10856.2	4840.1
Pyrazine, 3-ethyl-2,5-dimethyl-	630.8	839.3	406.2	274.1	20.7	13.3	-	-	451.8	-	22.2	-	-	14.5	25.8	30.4	-
1-Octanol	480.2	1035.8	164.0	139.5	24.8	28.4	-	-	349.9	41.1	28.2	56.8	41.9	52.3	154.6	87.5	55.9
linalool	-	-	-	-	-	754.0	4776.0	1372.1	-	-	-	1801.1	1334.2	2176.4	-	-	-
1-Terpinen-4-ol	-	-	-	-	-	345.1	1418.7	690.4	-	-	-	1745.0	987.3	1895.8	-	-	-
caryophillene	-	-	-	-	-	-	2129.0	1150.1	-	22.4	-	980.9	1964.4	1241.8	-	-	-
Benzaldehyde	113.6	164.1	282.9	173.1	166.3	192.8	72.8	42.8	158.2	41.5	162.6	147.6	124.7	95.4	100.4	80.1	67.5
Benzyl Alcohol	67.5	129.0	123.7	52.7	25.3	11.0	36.1	36.8	86.4	11.0	16.3	158.7	-	282.4	13.8	35.4	48.8
Cyclohexene, 1-methyl-3-(1-methylethenyl)-,	242.7	1718.2	1434.3	413.8	378.7	-	-	30.4	215.6	-	273.0	852.8	440.6	970.7	47.2	133.7	98.0
Thiophene-2-acetic acid, dodec-9-ynyl ester	484.8	4602.0	3754.6	1180.0	1064.9	116.5	64.7	91.4	626.5	145.8	690.6	475.9	830.5	890.3	121.4	454.5	218.0
1,2,4-Triazol-4-amine, N-(2-thienylmethyl)-	467.6	1215.5	1396.8	236.8	282.4	166.4	110.4	28.3	443.9	104.1	189.0	150.5	245.3	157.2	130.2	383.1	204.1
Cyclooctane	1013.2	1781.6	724.0	303.5	52.5	83.0	29.8	62.6	994.4	117.4	96.4	-	-	-	647.2	605.8	256.7
Cyclododecane	362.3	789.9	494.9	285.4	26.1	43.5	-	40.9	710.8	-	68.6	183.4	67.5	67.7	130.3	135.0	86.1
Octanoic acid	207.9	651.5	456.4	162.1	92.9	24.5	23.8	-	218.6	-	94.1	58.1	41.1	45.9	33.1	68.7	47.5
Thymol	-	-	3230.3	171.2	-	8128.0	7567.1	2231.8	22527.7	9312.0	8728.5	780.9	694.8	1288.4	-	-	-
Carvacrol	-	-	26073.4	24424.1	8549.6	234.3	8904.9	2533.4	-	-	-	5540.3	7543.9	5974.7	-	-	-

The results are means of three independent experiments. The coefficients of variability, expressed as percentage ratios between the standard deviations and the mean values, ranged between 5 and 10%.
- Undetectable level

Table 7.11- *Major volatile compounds (expressed as peak area × 10^5 detected for* Salmonella enteritidis *E5 in relation to the different stress conditions*

	Control	Ethanol 1%	Carvacrol			Oregano			Thyme EO			Thymol		
			40 ppm	70 ppm	100 ppm	60 ppm	100 ppm	150 ppm	50 ppm	90 ppm	135 ppm	40 ppm	70 ppm	100 ppm
Propane, 2-etoxy, 2-methyl	97.4	137.7	6.5	11.7	3.9	25.5	6.8	6.5	-	-	10.7	34.4	29.7	12.3
2,4-Dimethyl-1-heptene	138.4	121.1	30.1	10.3	19.8	9.4	-	-	13.3	4.9	13.7	12.5	9.5	10.1
Ethyl alcohol	330.4	4700.5	2414.1	4148.9	3138.8	2595.5	3532.8	3519.6	4113.1	2629.4	2554.7	2849.9	2759.2	2595.4
Propanoic acid, 2,2 dimethyl, propyl ester	136.0	163.0	32.6	16.2	17.4	21.1	9.5	7.1	21.6	10.4	12.7	27.3	16.0	10.8
Undecane, 6,6-dimethyl-	115.1	214.4	41.4	25.1	97.0	-	-	-	-	-	-	32.6	23.2	15.7
1-pentanol	186.8	169.9	21.5	20.7	17.1	-	-	-	22.6	10.0	-	37.4	16.1	14.2
Eucalyptol	-	-	-	-	-	197.2	196.4	291.9	1700.0	1639.7	1790.0	-	-	-
2-Heptanone, 4-methyl	129.0	141.5	28.9	18.4	24.8	-	-	-	-	-	-	32.1	22.5	13.2
p-cimene	-	-	-	-	0.0	1434.6	1871.1	3230.6	2421.7	2321.8	1856.1	-	-	-
3-Heptene, 2,2,4,6,6-pentamethyl-	111.9	120.9	105.1	120.4	232.6	-	-	-	-	-	-	125.7	121.6	22.3
3-Heptene, 2,2,4,6,6-pentamethyl-	193.2	279.6	105.8	84.6	228.4	61.3	128.9	78.2	145.0	36.6	103.5	126.3	120.3	25.2
Pyrazine, 2,5-dimethyl-	292.9	375.5	176.0	336.2	154.7	156.2	227.7	267.6	269.8	-	74.7	207.4	133.9	142.0
Nonanal	111.2	207.6	53.3	87.1	127.0	41.4	80.0	30.6	107.4	44.1	37.2	43.9	66.5	36.4
1-Octen-3-ol	-	-	-	-	-	753.6	1053.1	1318.3	1365.2	1026.8	1070.5	-	-	-
linalool	-	-	-	-	-	214.7	210.2	265.1	7134.7	5385.1	5402.6	-	-	-
Benzaldehyde	117.9	162.4	142.3	263.5	152.8	71.8	124.5	140.7	-	-	-	105.2	123.9	146.8
1-Octanol	111.8	145.9	34.1	33.3	19.5	-	-	-	-	-	-	46.2	29.5	21.2
1-Terpinen-4-ol	-	-	-	-	-	1501.1	1698.4	2828.4	1326.5	1211.8	1205.8	-	-	-
caryophillene	-	-	-	-	-	539.0	1571.5	3620.8	1736.5	2674.4	2589.7	-	-	-
Cyclohexene, 1-methyl-3-(1-methylethenyl)-, (ñ)-	268.9	582.8	240.1	392.4	486.3	155.9	244.2	-	312.1	167.4	181.6	319.5	256.9	186.1
Thiophene-2-acetic acid, dodec-9-ynyl ester	710.9	1478.2	656.4	1039.6	1150.1	595.1	874.4	356.3	1017.7	802.4	826.0	856.2	702.9	513.9
1,2,4-Triazol-4-amine, N-(2-thienylmethyl)-	253.5	559.9	260.5	535.1	600.3	196.7	425.3	390.2	386.7	212.5	263.1	338.9	308.7	290.8
Cycloctane	144.8	123.4	67.7	51.5	22.3	75.8	63.1	44.9	52.1	54.7	38.0	73.9	57.2	43.9
thymol	-	-	1095.3	150.9	188.0	4780.3	7839.6	9351.4	10390.2	7283.3	7459.5	8963.1	10321.9	10618.5
Carvacrol	-	-	10098.0	17270.9	16069.4	9206.9	15305.7	16709.4	11859.9	8246.9	8480.8	252.0	170.8	109.8
Durenol	66.1	162.3	111.8	86.1	94.6	118.7	207.5	326.7	169.8	158.6	216.1	71.9	74.3	63.3
Phenol, 4-(1,1,3,3-tetramethylbutyl)-	159.8	200.6	80.4	144.2	200.5	80.7	174.6	155.5	157.3	89.0	103.6	127.9	119.0	79.3

The results are means of three independent experiments. The coefficients of variability, expressed as percentage ratios between the standard deviations and the mean values, ranged between 5 and 10%.
- Undetectable level

Thymol, carvacrol, caryophillene, *p*-cymene, *γ*-terpinene, 1-terpinen-4-ol and linalool characterized the volatilomes of cells treated with oregano and thyme EOs. On the other hand a wide literature shows that they are the main components of these essential oils (Daferera et al., 2000).

To better understand the effects of the tested antimicrobial on the volatilome of *L. monocytogenes*, *E. coli* and *S. enteritidis* exposed during the growth to different sub-lethal concentrations the chosen compounds, a principal component analyses (PCA) was performed on the volatile molecule profiles without considering the added antimicrobials and their detoxification products. Although the volatile molecule profiles differed with the species, the PCA results showed clearly that the antimicrobials used induced specific changes in the compound release independently on their concentrations. In fact, the samples grouped generally in relation to antimicrobial used independently on the species considered. More specifically, the PCA loading plots for the two main factors explaining the variability of volatile molecules of late exponential phase cells of *L. monocytogenes* in relation to the stress condition applied during the growth are shown in Figure 7.4. All the samples were mapped in the space spanned by the first two principal components PC1 versus PC2. PC1 accounted for 37.81% of the variability, and PC2 for 20.69%.

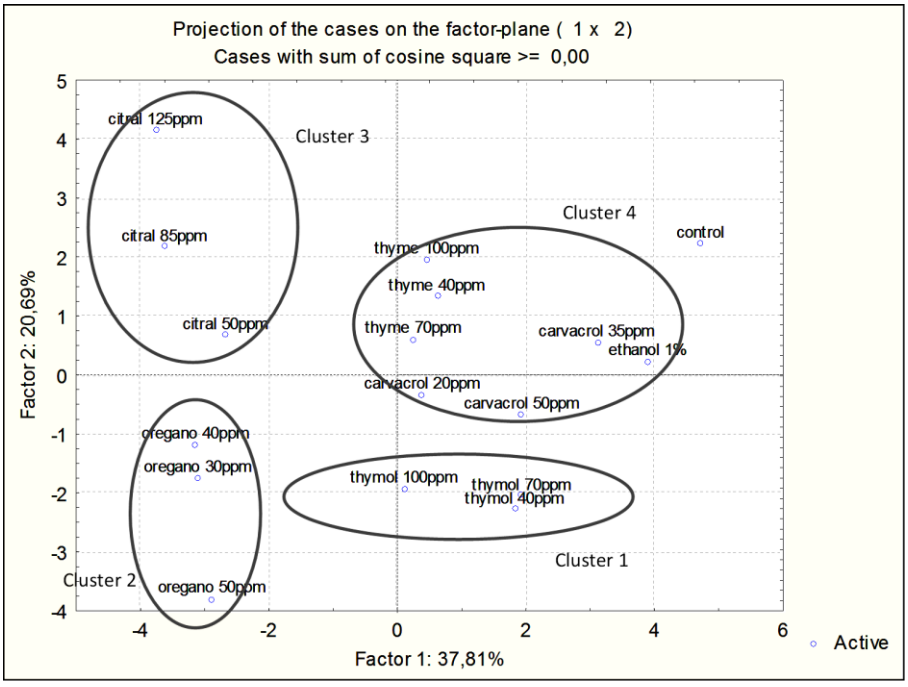


Figure 7.4- *Principal component analysis (PCA) loading plots of the two-first factors relative to the volatile molecules profile of* Listeria monocytogenes *Scott A in relation to the stress condition added (without considering the added antimicrobials and their detoxification products);*

The samples were grouped mainly on the basis of the added molecules independently on the concentrations added. Four clusters were evident: cluster 1 included all the samples added with thymol; in cluster 2 the samples treated with oregano EO independently on its concentration were present; cluster 3 grouped all the samples added with citral. Cluster 4 grouped the samples added with 1% ethanol, carvacrol and thyme EO. The control sample was not included in any cluster.

In Figure 7.5, the PCA loading plots relative to *E. coli* in relation to the stress condition applied during the growth are shown. In this case, PC1 accounted for 34.40% of the variability, and PC2 for 23.89%. Also in this case four clusters were evident: cluster 1 including all the samples added with 2-(E)-hexenal; in cluster 2 grouping the samples treated with thyme EO independently on its concentration; cluster 3 clustering all the samples added with oregano and the samples supplemented with the highest concentrations of thymol and carvacrol (100ppm); cluster 4 including the samples added with 1% ethanol, 40 and 70 ppm of carvacrol and 40 ppm of thymol. The control and the sample added with 70 ppm of thymol were not included in any clusters.

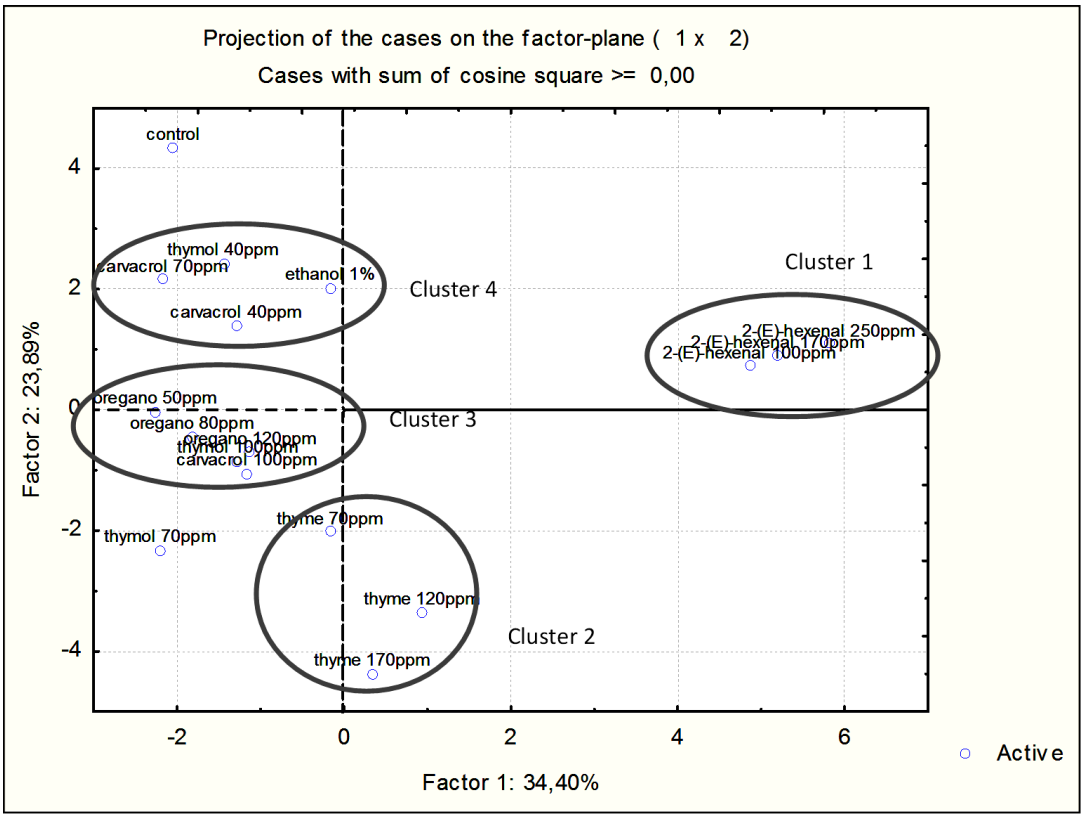


Figure 7.5- *Principal component analysis (PCA) loading plots of the two-first factors relative to the volatile molecules profile of* Escherichia coli *555 in relation to the stress condition added (without considering the added antimicrobials and their detoxification products);*

In Figure 7.6, the PCA loading plots for *S. enteritidis* are reported. In this case, PC1 and PC2 accounted for 50.10% and 20.80% of the variability, respectively. The samples were grouped mainly on the basis of the added molecules independently on the antimicrobial concentrations in two clusters. The first cluster included all the samples added with thymol and carvacrol; while the second grouped the samples treated with oregano and thyme EO independently on the concentration employed. The control sample and the sample added with 1% of ethanol were not included in any cluster.

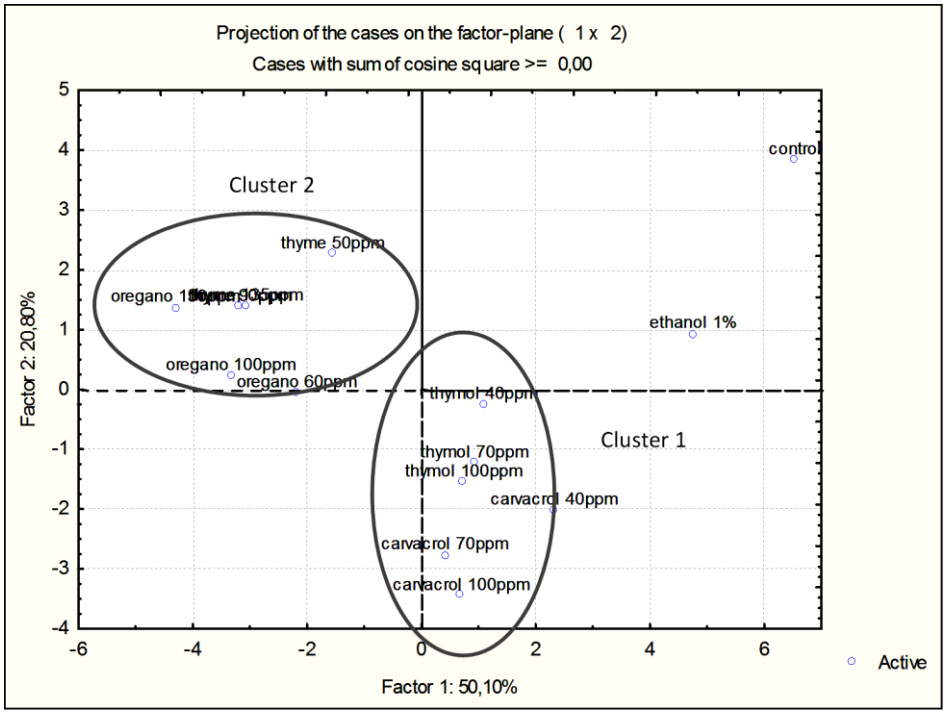


Figure 7.6- *Principal component analysis (PCA) loading plots of the two-first factors relative to the volatile molecules profile of* Salmonella enteritidis *in relation to the stress condition added (without considering the added antimicrobials and their detoxification products);*

4. Discussion

Microbial cells have adopted proficient defence systems to survive with a variety of physicochemical adverse conditions and to adapt to the environmental stresses. Particularly, essential for bacterial cells is to retain integrity and functionality of the membrane in response to environmental stresses. In presence of stresses, microbial cells respond by modulating the ratio of saturated to unsaturated FA, cis to trans unsaturation, branched to unbranched structure and type of branching and acyl chain length (Russel, 1984). The modulation mechanisms are different

depending to the species, the strains and the physiological state of the cells (Rock & Cronan, 1996).

In the considered microorganisms a uniform response was not recognized due to the fact that they belonged to different species. However, a general increase of the UL in presence of the tested molecules was observed independently on the species. In particular, a marked increase of C16:1 Z9 and C18:1 E9 was observed in Gram-negative bacteria *Salmonella enteritidis* and *Escherichia* coli independently on the supplemented molecule and the concentration added; while in case of Gram-positive *Listeria monocytogenes* a remarkable increase of C18:1z9 and C14:1e11 was evident in all the treated samples. Therefore, in presence of environmental stresses is clear the role of specific unsaturated FAs. The crucial role of unsaturated FAs has been reported by several works and in response to several different stresses, including low or high growth temperatures, oxidative stress, acid stress and ethanol and salt addition stress and high pressure homogenization (Chatterjee et al. 2000; Streit et al. 2008; Montanari et al. 2010; Wu et al. 2012; Tabanelli et al. 2013). Additionally, Patrignani et al. (2008) demonstrated the role of C18:1 and C18:2 in the resistance of some pathogenic species to antimicrobials, such as hexanal and (E)-2-hexenal. Di Pasqua et al. (2006) detected an increase of some UFAs and of the membrane fluidity, in *E. coli* and *B. thermosphacta* grown in the presence of sublethal concentrations of thymol, limonene, carvacrol, eugenol and cinnamaldehyde. On the other hand, is well-documented that UFAs provide to the membrane a high level of fluidity (Bayer et al., 2000).

The increase of trans isomers observed both in *E. coli* and *S. enteritidis*, seems to play a key role in presence of chemical stress. The findings of this study confirmed those of Patrignani et al. (2008), who found that the increase of C18:1trans both in *E. coli* and *S. enteritidis* cells exposed to ethanol, hexanal and 2-(E)-hexenal. In fact, the isomerization of double bonds is described to confer chemical stability and protection to the membrane against toxic compounds (Härtig et al., 2005). Cyclization and isomerization of membrane FA was shown to be the main response mechanisms of *Escherichia coli* to acidic growth conditions (Gianotti et al. 2009).

Regarding to cyclic fatty acids (CFA) in Gram-negative bacteria, a different modulation on the basis of the added molecule and the species was detected. The addition of thymol or carvacrol did not affect or caused a decrease of the detected CFA compared to the control, while the addition of oregano caused both in *S. enteritidis* and *E. coli* a marked increase of the relative percentages of C12cyc but coupled to a reduction of C17cyc and C19cyc; whereas the supplementation of thyme EO provoked in *Salmonella* a decrease of CFA in *E. coli* and a slight increase of C17cyc and C19

cyc relative percentages. Patrignani et al (2008) showed general reduction of CFA in *E. coli* and *S. enteritidis*, growth in presence of sublethal concentrations of hexanal and 2-(E)-hexenal.

It is well-documented in literature that the modulation of the synthesis of CFA is one of the main response of Gram-negative bacteria to adverse environmental conditions (Yuk & Marshall, 2004; Grogan & Cronan, 1997). The literature data shows that they play a key role also in the stress response mechanisms of Gram-positive bacteria (Gomez-Zavaglia et al. 2000; Tabanelli et al. 2013). In fact, Gomez-Zavaglia et al. (2000) reported that these CFAs enhanced the stress tolerance of *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus* and *L. acidophilus*, as well as that the amount of CFAs in the membranes of numerous LAB increased under various stress situations (Guillot et al. 2000; Beal et al. 2001). Tabanelli et al. (2013) showed, studying the response mechanisms of probiotic lactobacilli to a sub-lethal treatment at high pressure homogenization, that the content of CFA in relation to pressure applied varied in relation to species and strain. The ability to maintain the proper membrane fluidity in response to changing environmental conditions is fundamental for cell surviving and adaptation. Regulation of membrane fluidity through fatty acid alteration is a way for the microbial membrane to restore the balance between bilayer and non-bilayer forming lipids when challenged with environmental stresses and to maintain proper membrane structure and function (Denich et al., 2003).

Several authors (Fozo et al., 2004; Di Pasqua et al., 2006) have reported that increased of fatty acid length is another important membrane modification to rise survival in adverse environments such as acidic conditions or in presence of antimicrobial compounds. Also in our experimental conditions, an increase of the CL in all the treated of *E. coli*, *S. enteritidis* and *L. monocytogenes* was observed. These FA modifications probably compensated and overcame the fluidizing effect of the increase of unsaturation level. The only exceptions were represented by the cells of *S. enteritidis supplemented with oregano*, and *L. monocytogenes* supplemented with ethanol or thymol at different concentrations. However, *Salmonella enteritidis* cells supplemented with oregano showed with respect to the other treated samples were the highest concentration of C12 cyc and lower concentration of trans-isomers associated to the highest unsaturation level. In this case the fluidizing effects of UL is mainly compensated by the trans-UFA increase. In fact, trans UFA with their long linear structure behave more like saturated FA that lie in a linear manner, taking up less volume and creating a more ordered membranes (Diefenbach et al., 1992). This samples evidenced also a high level of cyclic FA. However, the literature data concerning the role of cyclic acid in the membrane fluidity are quite contrasting. In fact, some authors attributed to

the presence of a cyclopropane ring within membrane FA an increase of stability of the structural and dynamic properties of biological membrane and a decrease of fluidity (Grogan & Cronan, 1997). On the contrary, other authors reported that cyclopropane fatty acids confer fluidity upon the cell membrane and assist in tolerance towards disturbance factors (Denich et al., 2003). In particular, Denich et al. (2003) showed as cyclic FA, analogously to branched FA, increase the fluidity of cytoplasmic membrane as they retain the ability to slide past each other as they cannot form crystalline structure. Also Gianotti et al. (2009) showed the fluidizing effect of cyclic FA in *E. Coli* grown in acidic conditions.

L. monocytogenes supplemented with ethanol or with different concentration of thymol probably guaranteed the proper membrane fluidity also with minor CL because characterized by a minor UL with respect to the other treated samples. On the other hand the samples supplemented with ethanol and thymol cauterized together sharing the same response mechanisms.

Also the GC-MS-SPME analyses showed that the supplementation of growth media with natural antimicrobials such as cital, 2-(E)-hexenal, carvacrol, thymol, oregano and thyme EO modified significantly the volatile molecule profiles of *L. monocytogenes*, *E. Coli* and *S. enteritidis*. In fact, a dramatic increase of nerol, geraniol, 2-(*E*)-hexen-1-ol, 2-(Z)-hexen-1-ol, deriving from the detoxification of citral and 2-(E)-hexenal, was observed. On the other hand the PCA performed on the volatile molecule profiles without the from added antimicrobials and their detoxification products was able to group the samples mainly in relation the chemical stresses applied, suggesting specific response mechanisms for each microorganism and antimicrobial used. In fact, specific volatile profiles were recorded in relation to chemical stress applied and target microorganism. A uniform response was not evidenced probably due to differences in metabolic pathway of the microorganisms considered and the differences in their modulation in response to chemical-physical and environmental conditions.

In conclusion the findings of this work contribute to the comprehension of the volatilome and membrane FA modulation mechanisms used by the different microorganisms (*L. monocytogenes*, *E. coli* and *S. enteritidis*) in relation to the exposure to sublethal concentrations of 2-(E)-hexenal, citral, carvacrol, thymol, oregano and thyme EO. However, to clarify if the changes in membrane FA compositions and volatilome induced by the natural antimicrobials considered are the consequences of or the trigger for stress-related gene expression a transcriptome analysis is necessary.

CHAPTER 8

Study of the response mechanisms of *Escherichia coli* and *Lactococcus lactis* to Thyme essential oil, carvacrol, 2-(E)-hexanal and citral

Abstract

Essential oils are produced by plants as secondary metabolites for plant defense and some of these are well known for their antimicrobial properties. In *vitro* studies showed that thyme EOs and two of its main components, such as carvacrol and thymol, are characterized by a strong antimicrobial activity. Also volatile aldehydes such as 2-(*E*)-hexenal and citral, which are components of the aroma of many fruits and vegetables, are characterized by a strong antimicrobial activity both in model and food systems. Moreover, Essential oils and some of their components are generally recognized as safe (GRAS). Their application as food preservatives is very promising but it requires a deeper knowledge about the microorganisms they can target, their interaction with food matrix components and their modes of action. In fact, the mechanisms of action of most EOs is not or not fully understood. In order to more consciously use essential oils and their components in the food industry it is necessary to better comprehend the stress response induced by the addition of these natural antimicrobials on pathogenic and spoilage microorganisms as well as on microorganisms used as starter or biocontrol agents in food products. In this context, the main aim of this work was to study the stress response to sublethal concentrations of thyme essential oil, carvacrol, citral and 2-(E)-hexenal in the two model bacteria, *Escherichia coli* and *Lactococcus lactis* using DNA microarray technology. The data obtained proved that the addition of sublethal concentrations of the natural antimicrobials employed here did not strongly affect global gene expression in *L. lactis* NZ9700 while these treatments caused a major response in *E. coli* K12 for all antimicrobials used. In the latter, the modification of the expression in genes involved in fatty acid biosynthesis suggestsing that the cytoplasmic membrane of *E. coli* is the major cellular target of essential oils and their components.

1. Introduction

Essential oils (EO) are aromatic and volatile compounds extracted from the whole plant as well as from plant material such as flowers, roots, leaves, seeds, peel, fruits and wood (Hyldgaard et al., 2012). They are produced by plants as secondary metabolites for plant defense and some of these EOs are well known for their antimicrobial properties (Fraenkel, 1959; Tajkarimi et al., 2010). The historical use of EOs was in medicine, perfumery, cosmetics, and they are also added

to foods as part of spices or herbs. Generally EOs contain 20–60 constituents at different concentrations. EOs are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts (Bakkali et al. 2009).

In *vitro* studies showed that thyme EOs possess antimicrobial activity against a broad spectrum of Gram-negative or Gram-positive bacteria as well as yeasts and moulds (Solomakos et al., 2008; Burt, 2004; Bagamboula et al., 2004). Carvacrol is, in addition to thymol, one of the main components of thyme and oregano EOs; it is a phenolic monoterpenoid characterized by a strong antimicrobial activity against a wide range of pathogenic microorganisms (Bagamboula et al., 2004; Zhou et al., 2007, Oussallah et al., 2007), fungi and insects (Kordali et al., 2008).

Volatile aldehydes such as 2-(*E*)-hexenal and citral, which are components of the aroma of many fruits and vegetables, are characterized by a strong antimicrobial activity both in model and food systems (Gardini et al., 2001; Lanciotti et al., 2004). In particular, antimicrobial action against bacteria, yeasts and moulds in different conditions has already been demonstrated for citral (3,7-dimethyl-2-7-octadienal), an acyclic α,β-unsaturated monoterpene aldehyde that exists as the 2 isomers geranial and neral and naturally occurs in citrus essential oils. (Belletti et al., 2007, 2008; Caccioni and Deans, 1993; Rivera-Carriles et al., 2005; Wuryatmo et al., 2003).

Moreover, EOs and some of their components are generally recognized as safe (GRAS) (Kim et al., 1995; Burt, 2004; Moreira et al., 2005). In addition, some essential oils appear to exhibit particular medicinal properties that have been claimed to cure some organ dysfunctions or systemic disorders (Hajhashemi et al., 2003; Perry et al., 2003; Bakkali et al., 2008).

EOs are used in the food industry as flavoring agents since several years. Because of the antimicrobial properties of some of the EOs (Dorman and Deans 2000; Cosentino et al., 2003; Smith-Palmer et al., 1998; Kalemba and Kunicka 2003), their application as food preservatives is very promising but it requires a deeper knowledge about the microorganisms they can target, their interaction with food matrix components and their modes of action. In fact, the mechanisms of action of most EOs is not or not fully understood (Hyldgaard et al., 2012).

Given their structural differences and the presence of different functional groups the mechanism of the antibacterial activity of the various essential oil components will most likely not be the same and there may be several specific targets in the cell (Burt, 2004). Generally, it is accepted that EOs and their active molecules can lead to degradation of the cell wall, damage of the cytoplasmic membrane and membrane proteins, leakage of cellular contents, coagulation of

cytoplasm, depletion of the proton motive force, or more general perturbation of energy metabolism (Burt, 2004; Picone et al. 2013). Evaluating the effect of carvacrol on the *Escherichia coli* 555 metabolome using 1H-NMR spectroscopy, Picone et al. (2013) noticed a shift from respiration toward fermentation as the concentration of carvacrol increased.

In order to more consciously use essential oils and their components in the food industry it is necessary to better comprehend the stress response induced by the addition of these natural antimicrobials on pathogenic and spoilage microorganisms as well as on microorganisms used as starter or biocontrol agents in food products. It is well known that microorganisms respond to a wide range of stresses by regulating gene expression and protein profiles. Microorganisms come across several different stress conditions in foods, particularly minimally processed foods. Stress responses in pathogens can allow survival under more stringent conditions, augment resistance to consequent processing conditions, and/or increase virulence (Chung et al., 2006). Consequently, understanding the effects of stress on the physical tolerance of pathogens is important in order to evaluate and minimize the risk of food-borne illness (Chung et al., 2006). Moreover, stress responses in potential starter and/or biocontrol cultures may result in a decrease of survival and technological performances in food industry (Leroy & deVuyst, 2004; .Guchte et al., 2002).

The main aim of this work was to study the stress response to natural antimicrobials in the two model bacteria, *Escherichia coli* and *Lactococcus lactis* using DNA microarray technology. The effects on whole-genome gene expression (the transcriptome) of *E. coli* and *L. lactis* of sub-lethal concentrations of thyme essential oil and some of the major components of essential oils such as carvacrol, citral and 2-(E)-hexenal have been studied in depth using home-made as well as commercial DNA microarray slides.

2. Material and Methods

2.1 Natural antimicrobials

Citral, 2-*(E)*-hexenal, and carvacrol were purchased from Sigma-Aldrich (Milano, Italy). Thyme essential oil was obtained from Flora s.r.l. (Pisa, Italy) and first characterized by solid phase microextraction combined to gas-chromatography and mass-spectometry GC/MS-SPME to know the exact composition of the oil (Chapter 4). The natural antimicrobials were stored at 4 °C.

2.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination.

For the determination of the MIC and MBC values of 2-(E)-hexenal, citral, carvacrol and thyme EO on the target microorganisms *Escherichia coli* K12 and *Lactococcus lactis* NZ9000, the method reported in Chapter 3 (paragraph 2.3) was used. Brain Heart Infusion (BHI, Oxoid Ltd. Basingstoke, United Kingdom) and M17(Oxoid Ltd., Basingstoke, England) +2% glucose broth (GM17) were used for *E. coli* and *L. lactis*, respectively. Three different inoculum levels (6, 4 and 2 log cfu/mL) were used and MIC values were determined after 18 and 24h while MBC values were determined after 24h of incubation at 37^{o}with shaking for *E. coli* and at 30^{o} for *L. lactis* (standing cultures).

2.3Treatments of bacterial cultures with natural antimicrobial compounds

The concentrations of 2-(*E*)-hexenal, citral, carvacrol and thyme EO employed on *E. coli* and *L. lactis* are reported in Table 8.1. Each compound was used at approximately ½ of the determined MIC values after 18h at an inoculum level of 2 log cfu/mL.

Overnight grown cultures were inoculated at a level of about 6 log cfu/mL in 1.0 L flasks, containing 800 mL of BHI or GM17 broth, respectively, for *E. coli* and *L. lactis*. Immediately after inoculation, the samples were incubated at 37^{o} or 30°C for *E. coli* and *L. lactis*, respectively. The growth rate was monitored by measuring the optical density at 600 nm (OD600) every 0.5 h using a spectrophotometer UV-1204 (Shimadzu, Kyoto, Japan), until an OD600 of 0.4 was reached. Then, the microbial cultures where aliquoted into 50 mL tubes and supplemented with the selected concentration of each compound conveyed through 1% ethanol. The experiments were repeated three times on different days, and for each experiment three tubes for each condition were used. Bacterial cultures to which 1% of ethanol was added served as controls.

Treatments were performed for 1h at 37°C or 30°C, respectively, for *E. coli* and *L. lactis*. From every condition, the cells from two samples of 50 mL were harvested by centrifugation (6,000/g for 5 min in an eppendorf centrifuge (Eppendorf, S.r.l., Hamburg, Germany) at room temperature. The pellets were immediately frozen in liquid nitrogen prior to storage at -80°C.The effects of the addition of the natural antimicrobials on the growth rate of the target microorganisms were monitored also after the treatment, by measuring the OD600every 0.5 h of one 50 mL culture for each condition.

Table 8.1- *Compounds and relative concentrations employed on* E. coli *and* L. lactis. *The treatments were performed when the OD600 of the cultures reached 0.4, and for a duration of 1h.*

	Thyme EO (ppm)	Carvacrol (ppm)	Citral (ppm)	2-(*E*)-hexenal (ppm)
***E. coli* K12**	125	60	500	200
***L. lactis* NZ9700**	12.5	25	150	100

2.4 DNA microarray analyses

Transcriptome analyses were performed essentially as described previously (Kuipers et al. (2002),,Ultimately, the labelled cDNAs of *L. lactis* NZ9700 were hybridized to full-genome DNA microarray slides of *L. lactis* MG1363(ref). The *E.coli* K12 cDNAs were hybridized to commercial *E. coli* gene expression 8×15K microarray slides (Agilent Technologies, Palo Alto, CA, USA). After washing, the slides were scanned by using an Agilent G2565CA microarray scanner (Agilent Technologies). For the two bacterial species, each treatment condition was compared to the control. A biological replicate of each comparison as well as a dye swap were performed. DNA microarray slide pictures were analyzed using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). The Limma R package (Smyth, 2005) was used to analyse the DNA microarray data using the 1% ethanol control as the common reference. Fold changes were considered to be significantly changed when the Benjamini-Hochberg adjusted p-value is ≤ 0.1.

2.5 Statistics tools

An in-depth analysis of the transcriptome data was performed with a variety of bioinformatics tools from the MolGen GENOME2D website (http://genome2d.molgenrug.nl). In order to compare the different treatments, the fold-change (FC) values were used.

3. Results and Discussion

3.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) evaluation.

The MICs and MBCs of citral, 2-(E)-hexenal, thyme EO or carvacrol against three density levels of the target microorganisms *L. lactis* NZ9700 and *E. coli* K12 were assessed after incubation at 30°C and 37°C, respectively, (Table 8.2). Pronounced differences in the MICs and MBCs were observed in relation to the substances and the inoculum level used for both bacterial species. Citral showed a low antimicrobial effectiveness against *E. Coli*; in fact, the MIC values were always higher than 2000 ppm, independent of the inoculation level. On the contrary, *L. lactis* MIC and MBC values ranged between 300 and 1200 ppm as a function of the initial inoculation level. In fact, the MIC after 18h and the MCB decreased from 1100 and 1200 ppm to 300 and 700 ppm in cultures of 10^6 and 10^2 cfu/mL respectively. The effects of inoculation level on MIC and MBC values has been described previously (Carson et al., 1995; Lambert et al. 2001; Burt, 2004). The bacteriostatic effect of citral was evident at the lowest inoculum level and with an inoculum of 2 log cfu/mL the MIC after 24h was 350 ppm higher than that after 16h. These different responses to essential oil or their components between the Gram-positive *L. lactis* and the Gram-negative *E. coli* has been reported in literature (Smith-Palmer et al., 1998; Marino et al., 2001; Delaquis et al., 2002). Gram-negative bacteria are generally more resistant to many compounds due to their outer membrane, which acts as an efficient permeability barrier against macromolecules and hydrophobic substances (Helander et al., 1997; Lanciotti et al., 2003; Chang & Cronan, 1999). The influence of the initial inoculum on MICs and MBCs was evident for the treatment with of 2-(E)-hexenal. This molecule showed a good efficacy against both target microorganisms. *L. lactis* and *E. coli* had MIC and MBC values ranging between 200-750 ppm and 350-600 ppm, respectively, depending on the initial inoculation level.

Carvacrol showed the highest efficacy against both target microorganisms. However the MIC and MBC values of *E. coli* were not affected by the inoculation level. For *L. lactis* the MIC at18h and the MCB decreased from 125 ppm to 50 ppm with inoculation levels lowered from 10^6 to 10^2 cfu/mL, respectively. Thyme EO strongly inhibited the growth of *L. lactis*. In fact, the MIC and MBC values were 25 ppm at the lowest inoculum level, while this value increased as the inoculum level increased. *E. coli* is more resistant to thyme EO with MIC and MBC values

ranging between 250 and 500 ppm in dependence of the inoculation level. MICs and MBCs values are reported in Table 8.2.

Table 8.2-*Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of citral, 2-(E)-hexenal, carvacrol and thyme EO against* E. coli K12 *and* L. lactis *NZ9700*[a]

Lactococcus lactis NZ9700									
cell concentration	6 log cfu/ml			4 log cfu/ml			2 log cfu/ml		
MIC/MBC	MIC 18h (ppm)	MIC 24h (ppm)	MBC 24h (ppm)	MIC 18h (ppm)	MIC 24h (ppm)	MBC 24h (ppm)	MIC 18h (ppm)	MIC 24h (ppm)	MBC 24h (ppm)
Carvacrol	125	125	125	75	100	100	50	50	50
T-2-Hexenal	700	700	750	400	700	700	200	400	450
Citral	1100	1200	1200	900	1100	1200	300	650	700
Thyme oil	150	175	175	50	75	75	25	25	25
cell concentration	6 log cfu/ml			4 log cfu/ml			2 log cfu/ml		
MIC/MBC	MIC 18h (ppm)	MIC 24h (ppm)	MBC 24h (ppm)	MIC 18h (ppm)	MIC 24h (ppm)	MBC 24h (ppm)	MIC 18h (ppm)	MIC 24h (ppm)	MBC 24h (ppm)
Carvacrol	125	125	125	125	125	125	125	125	125
T-2-Hexenal	500	575	600	375	425	450	350	400	425
Citral	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000
Thyme oil	375	475	500	300	375	425	250	275	300

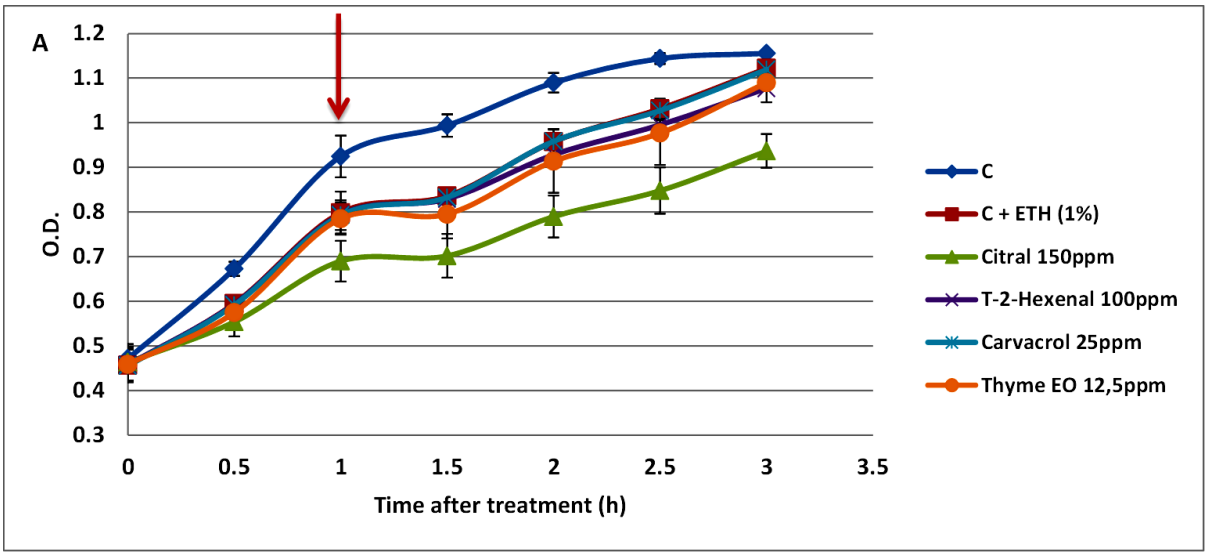
[a] MIC and MCB values are in ppm.

3.2 Transcriptional analyses of L. lactis and E. coli *treated with sub-lethal concentrations of natural antimicrobials*

The transcriptional response of *L. lactis* and *E. coli* to sub-lethal concentrations of the natural antimicrobials studied here was assessed by using a whole-genome DNA microarray approach. To reach this goal, each antimicrobial (2-(E)-hexenal, citral, carvacrol and thyme EO) was used at a concentration of about ½ of the MIC values for the two species. Treatments were performed for 1 h at 37 and 30°C for *E. coli* and *L. lactis*, respectively, on cells grown until was reached the middle of exponential growth phase, at an OD_{600} of 0.4 for both the strains. Since the antimicrobials were resuspended in 1% ethanol, the common reference was a bacterial culture treated with 1% ethanol. In order to verify the effects of the treatments on cell vitality, growth of the target microorganisms after the treatments was also monitored (Figure 8.1).

In *L. lactis*, the addition of sub-lethal concentrations of natural antimicrobials slightly reduced the maximum growth rate compared to the control (1% ethanol addition). Only the addition of 150 ppm of citral led to a marked reduction of the maximum growth rate The addition to *E. coli*, of 1% ethanol or 200 ppm of 2-(E)-hexenal did not affect the growth rate of the organism compared

to the control. On the contrary, carvacrol and, to a greater extent, thyme EO and citral strongly affected the maximum growth rate as well as the OD_{600} reached in stationary phase.



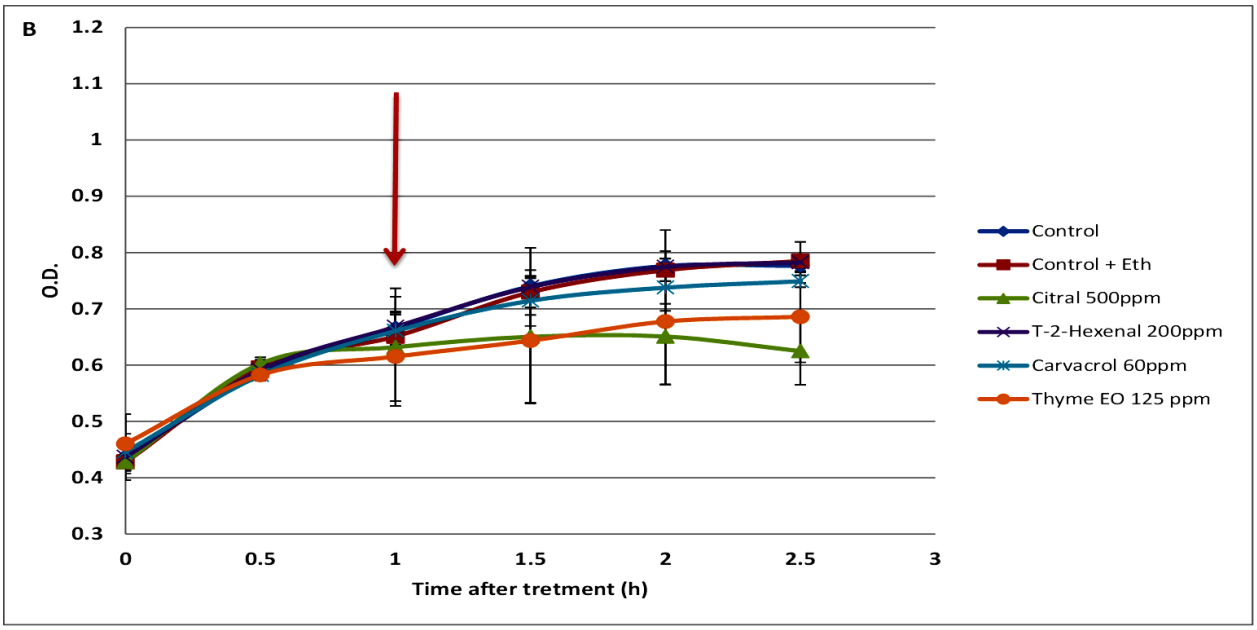


Figure 8.1-Effect of natural antimicrobial addition on the growth of *L.lactis* (A) and *E.coli* (B). L. lactis *NZ9700 growth after treatment with the indicated sub-lethal concentrations of natural antimicrobials. The control is represented by an* L. lactis *culture to which no addition was made(A).* E. coli *K12 growth after treatment with the indicated sub-lethal concentrations of natural antimicrobials. The control is represented by E. coli without any addition (B). The arrows indicate the point at which cultures were collected for micro arraying*

Growth was recorded as the change in OD600.

DNA microarray analyses were done on parallel cultures after 1 h. of treatment with the various compounds. At that point in time relatively little effects were seen on the growth of both bacterial species relative to the 1% ethanol control. The DNA microarray results showing the a

differences in the numbers of genes being up- or down regulated of the two target microorganism and in the response to the antimicrobial added are presented in Table 8.2.

Table 8.2-*Number of significantly (p<0.1) up- or down regulated genes in* L. lactis *NZ9700 (2A) and* E. coli *K12 (2B)*

***Lactococcus lactis* NZ9700**

2A	Citral	2-(E)-hexenal	Carvacrol	Thyme EO
UP	35	9	0	9
UNCHANGED	2337	2367	2349	2355
DOWN	7	3	30	15

Escherichia coli K12

2A	Citral	2-(E)-hexenal	Carvacrol	Thyme EO
UP	35	9	0	9
UNCHANGED	2337	2367	2349	2355
DOWN	7	3	30	15

Independent from the treatment applied, the gene functional categories involved in the response of *L. lactis* to the employed compounds were the energy metabolisms, purine/pyrimidine metabolism and fatty acid and phospholipid metabolism. With regard to the gene functional categories involved in the *E. coli* response to the added antimicrobials: energy metabolism, purine/pyrimidine metabolism, fatty acid and phospholipid metabolism, protein synthesis and DNA metabolism were affected the most.

The distribution of differentially expressed genes in relation to the treatment employed was examined to better understand the effect of each treatment (Figure 2). The Venn diagram obtained from the *L. lactis* data (Fig. 2 A) showed that most of the genes that were differentially expressed in the presence of one of the added antimicrobials were specific for that compound; only a few genes respond to the presence of each of two antimicrobials while no genes responded to each of three antimicrobials. All-in-all, the addition of the different natural antimicrobials did not strongly affect gene expression in *L. lactis* NZ9700 compared to the common reference (to which 1% ethanol was added). In fact, with the exception of only a few genes, the fold changes for *L. lactis* genes were lower than 3.0.

On the contrarily, the addition of the compounds to *E. coli* seems to lead to a response in gene expression that is partially similar for all antimicrobials used. In fact, as is evident from Figure

8.2 b, 70 genes are differentially expressed in all the four conditions. Moreover, approximately 31% of the differentially expressed genes were common in at least three conditions.

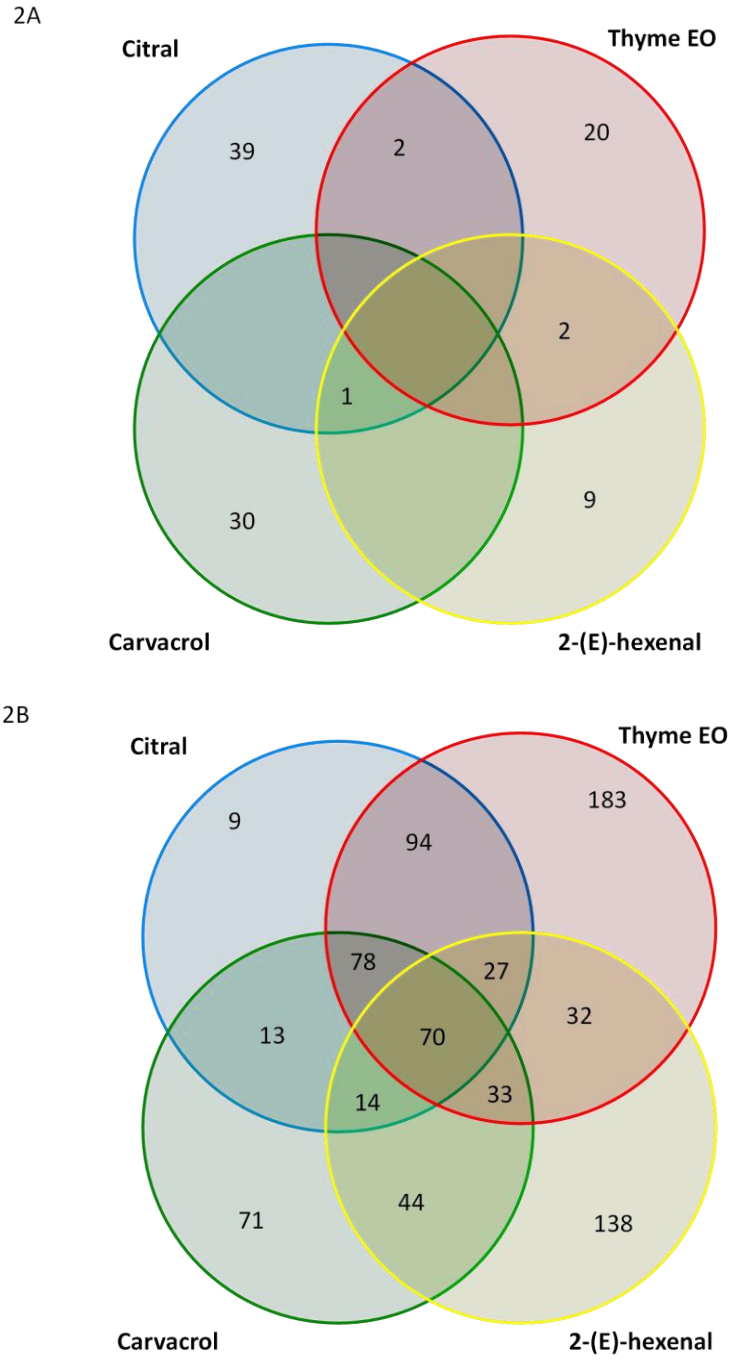


Figure 8.2-*Distribution of differentially expressed genes in* L. lactis *(A) and* E. coli *(B) in relation to the natural antimicrobial used. The Venn diagram (Kestler et al., 2005) reports the numbers of unique and common differentially expressed genes*

The response of *E. coli* to sub-lethal concentrations of thyme EO and citral are particularly similar, the numbers of differentially expressed genes common to both conditions being around

45%. Moreover, the gene functional categories involved in the response were similar for each of to the added compounds (Table 8.3).

The *E. coli* genes that are up- or down regulated at least 2-fold or more and with a p-value lower than 0.1 are reported in Table 8.3.

Table 8.3- *Selected genes up- or down regulated at least 2-fold in* E. coli *K12 after treatment with citral (500 ppm), 2-(E)-hexenal (200 ppm), carvacrol (60ppm) and thyme EO (125 ppm).)*[a]

	Fold Change				Description
Gene	Carvacrol	Thyme	Citral	2-(E)-hexenal	
Ribosome					
b0911 (rpsA)	1.84	2.07	1.76		30S ribosomal subunit protein S1 [b0911]
b3321 (rpsJ)	2.52	2.90	2.67	1.63	30S ribosomal subunit protein S10 [b3321]
b3297 (rpsK)	1.62	2.00	1.65		30S ribosomal subunit protein S11 [b3297]
b3342 (rpsL)	2.26	2.49	2.04	1.45	30S ribosomal subunit protein S12 [b3342]
b3307 (rpsN)	1.96	2.18	1.84	1.41	30S ribosomal subunit protein S14 [b3307]
b3165 (rpsO)	2.23	2.08	2.03		30S ribosomal subunit protein S15 [b3165]
b2609 (rpsP)	1.73	2.04	1.90	1.49	30S ribosomal subunit protein S16 [b2609]
b3311 (rpsQ)	3.02	2.84	2.64	1.56	30S ribosomal subunit protein S17 [b3311]
b4202 (rpsR)	1.84	2.48	1.88		30S ribosomal subunit protein S18 [b4202]
b3316 (rpsS)	2.21	2.48	2.07	1.35	30S ribosomal subunit protein S19 [b3316]
b0169 (rpsB)	2.20	2.23	1.37	2.14	30S ribosomal subunit protein S2 [b0169]
b3065 (rpsU)	1.82	2.35	2.22	1.50	30S ribosomal subunit protein S21 [b3065]
b3303 (rpsE)	2.05		1.90	2.15	30S ribosomal subunit protein S5 [b3303]
b4200 (rpsF)	1.64	2.23	1.93		30S ribosomal subunit protein S6 [b4200]
b3341 (rpsG)	1.68	2.09	1.85	1.50	30S ribosomal subunit protein S7, initiates assembly [b3341]
b3230 (rpsI)	2.33	2.09	2.65		30S ribosomal subunit protein S9 [b3230]
b1717 (rpmI)	2.61		2.00		50S ribosomal subunit protein A [b1717]
b3985 (rplJ)	1.98	2.28	2.02	1.48	50S ribosomal subunit protein L10 [b3985]
b3983 (rplK)	1.94	2.22	1.91		50S ribosomal subunit protein L11 [b3983]
b3231 (rplM)	3.05	7.35	2.78		50S ribosomal subunit protein L13 [b3231]
b3313 (rplP)	1.95	2.36	1.96		50S ribosomal subunit protein L16 [b3313]
b3294 (rplQ)	1.84	2.05	1.61	1.46	50S ribosomal subunit protein L17 [b3294]
b3304 (rplR)	2.22	2.90	1.92	1.46	50S ribosomal subunit protein L18 [b3304]
b3317 (rplB)	1.79	2.02	1.77		50S ribosomal subunit protein L2 [b3317]
b1716 (rplT)	2.00		2.18	2.47	50S ribosomal subunit protein L20, and regulator [b1716]
b3186 (rplU)	2.66		2.55	2.68	50S ribosomal subunit protein L21 [b3186]
b3318 (rplW)	2.07		2.06	2.37	50S ribosomal subunit protein L23 [b3318]
b2185 (rplY)	1.83	2.22			50S ribosomal subunit protein L25 [b2185]

b3185 (rpmA)	2.27	2.75	2.24		50S ribosomal subunit protein L27 [b3185]
b3312 (rpmC)	2.13	3.13	2.45	1.42	50S ribosomal subunit protein L29 [b3312]
b3320 (rplC)	2.19	2.54	2.31	1.45	50S ribosomal subunit protein L3 [b3320]
b3302 (rpmD)	1.78	2.09	1.82		50S ribosomal subunit protein L30 [b3302]
b1089 (rpmF)	2.71	2.33	2.63		50S ribosomal subunit protein L32 [b1089]
b3319 (rplD)	2.39	2.84	1.92		50S ribosomal subunit protein L4, regulates expression of S10 operon [b3319]
b3308 (rplE)	1.80	2.08	1.73		50S ribosomal subunit protein L5 [b3308]
b3305 (rplF)	1.95	2.19	1.86		50S ribosomal subunit protein L6 [b3305]
b3986 (rplL)	1.90	2.43	2.11		50S ribosomal subunit protein L7/L12 [b3986]
b4203 (rplI)	1.82	2.13	2.04		50S ribosomal subunit protein L9 [b4203]
Fatty acid metabolism					
b1241 (adhE)	2.06	1.27	1.74	1.66	CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase; pyruvate-formate-lyase deactivase [b1241]
b0356 (frmA)			2.01	3.57	alcohol dehydrogenase class III; formaldehyde dehydrogenase, glutathione-dependent [b0356]
b4042 (dgkA)	1.76	2.18	1.83	1.35	diacylglycerol kinase [b4042]
b3926 (glpK)	-1.47			-2.06	glycerol kinase [b3926]
b2243 (glpC)	-1.60	-1.66		-3.34	sn-glycerol-3-phosphate dehydrogenase [b2243]
b3426 (glpD)	-1.18			-2.89	sn-glycerol-3-phosphate dehydrogenase [b3426]
b1800 (yeaU)				3.08	putative tartrate dehydrogenase [b1800]
Energetic metabolism					
b2297 (pta)	2.00		2.04	1.75	phosphotransacetylase [b2297]
b1651 (gloA)		1.72		2.87	lactoylglutathione lyase [b1651]
b3403 (pck)	-1.64	-1.47		-2.13	phosphoenolpyruvate carboxykinase [b3403]
b0113 (pdhR)	1.51	2.04			transcriptional regulator for pyruvate dehydrogenase complex [b0113]
b0114 (aceE)	1.45	2.26			pyruvate dehydrogenase [b0114]
b0115 (aceF)	1.66	2.20			pyruvate dehydrogenase [b0115]
b2579 (yfiD)	1.57	1.56	1.44	2.58	putative formate acetyltransferase [b2579]
b0824 (ybiY)	-1.94	-1.98		-2.39	putative pyruvate formate-lyase 2 activating enzyme [b0824]
Purine, Pyrimidine metabolism and transcription					
b2146 (yeiT)	-1.75	-1.72		-2.38	putative oxidoreductase [b2146]
b3011 (yqhD)			5.13	8.36	putative oxidoreductase [b3011]
b3295 (rpoA)	1.82	2.01	1.71	1.60	RNA polymerase, alpha subunit [b3295]
b4238 (nrdD)				2.82	anaerobic ribonucleoside-triphosphate reductase [b4238]
b4244 (pyrI)	-2.10		-2.04	-2.05	aspartate carbamoyltransferase, regulatory subunit [b4244]
b1207 (prs)				-2.03	phosphoribosylpyrophosphate synthetase [b1207]
b2684 (mprA)		1.50	1.50	2.45	regulator of plasmid mcrB operon [b2684]

Hypothetical proteins

b1088 (yceD)	1.79	2.13	2.14	1.38	orf, hypothetical protein [b1088]
b1112 (bhsA)				5.51	orf, hypothetical protein [b1112]
b1179 (ycgL)	2.03				orf, hypothetical protein [b1179]
b1654 (grxD)			2.13	2.53	orf, hypothetical protein [b1654]
b3012 (dkgA)			1.36	2.25	orf, hypothetical protein [b3012]
b3207 (yrbL)	1.27	1.25	2.16		orf, hypothetical protein [b3207]
b3238 (yhcN)	1.73		2.41	7.04	orf, hypothetical protein [b3238]
b3914		4.29	3.82		orf, hypothetical protein [b3914]
b4050 (pspG)		2.47	1.80		orf, hypothetical protein [b4050]

heat shock, acid shock, proteasesand detoxification and protection

b1597 (asr)	2.20	5.01	2.10		acid shock protein [b1597]
b0605 (ahpC)				2.18	alkyl hydroperoxide reductase, C22 subunit; detoxification of hydroperoxides [b0605]
b0606 (ahpF)	1.69	2.80	2.03	1.49	alkyl hydroperoxide reductase, F52a subunit; detoxification of hydroperoxides [b0606]
b3686 (ibpB)		7.15	10.72		heat shock protein [b3686]
b3687 (ibpA)		2.35	3.43		heat shock protein [b3687]
b1305 (pspB)		2.92	2.19		phage shock protein [b1305]
b1307 (pspD)		2.57	1.63		phage shock protein [b1307]
b1304 (pspA)		4.10	2.39		phage shock protein, inner membrane protein [b1304]
b1306 (pspC)		2.05	1.64		phage shock protein: activates phage shock-protein expression [b1306]
b0161 (degP)	1.59	2.05	2.26		periplasmic serine protease Do; heat shock protein HtrA [b0161]
b1531 (marA)		2.07	2.76	3.80	multiple antibiotic resistance; transcriptional activator of defense systems [b1531]

Transport/binding proteins

b1247 (oppF)	1.73	1.72		2.05	homolog of Salmonella ATP-binding protein of oligopeptide ABC transport system [b1247]
b1244 (oppB)				2.12	oligopeptide transport permease protein [b1244]
b0904 (focA)	1.97		1.83	2.32	probable formate transporter [b0904]

Outer and inner cell membrane

b3035 (tolC)			5.96		outer membrane channel; specific tolerance to colicin E1; segregation of daughter chromosomes [b3035]
b0814 (ompX)	2.58	1.60	2.02	2.36	outer membrane protein X [b0814]
b2240 (glpT)	-1.57			-2.14	sn-glycerol-3-phosphate permease [b2240]
b0411 (tsx)		2.07	1.78		nucleoside channel; receptor of phage T6 and colicin K [b0411]

Amino acid metabolism

b3708 (tnaA)				-2.12	tryptophanase [b3708]
b2957 (ansB)				-2.16	periplasmic L-asparaginase II [b2957]
b1416				2.36	glyceraldehyde-3-phosphate dehydrogenase [b1416]
Secondary metabolisms					
b2153 (folE)		2.23	1.71	1.22	GTP cyclohydrolase I [b2153]
b4240 (treB)	-1.56			-2.02	PTS system enzyme II, trehalose specific [b4240]
Replication and repair					
b3179 (rrmJ)		2.43	2.57		cell division protein [b3179]
b4201 (priB)	1.64	2.30	1.76	1.34	primosomal replication protein N [b4201]
Other functions					
b0701	1.62	2.13	1.72		protein in rhs element [b0701]
b1902 (ftnB)	-1.67	-1.70		-2.09	ferritin-like protein [b1902]
b0849 (grxA)		2.85	2.48		glutaredoxin1 redox coenzyme for glutathione-dependent ribonucleotide reductase [b0849]
b3927 (glpF)	-1.52			-2.39	facilitated diffusion of glycerol [b3927]
b1743 (spy)		2.17	2.60		periplasmic protein related to spheroblast formation [b1743]
b1454 (yncG)				-4.19	putative transferase [b1454]
b2607 (trmD)	1.70	2.18	1.61	1.42	tRNA methyltransferase; tRNA [b2607]

[a], p-values are lower than 0.1

After all the *E. coli* treatments, an increase in expression in ribosomal subunit genes (*rps*, *rpm* and *rpl*) was evident. As can be seen in Figure 1b, the addition of the antimicrobials did not cause a growth arrest. Several authors have reported up- or down regulation of these genes under various stress condition. Bailey et al, (2009) observed a decrease in the expression of ribosomal subunit genes after an exposure of 30 min to triclosan. Downregulation of ribosomal protein genes (*rpl* and *rps*) was apparent in *Campylobacter jejuni*, after 15 min of exposure to osmotic stress, coincide with a temporary growth arrest, while the same genes later returned to steady-state or greater expression levels with the resumption of growth(Cameron et al, 2012). In fact, in our case, as possible to see in figure 1b, the addition of the antimicrobials did not cause an arrest of growth of *E. coli* (Fig 1B*).*

The perturbation in genes involved in fatty acids (FAs) biosynthesis, confirms what has been reported in Chapter 7. It is well known that one of the main targets of essential oils is the cytoplasmic membrane (Burt, 2004; Di Pasqua et al., 2007; Nazzaro et al., 2013). As reported in

Chapter 7, the addition of carvacrol, thyme EO and citral caused an increase of the unsaturation level as well as of trans-isomers in *E. coli* faty acids. The crucial role of unsaturated fatty acids has been reported in other studies and in response to several different stresses, including low or high growth temperatures, oxidative stress, acid stress and ethanol and salt addition stress the stress evoked by high pressure homogenization (Chatterjee et al. 2000; Streit et al. 2008; Montanari et al. 2010; Wu et al. 2012; Tabanelli et al. 2014). Moreover, the effect of essential oils and their components on the modulation of the synthesis of cyclic fatty acids is well documented in Gram-negative bacteria (Yuk and Marshall, 2004; Grogan and Cronan, 1997). Several authors (Fozo et al., 2004; Di Pasqua et al., 2006) have reported that also the increase in the length of fatty acids is another important membrane modification that might raise the survival in adverse environments e.g., of low pH or containing antimicrobial compounds.

The addition of natural antimicrobials also affected genes involved in energy metabolism. Picone et al. (2013) observed a shift from respiration to fermentation, upon carvacrol exposure. The inhibition of respiration in *E. coli*, together with K^+ leakage, was already observed by Cox et al. (1998) following to the exposure to sublethal concentrations of tea tree essential oil.

Interesting by we observed the upregulation of the *ompX* gene in *E. coli* treated with all the tested compounds (Table 8.3). The product of this gene seems to play a key role in the downregulation of porins in response to environmental stresses that induce its overproduction. On the other hand the effect of essential oils on the outer membrane of Gram-negative bacteria is well documented. Helander et al. (1998) showed the effect of essential oils on outer membrane permeability in Gram-negative bacteria: evidencing that monoterpenes components of essential oils such as carvacrol and thymol caused the disintegration of the outer membrane and release of outer membrane-associated material from the cells to the external medium.

In conclusion, it appears that the addition of sublethal concentrations of the natural antimicrobials employed here did not strongly affect global gene expression in *L. lactis* NZ9700 while these treatments caused a major response in *E. coli* for all antimicrobials used. In the latter, the modification of the expression in genes involved in fatty acid biosynthesis is in agreement with what is reported in literature and in Chapter 7 of this thesis and suggests that the cytoplasmic membrane of *E. coli* is the major cellular target of essential oils and their components.

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CHAPTER 9

CONCLUSIONS

The results obtained showed the beneficial effects of natural antimicrobials supplemented in dipping or washing phases for the improving of minimally processed fruits and vegetable safety and shelf-life. In fact, the addition of hexanal, citral and 2-(*E*)-hexenal, in sliced apples dipping solution, as well as thyme and oregano essential oils in lamb's lettuce washing solution, allowed to improve the shelf-life and safety of these kind of products packaged in ordinary atmosphere. In addition the results obtained showed that the shelf-life of minimally processed apples and lamb's lettuce can be further prolonged by combined use of natural antimicrobials in the dipping solution and packaging in modified atmosphere. Particularly, among the tested natural antimicrobials, 2-(*E*) hexenal in combination with citral or hexanal allowed the prolongation of apples shelf-life up to 35 days, without detrimental effects on safety and with a good retention of quality parameters such as colour, texture and volatile molecules profiles. Moreover, the addition of thyme essential oils in lamb's lettuce washing solution, after an optimization of the washing process and the use of an artificial modified atmosphere, permitted to prolong the shelf-life up to 14 days, without detrimental effects on the microbiological quality, the color and turgidity of products in comparison with chlorine.

Regarding the introduction of biocontrol agents in minimally processed products, the use of the strains *Lactobacillus plantarum* CIT3 and V7B3 respectively on apples and lettuce provided encouraging results concerning the safety and shelf life of minimally processed products considered. The results obtained are also more interesting because lactic acid bacteria are recognized as GRAS (Generally Recognized As Safe). The results also highlighted the importance of isolation and selection of biocontrol agents from commercial products of the same type. In fact, the good performance of the strains used was not only against deliberately inoculated pathogens, but also against spoilage microorganisms, these abilities have to be attributed to the capability of the strains to colonize the product and survive under stringent conditions of refrigerated storage. Also important is the capability of biocontrol agents to not affect the quality indexes of the product. The beneficial effects obtained by the use of the selected biocontrol agents were further increased combining them with natural antimicrobials. Also the use of the nisin producing *L. lactis* strain as biocontrol agent alone or in combination with natural antimicrobials showed enhanced potentials to increase the shelf-life and the safety of minimally processed apples and lamb's lettuce. Therefore, the selected biocontrol agents, and in particular its combination with

natural antimicrobials, may represent a good strategy to increase the safety and the shelf-life of minimally processed fruits and vegetables. Furthermore, since important health properties have been attributed to lactic acid bacteria, their use could also contribute to confer specific healthy properties to these products. However, the introduction of biocontrol agent can be further optimized, focusing on the level and mode of inoculation and to limit the negative effects observed on the color parameters.

Since the lack of knowledge of cell targets of essential oils represent one of the most important limit to the use of this molecules at industrial level, the results of this thesis may represent an useful tool to improve the knowledge about it. In fact, the results obtained evidenced that the tested molecules induced noticeable modifications of membrane fatty acid profiles and volatile compounds produced during the microbial growth. Although specific differences in relation to the species considered were identified, the tested compounds induced a marked increase of some membrane associated fatty acids, particularly unsaturated fatty acids, trans-isomers, and specific released free fatty acids. Also the trascriptome analyses of *E.coli* and *Lc. Lactis* cells, showed that the addition of sublethal concentrations of the natural antimicrobials employed here did not strongly affect global gene expression in *L. lactis* NZ9700 while these treatments caused a major response in *E. coli* K12 for all antimicrobials used. In the latter, the modification of the expression in genes involved in fatty acid biosynthesis suggesting that the cytoplasmic membrane of *E. coli* is the major cellular target of essential oils and their components.

Other regulation mechanisms, independently on the substances considered, involved in the stress response to the employed molecules were the energy metabolisms, purine/pyrimidine metabolism, fatty acid and phospholipid metabolism, protein synthesis and DNA metabolism. The comprehension of microbial stress response mechanisms can contribute to the scaling up of natural antimicrobials and bio-control agents at industrial level.

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