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THE USE AND CONTROL OF MICROORGANISMS TO IMPROVE THE QUALITY OF FOOD

IMPIEGO E CONTROLLO DEI MICRORGANISMI PER MIGLIORARE LA QUALITÀ DEGLI ALIMENTI

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ABSTRACT

In this present PhD thesis were examined and evaluated different methods for improving the quality of various food products.
The first work involved the study of antibacterial properties of the extract of the leaves of the plant *Cocculus orbiculatus*, against several serotypes of *Listeria monocytogenes*. The study of the composition of the extract showed the presence of 26 different compounds, thirteen of which have been extracted by hydro distillation and thirteen compounds by extraction with ethanol. Preliminary, also, the identification of the plant was carried out using molecular techniques. The results showed a bacteriostatic effect of the extract against the target organism under examination.

The work presented in the second chapter, is part of a research project on "Qualification of pig genetic resources aimed at enhancement of production for the Protected Designation of Origin of the dry-cured ham San Daniele Friuli (D.O.P. Prosciutto di San Daniele Friuli), the Protected Designation of Origin of the Gran Padano pig (D.O.P. Gran Suino Padano), and to the Protected Geographical Indication (I.G.P. Sauris) of fresh meat and sausages obtained and recognized with the brand AQUA of Friuli Venezia Giulia". It follows the chain of the pig from birth to slaughter. In particular, it was evaluated the impact of different pig breed on the final quality of the meat, using the same system of breeding and feeding. The breeds tested were Goland and Duroc per Large White mother. The evaluation was made by taking into account both, the meat (*M. longissimus dorsi* muscle) and San Daniele dry-cured ham, which is one of the transformed products. The results showed that there are no differences in the chemical-physical and microbiological characteristics of the meat as such, nor significant differences of physico-chemical characteristics of the dry-cured ham.

The third work involved the use of starter cultures of *L. curvatus* and *L. sakei* to improve the shelf-life of cooked ham, sliced and packaged under modified atmosphere packaging (MAP). The use of bioprotective cultures influenced the flavour, odour and colour stability of the sliced cooked ham. In addition, the growth of indigenous lactic acid bacteria was inhibited, preventing the formation of the white superficial slime and consequently extending the shelf-life of the product up to 30 days.

The fourth part of this thesis deals with the use of ozone for the decontamination of the equipment and tools used in food chain. Results obtained showed a great variability, depending on both, the type of the tested microorganisms (more or less-resistant, vegetative cells or spores) and the method used to deliver the ozone (wet and dry). As a result, an ozone concentration of 2 or 4 ppm allowed to obtain a reduction from 1 to 5 logs of the target microorganisms.

The last part of this work involved a mix of bioprotective cultures, which were *Lactobacillus carnosus*/*Lactobacillus sakei* + *Staphylococcus xylosus*, *Lactococcus lactis* subsp. *lactis*/*Lactobacillus sakei* + *Staphylococcus xylosus*, also in different ratio, for the improvement of the shelf-life of hamburger packed under modified atmosphere (MAP) and stored at 4 ± 2 °C. Data showed that inoculated cultures extended the hamburgers shelf-life by limiting the ammoniacal molecules production, the development of spoilage and pathogenic microorganisms, in particular *Brochothrix thermosphacta*, and avoiding the formation of the white slime on the surface of the hamburgers.

RIASSUNTO

Nella presente tesi di dottorato sono stati approfonditi e valutati metodi diversi per il miglioramento della qualità di diversi prodotti alimentari.
Il primo progetto ha previsto lo studio delle caratteristiche antibatteriche dell'estratto delle foglie della pianta *Cocculus orbiculatus,* nei confronti di diversi sierotipi di *Listeria monocytogenes*. Lo studio della composizione dell'estratto ha mostrato la presenza di 26 diversi composti, tredici dei quali sono stati estratti tramite idro-distillazione e tredici composti attraverso estrazione con etanolo. Preliminarmente, inoltre, l'identificazione della pianta è stata effettuata con tecniche molecolari. I risultati hanno mostrato un effetto batteriostatico dell'estratto nei confronti del microrganismo bersaglio in esame.
Il lavoro presentato nel secondo capitolo, rientra all'interno di un Progetto di Ricerca sulla "Qualificazione genetica dei suini finalizzata alla valorizzazione delle produzioni destinate alla D.O.P. Prosciutto di San Daniele Friuli, alla D.O.P. Gran Suino Padano, all'I.G.P. Sauris, alle carni fresche ed agli insaccati ottenuti e riconosciuti col marchio AQUA della Regione Friuli Venezia Giulia". Esso segue la filiera del suino dalla nascita fino al momento della macellazione. In particolare è stato valutato l'impatto della diversa razza suina sulla qualità finale della carne, a parità di sistema di allevamento ed alimentazione utilizzata. Le razze testate sono state Goland o Duroc x madre sempre Large White. La valutazione è stata fatta prendendo in considerazione sia la carne tal-quale (muscolo *M. longissimus dorsi*), che uno dei prodotti della trasformazione, il Prosciutto crudo San Daniele. I risultati mostrano che non ci sono differenze nelle caratteristiche chimico-fisiche e microbiologiche della carne tal quale, né differenze significative delle caratteristiche chimico-fisiche del prosciutto crudo.
Il terzo lavoro ha previsto l'utilizzo di colture starter di *Lactobacillus curvatus* e *Lactobacillus sakei* per il miglioramento della shelf-life di prosciutto cotto, affettato e confezionato in atmosfera modificata (MAP). Si è visto che le colture bioprotettive utilizzate hanno influenzato il sapore, l'odore e la stabilità del colore del prosciutto cotto affettato. Inoltre, hanno limitato la crescita di batteri lattici autoctoni, impedendo la formazione della patina bianca superficiale e allungando di conseguenza la shelf-life del prodotto fino a 30 giorni.
La quarta parte della presente tesi tratta dell'utilizzo dell'ozono per la decontaminazione di attrezzature e strumenti per uso alimentare. I risultati ottenuti mostrano una grande variabilità, che dipendono sia dal tipo di microrganismo testato (più o meno resistenti, cellule vegetative o spore), sia dal metodo di somministrazione dell'ozono (umido, secco). In ogni caso, l'utilizzo di 2 o 4 ppm di ozono hanno permesso di ottenere una decontaminazione variabile, da 1 e 5 log.
L'ultima parte ha previsto la valutazione di colture bioprotettive miste, quali: *Lactobacillus carnosus*/*Lactobacillus sakei* + *Staphylococcus xylosus*, *Lactococcus lactis* subsp. *lactis*/*Lactobacillus sakei* + *Staphylococcus xylosus*, anche con differenti rapporti delle specie in oggetto, per il miglioramento della shelf-life di hamburger confezionati in atmosfera modificata (MAP) e conservati a 4±2 °C. Dai dati è emerso che le colture inoculate possono prolungare la shelf-life, in quanto limitano la produzione di composti ammoniacali, sviluppi incontrollati di microrganismi alteranti e patogeni così come la crescita di *Brochothrix thermosphacta*, evitando la formazione di patina bianca sugli hamburger.

ABSTRAK

Dalam tesis program doktotal ini dilakukan pengujian dan evaluasi metode berlainan untuk meningkatkan kualitas beberapa produk makanan.
Karya pertama meliputi penelitian mengenai khasiat antibakteri dalam ekstrak daun *Cocculus orbiculatus* terhadap beberapa serotipe bakteri *Listeria monocytogenes*. Evaluasi komposisi kimia dari ekstrak tersebut menghasilkan 26 komponen berbeda, terdiri dari tigabelas komponen dihasilkan dengan teknik hidrodistilasi dan tigabelas komponen dihasilkan dengan metode ekstraksi etanol. Sebelumnya juga dilakukan identifikasi tanaman melalui pendekatan biologi molekuler. Hasil penelitian pada ekstak daun menunjukkan adanya aktivitas bakteriostatik terhadap target mikroorganisme yang diuji.
Penelitian dalam Bab 2, yang merupakan bagian dari riset pada proyek "Kualifikasi sumberdaya genetik pada babi, yang bertujuan untuk meningkatkan produksi ham dari Konsorsium Ham San Daniele Friuli (dengan Dilindungi Penunjukan Asal/Protected Designation of Origin-D.O.P), babi Gran Padano dengan label Dilindungi Penunjukan Asal/Protected Designation of Origin-D.O.P dan daging babi segar serta sosis dengan label Dilindungi Penunjukkan Geografi (Protected Geographical Indication /I.G.P. Sauris) di bawah merk AQUA Friuli Venezia Giulia". Dalam penelitian diikuti rantai hidup babi dari lahir hingga pemotongan. Khususnya, evaluasi pengaruh dua peranakan terhadap kualitas daging, sistem yang sama digunakan dalam evaluasi perkembangbiakan dan pemberian makanan. Dalam penelitian digunakan babi jantan Goland dan babi jantan Duroc yang dikawinkan dengan babi betina Large White. Evaluasi dilakukan pada daging pinggang (*M. longissimus dorsi* atau loin) yang merupakan produk transformasi dari ham dan ham San Daniele itu sendiri. Hasil penelitian menunjukkan tidak ada perbedaan pada karakteristik kimia-fisik dan mikrobiologis pada daging pinggang, maupun ham dari kedua jenis babi peranakan Goland dan Duroc.
Sedangkan penelitian dalam Bab 3, meliputi penggunaan kultur starter *Lactobacillus curvatus* dan *Lactobacillus sakei* untuk meningkatkan masa simpan irisan tipis ham panggang yang dan dikemas dalam Modifikasi Kemasan Atmosfer (Modified Atmosphere Packaging - MAP). Hasil penelitian menunjukkan bahwa penggunaan kultur bioproteksi mempengaruhi rasa, aroma dan memberikan warna yang stabil pada irisan ham panggang. Selain itu, kedua kultur tersebut juga menghambat pertumbuhan bakteri asam laktat tetap/normal (resident flora/indigenous/penghuni asli) pada ham, sebagai konsekuensinya mencegah pembentukan lendir putih dan oleh karena itu memperpanjang masa simpan produk hingga 30 hari.
Bagian keempat dari tesis ini berkaitan dengan pemanfaatan ozon untuk mendesinfeksikan peralatan dan alat yang bersentuhan dengan makanan. Hasil penelitian menunjukkan variasi luas, yang bergantung pada jenis mikroorganisme yang diuji (resistan atau kurang resistan, sel vegetatif atau berspora) dan metode administrasi ozon (metode basah atau kering). Setidaknya, penggunaan 2 atau 4 ppm ozon memungkinkan mendesinfeksi mikroorganisme dengan variasi penurunan jumlah bakteri sebanyak 1 hingga 5 log.
Pada bagian terakhir dari tesis ini, dilakukan evaluasi campuran kultur bioprotektif, yang terdiri dari *Lactobacillus carnosus*/*Lactobacillus sakei* + *Staphylococcus xylosus*, *Lactococcus lactis* subsp. *lactis*/*Lactobacillus sakei* + *Staphylococcus xylosus*, dalam ratio yang berbeda, yang bertujuan untuk meningkatkan masa simpan daging hamburger yang dikemas dalam Modifikasi Kemasan Atmosfer dan disimpan pada suhu 4±2 °C. Hasil menunjukkan bahwa kultur yang diinokulasikan pada daging hamburger dapat memperpanjang umur simpannya dengan membatasi produksi molekul amoniak, pertumbuhan mikroorganisme pembusuk dan pathogen yang tak terkendali, seperti *Brochothrix thermosphacta* dan mencegah pembentukan lendir putih.

RIASSUNT

In ta cheste tesi di dottorât e son stâs studiâs plui metodos pal mioramênt des qualitâs de robe di mangjâ.
In tal prin progjêt e son stades studiadis les caratteristichês antibatterichês dal estrât des fuees de plante *Cocculus orbiculatus* viers plui sirotipos di *Listeria monocytogenes*. El studi al mostre che e son stâs cjatâs 30 diviêrs composç, 13 cjatâs cule idro-distillazion e 17 cule estraziôn a etanolo. Par di pui, al inizi, e je stade fate le identificaziôn de plante cun tecniches molecolârs.
I risultâs e an mostrât un efiêt batteriostatic dal estrât viers il microrganismo bersaglio in questiôn.

Tal secont capitûl a si presente un lavôr che al fâs part dal progjêt di ricerche sule "Qualificazione genetica dei suini finalizzata alla valorizzazione delle produzioni destinât alla D.O.P. Prosciutto di S. Daniele del Friuli, alla D.O.P. Gran Suino Padano, all'I.G.P. Sauris, alle carni fresche ed agli insaccati ottenuti e riconosciuti col marchio AQUA della Regione Friuli Venezia Giulia". Al ten cont de vite dal purcìt di quânt che al nâs fin quânt che al vên macelât. E son stades studiades les diferences des diverses rasês di purcìs sule qualitât de cjâr final, cun sistemês di alevament e di alimentaziôn compains. Les rasês doprades e son *Goland o Duroc* x mari simpri *Large White*. Le valutaziôn e jê stade fate considerant sia le cjâr diretamenti (il muscul *M. longissimus dorsi*) che un prodôt lavorât come il Prosciût crut di San Denêl. Dai risultâs no si viodin diferences tes caratteristiches chimiches, fisiches e microbiologiches te cjâr e nancje tal prosciût crut.

Tal tierç lavôr e son stades doprades coltures starter di *L. curvatus* e *L. sakei* pal mioramênt de shelf-life dal prosciût cuet, tajât e impachetât in atmosfere modificade (MAP). Si e vedût che les coltures bioprotettives doprades e an cambiât el savôr, el odôr e le stabilitât dal colôr dal prosciût cuêt tajât. Par di pui e an fât calâ le crescince di batteris lattics autoctonos, fasint in mut che no si formi le lacje blancje sul prosciut e che si slungji le shelf-life fin a 30 dis.

El cuart toc di cheste tesi si ocupe dal ozono che al vên doprât pe decontaminaziôn dai imprêsç alimentars. Tai risultâs che son saltâs fûr a si viot che e jê une grose variabilitât; chest al dipent sia dal tipo di microrganismo testât (pui resistent o mancûl, cellules vegetatives o spores), sia di cemût che al ven doprât l'ozono (umit o sec). In ogni câs, 2 o 4 ppm di ozono e an permetût une decontaminaziôn che vâ dal 1 ai 5 log.

Tal ultin toc e son stades valutades les coltures bioprotettives mescedades, come: *Lactobacillus carnosus/Lactobacillus sakei* + *Staphylococcus xylosus, Lactococcus lactis* subsp. *Lactis/Lactobacillus sakei* + *Staphylococcus xylosus*, ancje cun rapuars differens tra chestes species, pal miorament de shelf-life dai hamburger confezionâs in atmosfere modificade e conservâs a 4 ± 2 °C. Dai datos si e vedut che les coltures e puedin slungjâ le shelf-life, dato che e limitin la produzion di armoniche mešedance e svilups incontrolâs di microorganisims alterans e patogenos e ancje le crescince di *Brochothrix thermospacta* evitânt che si crei le lacje blancje sui hamburger.

1. The in vitro antibacterial effect of *Cocculus orbiculatus* extract on the growth of *Listeria monocytogenes*.

1.1. Introduction

The moonseed family, Menispermaceae is distributed throughout, South America, Africa, and Asia. The members of this family are rich in alkaloids and are consequently used worldwide in traditional medicine and in the preparation of food and drink. The bisbenzylisoquinoline alkaloids are of interest to chemists, ethnobotanists and pharmacognosists, as many of these compounds exhibit antimalarial, antibacterial and cytotoxic activities. For example, bisbenzylisoquinolines found in the roots, stems and leaves of *Cyclea barbata* Miers are used for the treatment of fevers associated with malaria and possess other antimalarial activities (Guinaudeau et al., 1993; Lin et al., 1993). *Cissampelos sympodialis* Eichl is widely used in Brazilian folk medicine for the treatment of asthma, bronchitis and rheumatism and also has antileishmanial activity (Da Silva et al., 2012). *Stephania rotunda* Lour, has been primarily used in Cambodian folk medicine for the treatment of fever and malaria, and has also shown significant antiplasmodial activity against *Plasmodium falciparum* (article in press). Used as an ingredient in Thai cuisine, *Tiliacora triandra* has demonstrated antimycobacterial activity against multidrug-resistant of *Mycobacterium tuberculosis* (Sureram et al., 2012).

Figure 1.1 The tree of *Cocculus orbiculatus*

Figure 1.2 The young leaf of *Cocculus orbiculatus*

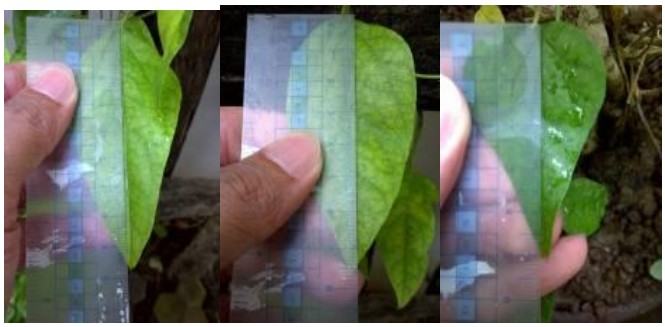

Figure 1.3 The leaves of *Cocculus orbiculatus*

Figure 1.4 The dry leaves of *C. orbiculatus*

This study focused on the compounds in the leaves of *Cocculus orbiculatus* (Figure 1.1-Figure 1.4), which appears to have antimicrobial activity. Gels (pudding) were prepared from the leaves for drinks or snacks and were observed daily. After 3 weeks of storage in closed containers in the refrigerator, no microbial activity, as evidenced by putrid odour, slimes or decay, was observed. The only activity observed was the gel drying. The gels were cooked without fire or boiling water. Approximately 300 g of leaves were ripped, and 1.000 ml of cold drinking water (warm or boiling water caused no jellying) was poured over the leaves, which were simultaneously squeezed by hand. The gels were then filtered through a sieve and refrigerated for 1 to 2 hours until they became firm and gelatinous (jelly). If the mix was allowed to stand at room temperature (27-29 ºC) after filtration, jellying required a longer time (approximately 4-5 hours) (Figure 1.5).

Figure 1.5 The preparation of gel from leaves *C. orbiculatus*.
The gel appearance after one week (7*) and three weeks (8*) of storage in closed container in the refrigerator.

C. orbiculatus has been used as an ingredient of *Fang-Ji*, which is a famous traditional Chinese medicine for the treatments of urocystitis, cold, malaria, fever and oedema (Chang and Wu, 2005). In addition, species of the genus Cocculus are used worldwide for the treatment of snake bites (Molander et al., 2012). Two amidic aporphine alkaloids isolated

from *C. orbiculatus*, (+)-laurelliptinhexadecan-1-one and (+)-laurelliptinoctadecan-1-one, were inactive against *Staphylococcus aureus* ATCC25932, *Escherichia coli* ATCC10536 and *Candida albicans* ATCC90028 (Nimgirawath et al., 2008).
The identification of entire plants and plant parts used and commercialised as food ingredients, medicines and antibacterial agents is necessary to provide precise and sufficient informations, to avoid confusion, and recognition of the correct plants can help confirm morphology-based identification. Some plants have similar morphological appearances in their leaves, and multiple synonymous names can lead to the misidentification and use of toxic species. At the beginning of this study, the leaves were identifed as *Cyclea barbata* L.Miers. This species is abundantly available and is used in traditional medicine for the treatment of fever, antimalarial and stomachache (Arkarapanthu et al., 2005). *Cyclea barbata* L.Miers is also used as an ingredient in gels and traditional commercialised drinks (street food), named 'Cincau'. The main ingredients of 'Cincau' are known to be *Cyclea barbata* and *Premna oblongifolia* Merr, but the use of *Cocculus orbiculatus* has not been reported.

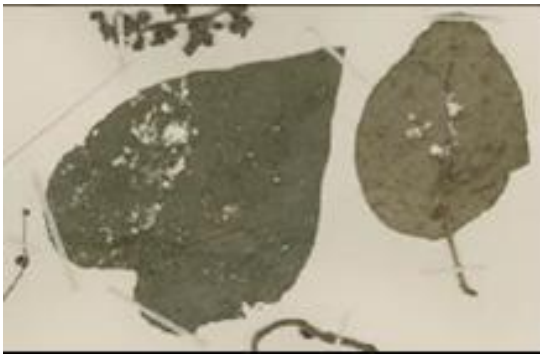

Figure 1.6 The herbarium of *Cyclea barbata* Miers L

Figure 1.7 The fresh leaves of *Cyclea barbata* Miers L

The dry leaves of *C. orbiculatus* (Figure 1.2 and Figure 1.3) have a different morphology than the herbarium specimen of *C. barbata* leaves (Figure 1.6), which are very similar to the leaves of *Cyclea elegans* (Figure 1.8), but less similar than *Cyclea polypetala* Dunn (Figure 1.9). Because of this confusion and uncertainty point, it is necessary to accurately identify the leaves of *Cocculus orbiculatus*.

Figure 1.8 The herbarium of *Cyclea elegans* King

Figure 1.9 The herbarium of *Cyclea polypetala* Dunn

Another studied case of plants with similar morphologies is that of *Illicum verum* (Chinese star anise) used for the treatment of infant colic, with its closely related species, *Illicum anisatum* (Japanese star anise), which has been documented to have both neurologic and gastrointestinal toxicities (Ize-Ludlow et al., 2004). Indeed, the substitution of plant parts from one species for another species based on the morphological appearance could cause serious adverse health effects.

One herbal term used in traditional Chinese medicine could actually refer to a different species. The primary source of this confusion is due to the pinyin (phonetic) spelling of the Chinese name used as the commercial trade name. The pinyin name for the commercial *Fang Ji* for *Aristolochia fangchi*: *Guang Fang Ji* is similar to "*Han Fang Ji* which is *Stephania tetrandra* (from the genus Menispermaceae). The substitution of *S. tetrandra*, which is used as an ingredients for herbal weight-loss, with *A. fangchi* may have occurred due to the similar pinyin name. The change in this herbal formula led to severe renal disease in consumers, which rapidly progressing to renal failure (the disease known as Aristolochic acid nephropathy (AAN), initially called as "Chinese-herb nephropathy" or CHN), thus necessitating dialysis or transplantation; in addition, *A. fangchi* has been reported to cause urothelial atypia (cell abnormalities in the bladder, ureters or renal pelvis) (Cosyns et al., 1999; Nortier and Vanherweghem, 2002; Vanherweghem et al., 1993). Plants members of the Aristolochia genus (family Aristolochiceae) contain aristolochic acid, while no aristolochic acid has been reported in the non-Aristolochia genus. The root, stem, leaves and fruit of *A. fangchi* could contain with aristolochic acid (Hashimoto et al., 1999), and in traditional medicine, Aristolochia species are used for the treatment of snake bites (Gupta and Peshin, 2012).

Thus, knowing only the trade name may lead to the incorrect identification of plant species. Some species are difficult to identify using only morphological characteristics (leaf shape and size) (Henderson and Martins, 2002), except by highly trained botanist. The DNA barcoding, a rapid and accurate molecular technique for species identification based on short and standardised DNA regions, provide a rapid and accurate, offer and would be an advantageous contribution to the discrimination of plant species (Feau et al., 2009).

The Consortium for the Barcoding of Life (CBoL) Plant Working Group has recommended the use of a universal DNA barcode for land plants and matK and rbcL as a core coding genes (Plant Working Group, 2009). In plants, amplifying a single barcode is not enough to differentiate between closely related species (Chase et al., 2007; Kress and Erickson, 2007; Lahaye et al., 2008; Taberlet et al., 2007).

Therefore, DNA sequence data from the internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) and two chloroplast DNA (cpDNA) regions, matK and rbcL, were used in this study for plant identification.

1.2. Materials and Methods

1.2.1. Plant material

C. orbiculatus leaves were collected at the beginning of October, 2010 from home gardens in Jakarta-Indonesia. After arrival in Italy, the leaves were dried at 50 ºC for 2 hours. The dried leaves were stored in the dark at room temperature until further usage and were ground into a powder with a ceramic mortar prior to the extraction. While, the fresh leaves were stored at -20 °C until further usage.

1.2.2. Supercritical CO_2 (SC-CO_2)

A laboratory-scale supercritical fluid extraction system was used for the SC-CO_2 extraction (Figure 1.10). The dried leaves were grounded with a ceramic mortar and transferred into the extraction column. Thirty grams of the powdered sample was loaded into the extraction vessel, and CO_2 was flowed through the system. The temperature set at 40 °C, the pressure was set at the 300 bar, and the carbon dioxide flow rate was 0.3 kg/hour, for 2 hours. Next, the extracted oil was separated by pressure reduction and collected in a cooler trap (-4 °C). The collected oil was stored at 4 °C until usage.

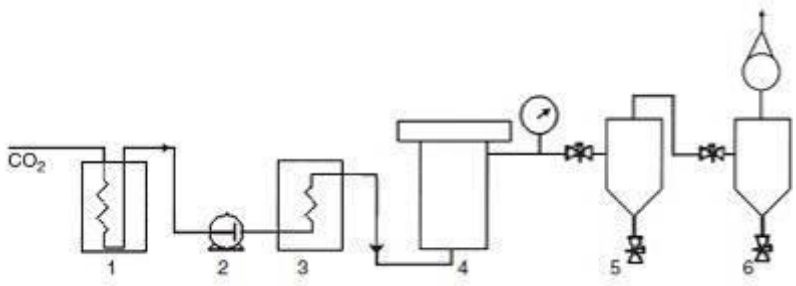

Figure 1.10 The scheme of the SC-CO_2 laboratory scale supercritical fluid extraction system: (1) solvent cooler; (2) pump; (3) heater; (4) extractor with capacity of 100 ml; (5,6) separator (Da Porto et al., 2009).

1.2.3. Preparation of extracts for analysis by GC-MS

Two different extraction methods and solvents (water and ethanol) were used to obtain the leaf extracts used for GC-MS analysis. The water and ethanol extraction methods were evaluated for the influence of their polarity in the extraction of compounds from the leaves.

1.2.4. Water extraction (Hydro distillation)

The water extraction was performed using a Clevenger apparatus. Ten grams of powdered dry leaves was loaded into the cell, which was then filled with 250 ml of milliQ-water (ultrapure laboratory-grade water), provided by Millipore (Q-POD, 0.22 μm). The heat-up time was 5 hours, the static extraction took place in closed-system valves, and the cell was rinsed (with 10 ml of hexane-Carlo Erba. Rodano. Italy). The essential oil was harvested, hexane was added to eliminate the water residue, and the oil was concentrated with N_2 gas.

1.2.5. Ethanol extraction

Five grams of powdered dry leaves was extracted with 37.5 ml of ethanol (Carlo Erba. Rodano. Italy) in a small Soxhlet apparatus for 3 hours. The extracts were harvested in ethanol and concentrated with N_2 gas.

1.2.6. Gas Chromatography-Mass Spectrometry (GC-MS)

The analysis was carried out using gas chromatography (Shimadzu. QP5050), fitted with a Shimadzu AOC-20i auto sampler and Shimadzu Class 5000 Chromatography Workstation software (Shimadzu, Italy) equipped with a fused silica capillary column (60 m long; 0.25 mm internal diameter fused silica capillary column; coated with a 0.25 μm film thickness). The extraction was injected into the GC-MS system in polar and non-polar fractions. High purity- helium (99.9995%) at a constant flow rate of 0.2 ml/min was used as the carrier gas, and the split ratio was 1/70 (v/v). For essential oils obtained by hydro distillation, an aliquot of 100 mg of extracted leaves was diluted with 1.0 ml of hexane, and 1.0 μl was injected into the GC-MS system. Meanwhile, 1.0 μl of the extract obtained by ethanol extraction was directly injected into the GC-MS system. The oven temperatures used for the analysis were set as follows: 45 ºC for 3 min, from 25 to 250 ºC at 3 ºC/min and holding at 250 ºC for 5 min. The injector, transfer line and ion trap temperatures were set at respectively, 250, 280 and 200 ºC. The electron impact (70 eV) spectra were recorded at 1 s/scan with a filament emission current of 10 μA. The identification of phytochemical compounds was based on both the comparison of the linear retention time (RT) (http://www.flavournet.org) and on the matching of mass spectra of the compounds with the reference mass spectra of two libraries (Wiley5 and Nist90) coupled with the software of GC-MS software and Adams' library (Da Porto et al., 2009).

1.2.7. Preparations of the bacterial cultures

Stock cultures of *L. monocytogenes* strains 1/2c, 150, and 2 XII from the culture collection of the Department of Food Microbiology at the University of Udine, were used in this study. These strains were maintained in Brain Heart Infusion broth (BHI) (Oxoid. Basingstoke. Hampshire. England) containing 10% (v/v) glycerol at -80 ºC. Prior to the experiment, stock cultures of *L. monocytogenes* were re-cultured in BHI broth and incubated at 37 ºC and centrifuged to obtain pellets. A stock culture (mother solution) was prepared by resuspending the pellets into 10 ml of sterile peptone water (8.5 g/l NaCl (J.T. Baker. Deventer. Holland) with 0.1 g /l peptone (Oxoid. Basingstoke. England). The optical density was measured at 600 nm (OD600) to obtain a value 0.1, which is corresponded to 10^7cfu/ml (colony forming unit). The mother solution was used directly used for inoculation of the leaf extracts into 96-wells plates. Viable counts were obtained by spread-plating 10-fold dilutions made in sterile peptone-water and plated onto BHI plates, which were incubated at 37 ºC for 24 h. After incubation, the number of bacteria was calculated and expressed as cfu/ml.

1.2.8. Assay of bactericidal effect

The bactericidal activity of the extract of *C. orbiculatus* extracts against *L. monocytogenes* was measured with liquid cultures in 96-wells plates. A two-fold dilution (1:1) of the extract was prepared by dissolving 1.5 mg of the extract (obtained by supercritical CO_2) in 1.5 ml of BHI broth. The dilutions were tested under the following 3 conditions:

- suspension of 1:1;
- suspension of 1:10 = 150 μl of 1:1 suspension and 1500 μl BHI broth;
- suspension of 1:100 = 15 μl of 1:1 aliquot and 1500 μl BHI broth.

Each series of aliquots (180 μl) was distributed into wells, and 20 μl of the mother solution was added to each well. Aliquots without the inoculum were treated as controls. BHI broth without extract or inoculum was included to verify the bactericidal effect on the growth of *L. monocytogenes*.

The 96-wells plates were read at 600 nm using an absorbance reader (Sunrise-Basic Tecan, TECAN Austria GmBH, Grodig, Austria), with the ambient conditions was set at 37 ºC for 24 to 48 h. The measured optical density values of the wells were transferred into an Excel worksheet.

1.2.9. Molecular identification

The molecular identification of the collected leaves was performed by DNA barcoding using the internal transcribed spacers (ITS) locus, maturase K (matK) and ribulose-1,6-bisphosphate carboxylase: RUBISCO (rbcL) chloroplastidial genes.
The DNA sequence data from the (ITS) of nuclear ribosomal DNA (rDNA) and two chloroplast DNA (cpDNA) regions (matK and rbcL) were used to identify the leaves.

1.2.10. Plant DNA extraction, barcode amplification and sequencing

DNA was extracted from fresh leaves using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, USA), according to the manufacturer's instructions. The DNA concentration for the extraction was measured with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific. EuroClone S.p.A Pero. Milan. Italy).

1.2.11. PCR and sequencing

The target of DNA regions (loci) were amplified using a GenAMP PCR System 9700 AB thermocycler, with different temperature profiles and PCR mix for each region.
The ITS region was amplified using the primers ITS4r (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) and ITS5af (5'-CCTTATCATTTAGAGGAAGGAG-3') (Stanford et al., 2000). The DNA was amplified by PCR using the following program: an initial activation step of 5 min at 95 ºC followed; by 8 cycles of 30 sec at 94 ºC, 30 sec at 63 ºC and 45 sec at 72 ºC, followed by 30 cycles of 30 sec at 94 ºC, 30 sec at 55 ºC, and 45 sec at 72 ºC, followed by a final termination of 10 min at 72ºC, and maintenance at 4°C. The PCR for the ITS region was performed in a 25 µl reaction volume containing 1 µl of the DNA template, 0.2 mg/ml BSA, X1 Buffer, 2% DMSO, 0.25 mM dNTP mix, 2.5 mM MgCl2, 0.3 µM ITS4 primer (Sigma Aldrich. Milan. Italy); 0.3 µM ITS5a primer (Sigma Aldrich. Milan. Italy), 1.5 U Taq polymerase (AmpliTag DNA Polymerase. Applied Biosystem. Grand Island. USA) and 25 µl of sterile Milli-Q water.
The matK region was amplified using primers 3F_KIMf (5'-CGTACAGTACTTTTGTGTTTACGAG-3') (1 µM) (Sigma Aldrich. Milan. Italy) and 1R_KIMr (5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3') (1 µM) (Sigma Aldrich. Milan. Italy) (Hollingsworth et al., 2009) in 20 µl reaction mixtures containing 1 µl of the DNA template, 0.1 mg/ml BSA, X1 Buffer, 4% DMSO, 0.20 mM dNTP mix, MgCl2 (at 3 different concentrations, 1.5 mM, 3.0 mM and 4.5 mM), 2.0 U Taq polymerase (AmpliTag DNA Polymerase. Applied Biosystem. Grand Island. USA), and sterile 20 µl of sterile Milli-Q water. The amplification was carried out with a GenAMP PCR System 9700 AB thermocycler, using the following conditions: a first cycle of 5 min at 95ºC followed by 35 cycles of 30 sec at 94 ºC, 20 sec at 52 ºC, and 50 sec at 72 ºC; followed by a final termination for 5 min at 72 ºC, and maintenance at 4 ºC.
The rbcL region was amplified by PCR in a final reaction volume of 20 µl. The reaction mixture contained: 1 µl of the DNA template, X1 Buffer, 0.4 mM dNTP mix, 2.5 mM MgCl2, 0.5 µM rbcLa f primer (5'-ATGTCACCACAAACAGAGACTAAAGC-3') (Kress and Erickson, 2007), 0.5 µM rbcLa_rev primer (5'-GTAAAATCAAGTCCACCRCG-3') (Hollingsworth et al., 2009), 1.0 U Taq polymerase (AmpliTag DNA Polymerase. Applied Biosystem Grand Island. USA), and 20 µl of sterile Milli-Q water. The amplification was performed with a GenAMP PCR System 9700 AB thermocycler with an initial activation

step for 5 min at 95 ºC followed by 5 cycles of 30 sec at 94 ºC, 1 min at 55 ºC, and 1 min at 72 ºC, followed by 30 cycles of 30 sec at 94 ºC, 1 min at 54 ºC, and 1 min at 72 ºC with a final termination of 10 min at 72 ºC, and maintenance at 4 ºC.
Negative (no-template) and positive (DNA of *Helianthus spp*) controls were included in each PCR run.
After amplification, the DNA products were subjected to electrophoresis in a 0.8% agarose gel using 1X TBE buffer (Sigma Aldrich. Milan. Italy) containing gel red (as a substitute for ethidium bromide) and were visualised and photographed under UV light in a chamber (Syngene). A 100 bp DNA ladder (N3231L. New England BioLabs.Inc) was used as the fragment size marker.
The amplification (PCR) products were purified with a DNA purification kit QIAquick (PCR Purification Kit 250. Qiagen GmbH. Hilden. Germany). Reactions including 15 µl of each purified PCR product and the same primers used in the PCR were prepared for sequencing following the Sample Submission Guide for custom DNA sequencing in the tube format and were sent to the sequencing facility at eurofins ǀmwg operon (www.eurofinsdna.com). The representative nucleotide sequences of the ITS region and two cpDNA (matK and rbcL) were used. Plant identification was aligned and attempted using the BLAST program from NCBI. Matching accessions were those presenting the highest maximum score values.

1.3. Results and Discussion

1.3.1. Bactericidal activity

The oil of *C. orbiculatus* has demonstrated bactericidal activity against *L. monocytogenes* strains 1/2c, 150 and 2XII as reported in Figure 1.11-Figure 1.13.
According to the trend of the growth curve, a difference in the level of contamination was already observed at time 0. There were no growth on *L. monocytogenes* strain 1/2c and 150 at a concentration 1:1 and this condition remained constant over 24 h. The growth of *L. monocytogenes* strain 1/2c at a concentration of 1:10 was slowed down, but at a concentration of 1:100 was not affected by the presence of the oil.
The oil only slowed down the growth of *L. monocytogenes* strain 150 at a concentration of 1:10 and 1:100.

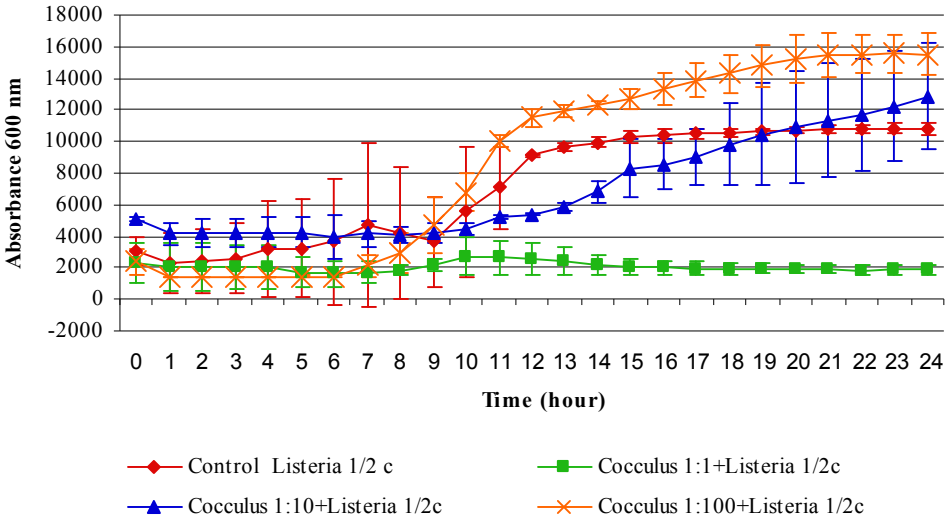

Figure 1.11 The bactericidal activity of different concentrations of the extract *Cocculus orbiculatus* on *L. monocytogenes* strain 1/2c

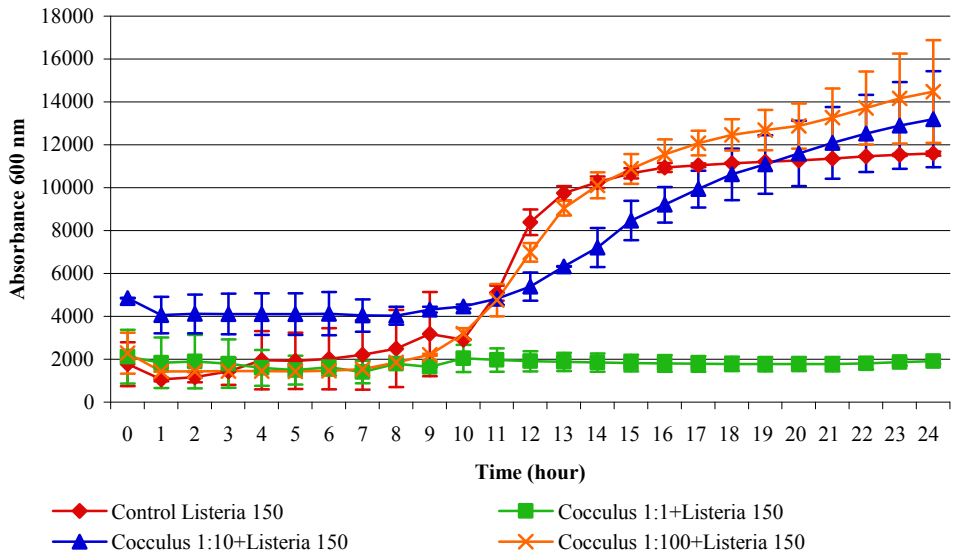

Figure 1.12 The bactericidal activity of different concentrations of the extract *Cocculus orbiculatus* on *L. monocytogenes* strain 150

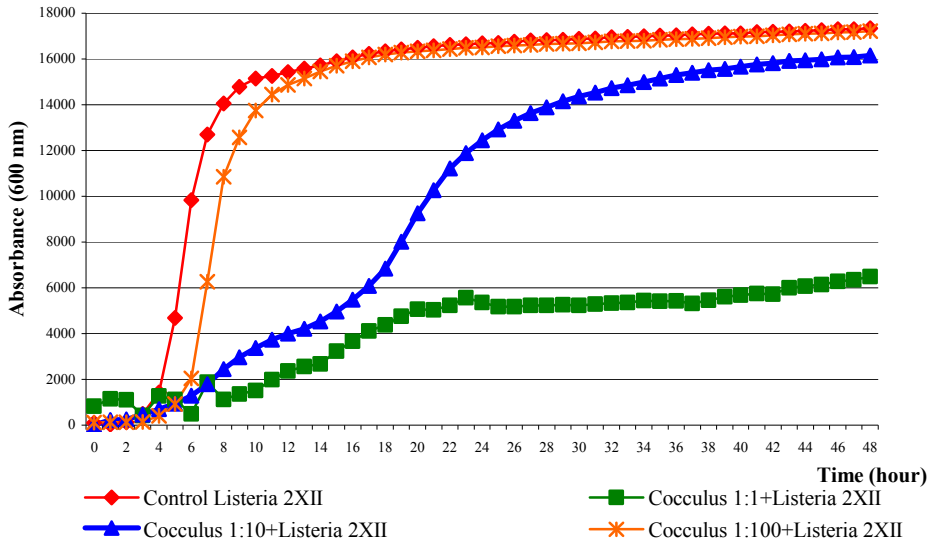

Figure 1.13 The bactericidal activity of different concentrations of the extract *Cocculus orbiculatus* on *L. monocytogenes* strain 2XII

The oil at a concentration of 1:1 was only slowed down growth of *L. monocytogenes* strain 2XII. The growth of strain 2XII at a concentration of 1:10 was also only slowed down, while at a concentration of 1:100 was not reduced or even slowed down.

1.3.2. GC-MS analysis

The GC-MS analysis of the n-hexane fraction from the hydro distillation enabled the identification of 13 components, which were found to be a complex mixtures of alkaloids, aromatic compounds and pheromones; their retention times and per cent areas (area%) given in Table 1.1. The major constituents of the essential oil, extracted by hydro distillation were found to be citronellyl butyrate (46.14%); 9,12-octadecadienal (15.60%); z-z-z-9,12,15-octadecatrienal (20.80%) and ethyl caproate (9.32%). As shown in Table 1.2, the chemical components of the samples obtained by ethanol extraction were more abundant and included 17 components (four of the components are the same with the components identified by hydro distillation), the major constituents of which were glyceryl alcohol (24.58%), palmitic acid isopropyl ester (20.28%), z-z-z-9,12,15-octadecatrienal (16.47%), trans-farsenol (9.57%), 9,12-octadecadienal (8.60%) and citronellyl butyrate(7.66%). Each of the components have been reported and demonstrated to possess a number of interesting and potent biological activities such as cytotoxic effects, antiplasmodial and antibacterial effects. Magnoflorin iodine (0.17%), has been shown to have in vitro antiplasmodial, antiamoebic and cytotoxic activities against *Plasmodium falciparum* (multidrug-resistant strain K1).
The bisbenzylisoquinoline alkaloid berbamine (BBM) (3.00%), has been used as an anticancer drug in China and for the treatment of various aliments in Europe. Berbamine derived from the plant *Berberis amurensis* (Xiaboan) (Cui et al., 2012), has been reported to

have moderately cytotoxic activities (IC50:0.968 μM) and high antiplasmodial activity against *Plasmodium falciparum* (Wright et al., 2000), to induce apoptosis in human hepatoma, colorectal adenocarcinoma and leukaemia cells and to suppress the growth of human A549 lung cancer cells (Duan et al., 2010). For decades, berbamine, a small compound naturally occurring in *Berberis amurensis*, has been known to stimulate normal hematopoiesis and to enhance immune function in cancer patients. Berbamine has also been shown to excert cytotoxic activity against the chronic myeloid leukaemia (CML) cell line KU812 and to suppress the proliferation and induced apoptosis of KU812 leukaemic cells. Most CML patients have abnormally high basophil counts in their peripheral blood or bone marrow, especially in those suffering blast crises. KU812 is a human basophilic leukaemia cell line established from the peripheral blood of a patient in CML blast crisis. These findings suggest that berbamine is a promising drug for the treatment of patients in advanced stages of CML (Liang et al., 2011).

Berbamine, from the Chinese herbs, *Berberis amurensis*, has potent anti-inflammatory properties and can suppress the growth, migration and invasion of highly-metastatic human breast cancer cells (Weber et al., 2012). In traditional Chinese medicine, berbamine is used primarily to stimulate normal haematopoiesis in clinic. The anticancer activity of berbamine against human hepatocellular carcinoma (HCC) HepG2 has been demonstrated in vitro in a study in which, berbamine decreased the cell growth in a dose-dependent manner with an IC50 value of 34.5 ± 0.5 μM (Wang et al., 2009).

Papaverine (0.19%), a major benzylisoquinoline alkaloid in opium poppy (*Papaver somniferum*. L), is used as a vasodilator (an agent to widen blood vessels), as a smooth vessel relaxant in microsurgery, to treat erectile disorders, and as an antispasmodic (in the prevention of spasms) for the treatment of intestinal and urinary tract spasms, bronchial asthma, renal and biliary colic, pulmonary arterial embolism, migraine headaches and schizophrenia (Desgagné-Penix and Facchini, 2012). Papaverine is a pharmaceutically significant alkaloid also identified in empty poppy capsules (poppy straw) of *Papaver somniferum*. L (opium poppy)(Stranska et al., 2013).

Papaveraldine (0.54%), is a product of the oxidation of papaverine and is biologically similar to papaverine in some respects. Papaveraldine has shown activity against barium chloride- and acethylcholine-induced spasms, is protective against histamine-induced bronchospasms, and has little or no analgetic activity after oral administration in rats (Weisbach et al., 1968).

(S)-Isoboldine (2.11%) is also known as Isoboldine, (+)-Isoboldine, 2, 10-Dimethoxy-6aalpha-aporphine-1,9-diol, C09541, 3019-51-0 (PubChem, 2012d). Isoboldine ($C_{19}H_{21}NO_4$) was also isolated and indentified from the leaves of *Cocculus laurifolius* D.C, using chloroform extraction. The hydrodistilled and ethanol extracts of *C. laurifolius* have killing activity against *Xanthomonas campestris*, *Bacillus subtilis* and *Streptomyces albogriseolus* subsp.89 (Tsakadze et al., 2005).

The methanolic extract of *Beilschmiedia alloiophylla* and hexane extract of *Beilschmiedia kunstleri* also isolated isoboldine, which is exhibited acetylcholinesterase (AChE) inhibition activity at IC_{50} values 10±0.6 μM. The application of AChE inhibitor is to treat Alzheimer's disease, anti-α-glucosidase (yeast) with IC_{50} value 130.8±3.4 μM (the α-glucosidase inhibitor is used in the treatment of type 2 diabetes), anti-leishmanial with IC_{50} value 35.0 μM and anti fungal activity against *Candida albicans* with minimum inhibition concentration: 32.0 μg/ml(Mollataghi et al., 2012).

The alkaloid, (S)-Isoboldine was identified from heavenly bamboo (*Nandina domestica* Thumb), too. The fruit of this plant has long been used to treat asthma, whooping cough, pharyngeal tumours and uterine bleeding in Japan. While in China, the leaves and stems are used as medicine (Iwasa et al., 2008).

The bisbenzylisoquinoline alkaloid bisnorthalrugosine (0.18%) is known to possess tumour inhibitory activities. Bisnorthalrugosine was also identified in the extract of the stems of *Pycnarrhena ozantha* Diels (Abouchacra et al., 1987).
Isopinocampheol (0.79%) is known to possess insect antifeedant activities (Miyazawa et al., 1997; Noma et al., 2010) and is one of the components of hyssop (*Hyssopus officinalis*) oil. When used alone, isopinocampheol reduced the growth of the pathogenic plant fungi, *Pyrenophora avenae*. When used in combination with other components of hyssop oil such as L-bornyl acetate and pinocamphone, isopinocampheol has been shown to completely inhibited fungal growth (Letessier et al., 2001).
Furfuryl alcohol, which is also one the Maillard reaction products has been found can become a DNA-reactive intermediate that has a mutagenic, toxic and carcinogenic effects (Akillioglu et al., 2011). A study done by Sujatha has shown cytotoxic potential of furfuryl alcohol (Sujatha, 2008).
The biological activities of tri-O-ethylcycleacurine (0.33%) are not yet known.
Trans-geraniol (0.34%), which is also as one of the main constituents of the oil of lemon grass (*Cymbopogon flexuosus*) has antimycotic activity against the human pathogenic fungi, *Fusarium oxysporum* and *Trichophyton mentagrophytes* (Pandey et al., 2003). Trans-geraniol or geraniol is used to flavour beverages, candies, ice creams and baked goods and has acaricidal activities against the food mite, *Tyrophagus putrescentiae*, effectively controlling its populations based on the lethal dosage (LD50) value of geraniol (1.95 μg/cm3). As one of the most toxic compounds in the oil of *Pelargonium graveolens*, geraniol may be useful to replace benzyl benzoate, the commercial acaricide (Jeon et al., 2009).
Trans-geraniol is the principal compound (16.54% of the concentrated essential oil) in the essential oil of *Cymbopogon distans* and showed strong repellent activity against the booklouse (*Liposcelis bostrychophila*) at the lowest assayed concentration tested (1.6 nl/cm2). Significant (44%) repellency was still observed at 4 h after exposure. At a concentration of 26 nl/cm2, trans-geraniol exhibited the same level of repellency as the commercial repellent N,N-diethyl-3-methylbenzamide (DEET) against booklouse. The crude essential oil of *C. distans*, containing 0.16 nl/cm^2 of trans-geraniol, exhibited strong repellency against the red flour beetle (*Tribolium castaneum*) at 4 h after exposure, while DEET showed repellency only at the highest concentration of 16 nl/ cm^2. Trans-geraniol was also demonstrated to repel mosquitoes, sand flies, human body lice, aphids and stored-produced insects (Zhang et al., 2011).
Trans-2-decenal (1.40%), which is another component of coriander essential oil from *Coriandum sativum*, trans-2-decenal, showed 100% nematicidal activity against the pine wood nematode *Bursaphelenchus xylophylus* at 2.0 mg/ml (Kim et al., 2008). The biological activity of trans-2-decenal, seems to pose a health risk to humans. Epidemiological studies indicated that there is an increased risk of respiratory tract cancer among cooks and bakers, and the cooking oil fumes are believed to cause this risk. When analysed and identified by GC-MS, the cooking oil fumes were found to contain trans-2-decenal. The cytotoxicity of the methanolic extract of fumes from heated soybean oil, sunflower oil and lard, was evaluated using MTT assays (mitochondrial-dependent reactions used to determine cellular respiration as an indicator of cell viability) and generally showed cytotoxicity towards human A-549 lung carcinoma cells at concentrations of 100-200 μg/ml. The cell viability decreased with increasing concentrations of oil fumes from soybean oil, sunflower oil and lard (Dung et al., 2006).
Decanal (0.30%), also known as decanaldehyde, capraldehyde, 1-decanal and n-decanal, which is also one of the main constituents of cold-pressed terpeneless Valencia oil. The antimicrobial activity of decanal, expressed as inhibition zones by the vapour diffusion

assay, has been demonstrated against *Listeria monocytogenes* and *L. innocua* (Shannon et al., 2011). Decanal from the sweet orange oil, has showed inhibition and bactericidal activity against *Escherichia coli* (ATCC 10536), *Staphylococcus aureus* (ATCC 29213), *Saccharomyces cerivisiae* (ATCC 9080), *Penicillium citrinum* (ATCC 9849) and *Aspergillus niger* (ATCC 16404) (Liu et al., 2012). n-decanal was identified as one of the volatile constituents from the essential oil of propolis showed antimicrobial activity against *Enterobacter cloaceae* (ATCC 13047), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *S. epidermis* (ATCC 12228), *Pseudomonas aeruginosa* (ATCC 227853), *Klebsiella pneumoniae* (ATCC 13883), *Candida albicans* (ATCC 10231), *C. tropicalis* (ATCC 13801), *C. glabrata* (ATCC 28838) (Melliou et al., 2007).

C. orbiculatus contains the fatty acid n-capric isoprophyl ester (0.31%), also known as capric acid, decanoic acid, isopropyl decanoate and isopropyl caprate (PubChem, 2012e). Capric acid, is a second medium-chain fatty acid and is an important components of a normal diet. The manipulation of capric acid content of the diet may influence bone resorption, bone formation and bone mass/density and reduce bone turnover. Recent studies have shown that fatty acids have important therapeutic implications for the treatment of bone diseases, associated with excessive osteoclastogenesis, such as induced rheumatoid arthritis and osteoporosis (Park et al., 2011).

Decanoic acid was also identified in the herb oil of *Ducrosia anethifolia* (DC.) Boiss. (family Apiaceae) and is produced by hydrodistillation of the leaves, stems and fruit. In Iran, this plant is used to improved the smell of foods and drinks, and it is used in traditional medicine it is used to cure catarr, headaches and backaches. In Karoon, the seeds are given to children as an infusion in cases of colic (Janssen et al., 1984).

When used alone at 45.3 μg/ml, capric acid extracted from the non-pathogenic yeast *Saccharomyces boulardii*, showed an inhibitory activity against hyphae formation of the yeast pathogen *Candida albicans* (Murzyn et al., 2010).

Dietary strategies to reduce methane production from dairy industries, showed that the addition of 40 mg of capric acid on the β-cyclodextrin carrier when added to 60 ml of medium (0.7 g/l or 139 g/kg of substrate) induced a 60% reduction in methane production in vitro (van Zijderveld et al., 2011).

A comparative evaluation of the dietary effects of soyphospolipids containing at 10% capric acid, significantly decreased the serum levels of total cholesterol (TC), triglycerides (TG), very low density lipoprotein (VLDL)-cholesterol and LDL-cholesterol in the rats. There was also a significant decrease in the serum levels of high density lipoprotein (HDL)-cholesterol level in the rats feds soybean oil with soyphospholipids containing 5% capric acid (Dasgupta and Bhattacharyya, 2009).

The medium-chain fatty acid of capric acid (C10), exhibited inhibition against the oral bacteria *Streptococcus mutans*, *Streptococcus gordonii*, *Streptococcus sanguis*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Huang et al., 2011).

Dietary supplementation with 10 g/kg of capric acid significantly reduced the number of coliform bacteria in the faeces of weaned rabbits (35 days old) artificially infected with 10^9 enteropathogenic *Escherichia coli* O103 cells. At 6 days post-inoculation, capric acid decreased the faecal output of coliform from 10.18±0.62 to 8.04±0.50 log cfu/g. When the surviving rabbits were slaughtered at 53 days of age, the number of coliform bacteria in the caecum was significantly reduced from 8.71 log cfu/ml (before infection) to 5.83 log cfu/ml (after infection) (Skřivanová et al., 2008).

Ethyl caproate (9.32%), is recognised as an important apple-like flavour in sake and is one of the most important sake flavour components produced by the yeast, *Saccharomyces cerevisiae* (Arikawa et al., 2000; Aritomi et al., 2004; Asano et al., 2000; Furukawa et al., 2003). An appropriate concentration of ethyl caproate works to high rating in sensory

evaluations of sake. The interesting fact is that ethyl caproate work synergistically with capric acid and is a precursor of ethyl ester in sake; thus, the content of caproic acid in sake is highly correlated with that of ethyl caproate (Kuribayashi et al., 2012).
2-undecanol (4.23%), also known as methyl nonyl carbinol, 2-hendecanol and undecan-2-ol (PubChem, 2012a). 2-undecanol is the major components in the mandibular glands of ants from species *Tetramorium termitodium* (Longhurst et al., 1980). 2-undecanol was identified in the essential oil extracted from the fresh leaves of wild and cultivated plants of *Ruta chalepensis*, too. Its essential oil showed larvicidal and repellent activity against the Asian tiger mosquito, *Aedes albopictus*. The medical importance of *A. albopictus* is mainly due to its ability to serve as a vector and transmit many viruses, including dengue, yellow fever, West Nile, Japanese encephalitis and St. Louis encephalitis and chikunguya (Conti et al., 2012).
Methyl pentadecanoate, also known as pentadecanoic acid, has been identified as a potential therapeutic candidate in traditional Chinese medicine (TCM) and can reduce the activities of phospodiesterase 4D (PDE4D) and 5-lipoxygenase-activating protein (ALOX5AP), as two of the major risk factors in ischemic stroke risks. Uncontrolled PDE4D activity often leads to cAMP-induced stroke and cardiovascular diseases. In addition, the overexpression of ALOX5AP has been shown to play a major role in the inflammatory pathway that could induce the development of atherosclerosis and stroke (Chen et al., 2011). Gas chromatography/mass spectrometry analysis of the flesh and peel extract of mango (*Mangifera indica* L.) detected high amounts of pentadecanoic acid and (Z,Z)-9-12-octadecadienoic acid. The peel extract was found to have antioxidant activity and significantly inhibited the proliferation of human cervical carcinoma Hela cells in a dose-dependent manner (Ali et al., 2012).
Glyceryl alcohol (24.58%), also known as glycerol, glycerin, glycerine and 1,2,3-propanetriol (PubChem, 2012c). Glycerol is used as a plasticizer for the formulations of the edible coatings or films for food packaging (Flores et al., 2007; Núñez-Flores et al., 2013; Ramos et al., 2012).
Lauraldehyde (2.6%), also known as dodecanal, lauric aldehyde, lauryl aldehyde, dodecyl aldehyde, dodecanalaldehyde, n-dodecanal and 1-dodecanal, has also been identified in the essential oil of *Coriandum sativum* L. (Apiaceae), obtained by hydro-distillation. Lauraldehyde has demonstrated antibacterial activity against gram-positive (*Staphylococcus aureus* and *Bacillus* spp.) and gram-negative (*Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumonia*, *Proteus mirabilis*) bacteria and antifungal activity against *Candida albicans* at a concentration of 32.5 x 10^2 µg. The minimum inhibitory concentration (MIC) of oil for gram-positive bacteria was 108 mg/ml and that for gram-negative bacteria ranged from 130 to 217 mg/ml. The MIC for *C. albicans* is 163 mg/ml (Matasyoh et al., 2009).
The formulation containing dodecanal showed the ability to prevent wood decay by inhibiting the growth of the brown-rod fungi, *Tyromyces palustris* and the termite, *Captotermes formosanus*, in the decay resistance test (Kartal et al., 2006).
The herb oil of *Ducrosia anethifolia* (DC.) Boiss., also contains of n-dodecanal as one of its main components in addition to decanoic acid, as mentioned above. Antimicrobial screening experiments measuring the inhibition zones due to the herb oil and its components have shown that n-dodecanal is mainly active against *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*, and the yeasts, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Epidermophyton floccosum* (Janssen et al., 1984).
Olfactometer experiments have shown that dodecanal, a sex pheromone produced in the cocoons of the female parasitic wasp *Cephalonomia tarsalis*, allows male wasps to find their mates. *C. tarsalis*, is a specialist parasitoid of the larvae of the sawtoothed grain beetle, *Oryzaephilus surinamensis*, and the merchant grain beetle, *Oryzaephilus mercator*. Wasps use chemical cues to locate their host larvae attack, and paralyse the host, and oviposit on

the outside of the host. The larvae feeds ectoparasitically on the host and pupates in a cocoon next to it. Because both host species (*O. surinamensis* and *O. mercator*) are worldwide pests of stored grain and other commodities, *C. tarsalis* is used for their biological control in Europe (Collatz et al., 2009).

The bisbenzylisoquinoline alkaloid 12-O-dimethylcoclobin (0.39%) has been isolated from the stem bark of *Guatteria guianensis* (family Annonaceae) (Berthou et al., 1989). The constituent at C(12) is phenolic (Schiff, 1999), but its biological activity from *G. guianensis* has not been reported. Some Guatteria species have been used in folk medicine to treat various diseases and malaria (Coe & Anderson. 1996, Mahiou. 2000). Some researchers have evaluated the antiplasmodial activity of the isoquinoline alkaloid, aphorphine, in vitro. Alkaloids isolated from the dry-leaves of *G. australis*, have been shown to be active against a chloroquine-resistant (K1) strain of *Plasmodium falciparum* with an IC_{50} of 0.3 μg/ml and have demostrated activity against the chloroquine-sensitive strain of *Plasmodium falciparum*, with an IC_{50} of 1.8 μg/mL of *Plasmodium falciparum*. The alkaloids of *G. australis* represent an interesting target for the isolation of new antimalarial agents. The methanol extract of *G. amplifolia* presented an IC_{50} of 1.9 for the sensitive strain of *P. falciparum* and 1.5 μg/ml for the sensitive and resistant strains of *P. falciparum*, respectively. The ethanol extract of *G. schomburgkiana* was active only when tested against the resistant strain of *P. falciparum*, showing an IC_{50} of 4.0 μg/ml. The total alkaloid extract from *G. lehmannii* demonstrated no activity against a chloroquine-resistant strain (IC_{50} = 143.5 μg/ml), while the majority of the bisbenzylisoquinoline alkaloids isolated from *G. boliviana* were highly active against both chloroquine-resistant and -sensitive strains of *P. falciparum,* with IC_{50} values lower than 0.2 μg/ml (Fischer et al., 2004).

G. australis also showed effective against *Tripanosoma cruzi*, a parasite causing infection of Chagas disease or American trypanosomiasis (Tempone et al., 2005). The alkaloids fraction of *G. foliosa* Benth L. and *G. schomburgkiana* Mart showed leishmanicidal activity, in vitro, against protozoa *Leishmania amazonensis*, *L. braziliensis* and *L. donovani*. In which these species of genus Leishmania are causative agents of human leishmaniasis (Rocha et al., 2005).

Citronellyl butyrate (46.14% by hydrodistillation and 7.66% by hexane extraction),also known as rhodinyl butyrate, citronellyl n-butyrate, natural rhodinol, butanoic acid, and citronellyl butanoate, is used widely in the food, cosmetic and pharmaceutical industries to provide flavour and fragrance. This compound, used for its flowery-fruity notes, is produced by *Mucor miehei* (De Castro et al., 1997; Shieh and Lou, 2000), *Rhizopus* sp (Alves Macedo et al., 2003; Melo et al., 2005), *Candida cylindraceae* (Walter et al., 1989), *Candida antarctica* (Lozano et al., 2007), *Pseudomonas sp* (Yee et al., 1997), *Candida rugosa*, *Pseudomonas fluorescens* and *Rhizopus japonicus* (Wang and Linko, 1995).

The antimicrobial activity of citronellyl butyrate, expressed as inhibition zones by the agar diffusion disc method, has been demonstrated against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*e, *Salmonella spp* and *Candida albicans*, although no inhibition was observed against *Escherichia coli* and *Proteus vulgaris* (Sestraş et al., 2012).

Citronellyl butyrate has also been identified in the essential oil (herb oil) of the rose-scented geranium *Pelargonium sp.* (family Geraniaceae), which is a multi-harvest, high-value, aromatic plant. Its essential oil is widely used in the fragrance industry for aromatherapy and for the extraction of commercial rhodinol (mixture of linalool, citronellol, and geraniol) (Rajeswara Rao et al., 2002; Rajeswara Rao et al., 2000).

There is an increasing agricultural need for the development of new pest control methods that are nonlethal and safe for the environment and target organisms. At low concentrations (0.1 ppm), citronellyl butyrate has shown promise as a repellent, with activity against the exotic avian pest, European Starling (*Sturnus vulgaris*) in North America. Treatment of the

feeds with citronellyl butyrate for European Starling (at a concentration of 0.1 parts citronellyl per 1 part ethanol) decreased the consumption by approximately 90% (Hile, 2004).

Palmitic acid isopropyl ester (20.28%), also known as isopropyl palmitate, hexadecanoic acid, 1-methylethyl ester, isopropyl hexadecanoate (PubChem, 2012f). Isopropyl palmitate also yielded from fractionation of palmitic acid of palm oil and is used in bath oils, aerosol hair conditioners, fluid hair conditioners, hair grooming aids, hand creams, lotions and rouges (Kalustian, 1985). Isopropyl palmitate used as an excellent solvent for products contain with mineral oil, silicone and lanolin. Its good characteristic in absorption make isopropyl palmitate useful in cosmetics and tropical products which are require a good absorption through the skin (Bhatia et al., 2006).

trans-Farnesol (9.57%), also known as farnesol, farnesyl alcohol, trans,trans-Farsenol and (E,E)-Farnesol (PubChem, 2012b) which is also one of the main constituents of the flower essential oil of German chamomile (*Matricaria recutita*). The antimicrobial activity of decanal, expressed as inhibition zones by the vapour diffusion assay, showed inhibition on the growth of pathogenic dermatophytes, *Microsporum canis*, *M. gypseum*, *Trichophyton tonsurans*, *T. mentagrophytes* and *T. rubrum* and opportunistic saprophytes, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Trichoderma harzianum* and *Fusarium oxysporum* (Jamalian et al., 2012). trans,trans-farnesol was also identified from the glands of Japanese bumblebees, *Bombus* (*Diversobombus*) *diversus diversus* and *Bombus* (*Diversobombus*) *diversus tersatus,* this chemical play an important role in reproductive isolation (Kubo and Ono, 2010). Farnesol posses anti-tumour potential, a study by Au-Yeung (2008) indicate that farnesol induced apoptosis involve caspace 3 activation, PARP cleavage, nuclear chromatin condensation, down regulation of Bcl-xL and surviving expression (Au-Yeung et al., 2008). Farnesol is a fragrance ingredient used in cosmetics, fine fragrances, shampoos, toilet soaps as well as in non-cosmetic products like household cleaners and detergents. Its use worldwide is approximately 1-10 metric tonnes per year. Farnesol was approved as GRAS by Food and Drug Administration (FDA). The maximum skin level from the use of farnesol in formulae subjected for fine fragrances is defined by the IFRA Standard (Lapczynski et al., 2008).

The biological activity of 9,12-octadecadienal (15.60% by hydrodistillation and 8.60% by hexane extraction) is not well understood. The major constituents of the oil from the barberry (*Bereberise integrrima*) fruit, obtained by n-hexane and analysed by GC-MS, are (Z,Z) 9,12-octadecanoic acid (11.92%) and 9,12,15-octadecatrienal (7.77%). This oil is used in traditional Iranian medicine to cure diarrhoea, to strengthen the stomach, liver and heart, to eliminate excess bile, relieve thirst, and to reduce stomach heat, internal inflammation and blood ebullition (Halimi et al., 2011).

The volatile aroma from the fruit the bottle gourd (*Lagenaria siceraria* Molina (LS)), also known as *Lagenaria leucantha* Rusby and *Lagenaria vulgaris* Seringe (family Cucurbitaceae), has been isolated by steam distillation and analysed by GC-MS. 9,12-octadecadienal was identified as one of the glycosidic precursors which dominates the volatile aroma profile and contributes to the fruity, floral and citrus odour. In India, the fruit pulp is used as an emetic and purgative agent and is also used for its cooling, diuretic, antibilious and pectoral properties. The boiled fruit pulp in the oil is used to treat rheumatism and insomnia. The fruit is edible and has traditionally been utilised for the treatment of jaundice, diabetes, ulcer, piles, colitis, insanity, hypertension, congestive cardiac failure and skin disease. The methanolic extract of the fruit flesh showed antimicrobial activity against *Pseudomonas aeruginosa* and *Streptococcus pyogenes*, but not against *Staphylococcus aureus* or *Escherichia coli*, in the agar-well diffusion method (Prajapati et al., 2010). Additional research is needed to evaluate if 9,12-octadecadienal has curative-medicative and antimicrobial properties.

Z-Z-Z-9,12,15-octadecatrienal (20.80% by hydro distillation and 16.47% by hexane extraction) is also known as alpha-linolenic acid (ALA), linolenate, alpha-linolenate and 9,12,15-octadecatrienoic acid. As mentioned above, Z-Z-Z-9,12,15-octadecatrienal has been identified as a major component of barberry fruit, together with 9,12-octadecadienal, and has been used in folk medicine in Iran (Halimi et al., 2011).
Fatty acids are the major components of lipid molecules in milk and have different lengths and saturation levels, including medium, long-chain saturated, monounsaturated and polyunsaturated (PU) fatty acid. One of the PUFA in the milk of humans and other species is alpha-linolenic acid (Arsić et al., 2009; Wan et al., 2010).
The dietary intake of chia seeds (*Salvia hispanica* L.), which are rich in alpha-linolenic acid, prevented the onset of dyslipidaemia and insulin resistance in rats after three weeks of treatment. Long-term treatment led to the normalisation of metabolic abnormalities due to dyslipidaemia and insulin resistance without affecting the insulinaemia (Chicco et al., 2009).
Alpha-linolenic acid has been identified in the methanol extract of the fresh flower of sweet basil (34.3% of the total fatty acid), in the aerial parts of celery (6.7%) and sage (1.0%) and in the leaves of eucalyptus (8.7%). All of these extracts caused an inhibition of inflammation in the first hour of carrageenan-induced oedema in the rat paw, and this effect was significant up to 4 hours. The results indicated that extracts containing α-linolenic acid exhibit anti-inflammatory effects on the process of acute inflammation (Abdel-Moein et al., 2011).
Alpha-linolenic acid is also a major fatty acid in *Lavandula angustifolia*, *Lavandula latifolia*, *Lavandula stoechas* (fenchone), *Lavandula multifida*, and *Lavandula dentata* (Urwin and Mailer, 2008). The antimicrobial activity of the supercritical CO_2 extraction of *Lavandula angustifolia* against *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* was higher or equal to that of the hexane extract and hydrodistilled oil, based on the minimum inhibitory concentration assay (Danh et al., 2012). *Lavandula latifolia* being reported as 0.16% against Haemophilus influenza, 0.32% against *Streptococcus pyogenes* and *Staphylococcus aureus* and greater than 0.32% against *Escherichia coli*. *Lavandula stoechas* is traditionally used to treat headaches (Cavanagh and Wilkinson, 2002). The essential oil of Spanish lavender (*Lavandula stoechas*) demonstrated moderate activity against *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermis* ATCC 12228, *Streptococcus faecalis* ATCC 51299, *Salmonella typhimurium* ATCC 13311, *Klebsiella pneumonia*e ATCC 9997 and *Mycobacterium smegmatis* ATCC 607 (Teixeira et al., 2012). The main component of Spanish lavender (fenchone) essential oil has been reported to be a weak antibacterial agent but showed antibacterial effects against *Listeria monocytogenes* and *Escherichia coli* (Dadalioğlu and Evrendilek, 2004).
The essential oil of *Lavandula multifida* showed strong activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 (El Rhaffari et al., 2007), *Candida albicans* ATCC 10231, *Candida krusei*, *Candida guilliermondii* (isolated from cases of mucosal candidosis), *Candida tropicalis* ATCC 13803, *Candida parapsilosis* ATCC 90018, *Cryotococcus neoformans* CECT 1078, *Aspergillus flavus* F44 (isolated from bronchial secretions), *Aspergillus niger* ATCC 16404, *Aspergillus fumigatus* ATCC 46645, dermatophyte clinical strains isolated from nails and skin, *Epidermophyton floccosum* FF9, *Microsporum canis* FF1, *Trichophyton mentagrophytes* FF7 and the dermatophyte strains *Microsporum gypseum* CECT 2908, *Trichophyton mentagrophytes* var. interdigitale CECT 2958, *Trichophyton rubrum* CECT 2794 and *Trichophyton verrucosum* CECT 2992 (Zuzarte et al., 2012).
Based on the diffusion technique on solid agar, the essential oil of *Lavandula dentata* exhibited antimicrobial activity against *Salmonella* sp, *Neisseria meningitides*, *Enterobacter cloaceae*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Pantoea* sp, *Escherichia coli* ATCC 125922, *Proteus mirabilis*, *Staphylococcus aureus* ATCC 25923, *Streptococcus* sp,

Streptococcus pneumoniae, *Listeria monocytogenes* (Imelouane et al., 2009), *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* (Mothana et al., 2012), but not against *Pseudomonas aeruginosa* or *Candida albicans*.

Table 1.1 The compounds identified and the area percentage occurrence in the leaves oil of *C. orbiculatus* by hydrodistillation.

Number	Compound	RT	Media peak area (%)	SD	CV %
1	Magnoflurine Iodide[Al]	12.74	0.49	0.03	6.82
2	Papaverine[Al]	18.05	0.42	0.01	2.69
3	Isopinocampheol[Ar]	18.37	0.79	0.04	4.89
4	Tri-O-ethylcycleacurine[Al]	22.99	0.33	0.00	1.48
5	*trans*-geraniol[Ar]	25.77	0.34	0.03	9.79
6	*trans*-2-decenal[Ar]	26.81	1.40	0.05	3.71
7	n-capric isoprophyl ester[Ar]	34.70	0.31	0.02	4.97
8	Ethyl caproate[Ar]	35.84	9.32	0.03	0.36
9	Methy pentadecanoate[Ar]	36.75	1.50	0.02	1.04
10	Lauraldehyde[Ar]	42.78	2.56	0.01	0.21
11	Citronellyl butyrate[Ar]	44.67	46.13	0.93	2.01
12	9,12-octadecadienal[Ph]	56.72	15.59	0.23	1.47
13	z-z-z-9,12,15-octadecatrienal[Ph]	59.06	20.80	0.60	2.90

Table 1.2 The compounds identified and the area percentage occurrence in the leaves oil of *C. orbiculatus* by ethanol extraction.

Number	Compound	RT	Media peak area (%)	SD	CV %
1	Magnoflurine Iodide[Al]	12.75	0.17	0.00	1.71
2	Berbamine[Al]	15.29	3.00	0.08	2.81
3	12-0-demethylcoclobine[Al]	15.53	0.39	0.01	1.61
4	Papaverine[Al]	18.05	0.19	0.00	1.36
5	(S)-Isoboldine[Al]	18.17	2.11	0.07	3.20
6	Bisnorthalrugosine[Al]	18.73	0.18	0.02	9.84
7	Papaveraldine[Al]	19.30	0.54	0.02	4.29
8	Furfuryl alcohol[Ar]	21.54	0.29	0.01	2.93
9	Decanal[Ar]	28.71	0.30	0.01	4.15
10	Glyceryl alcohol[Ar]	38.10	24.58	1.61	6.57
11	Methyl nonyl carbinol[Ar]	39.48	1.44	0.05	3.72
12	Citronellyl butyrate[Ar]	44.67	7.66	0.31	4.09
13	Palmitic acid isopropyl ester[Ar]	49.89	20.28	0.32	1.56
14	*trans*-farnesol[Ar]	52.77	9.57	0.32	3.32
15	2-undecanol[Ar]	54.87	4.23	0.12	2.85
16	9,12-octadecadienal[Ph]	56.72	8.60	0.20	2.34
17	z-z-z-9,12,15-octadecatrienal[Ph]	59.06	16.47	0.16	1.00

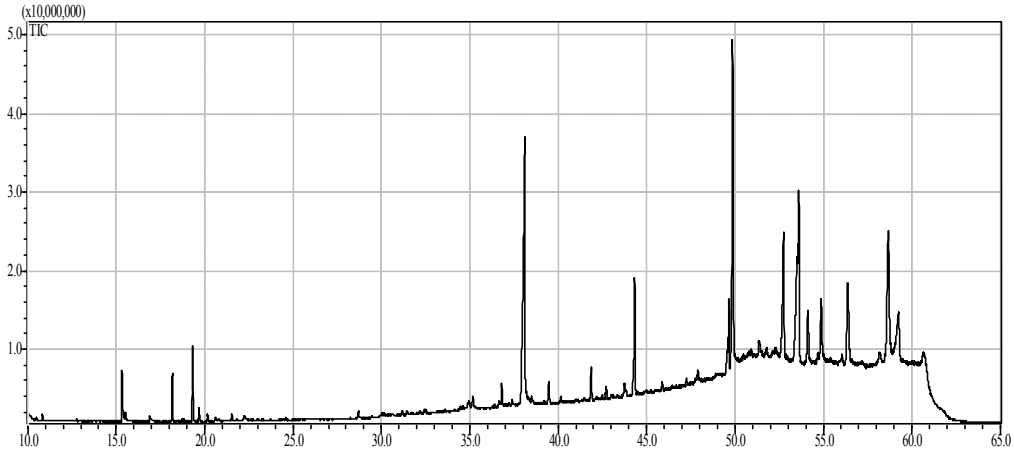

Figure 1.14 Gas chromatograph of essential oil of the leaves of *C. orbiculatus* obtained by hydro distillation.

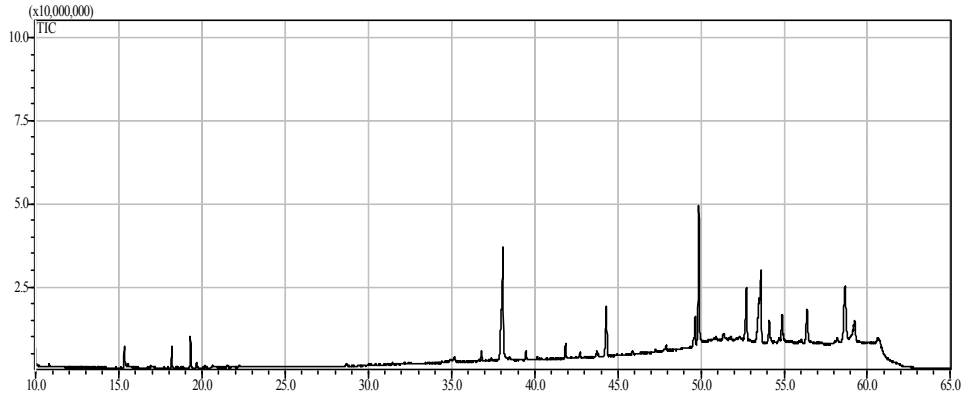

Figure 1.15 Gas chromatograph of essential oil of the leaves of *C. orbiculatus* obtained by ethanol extraction.

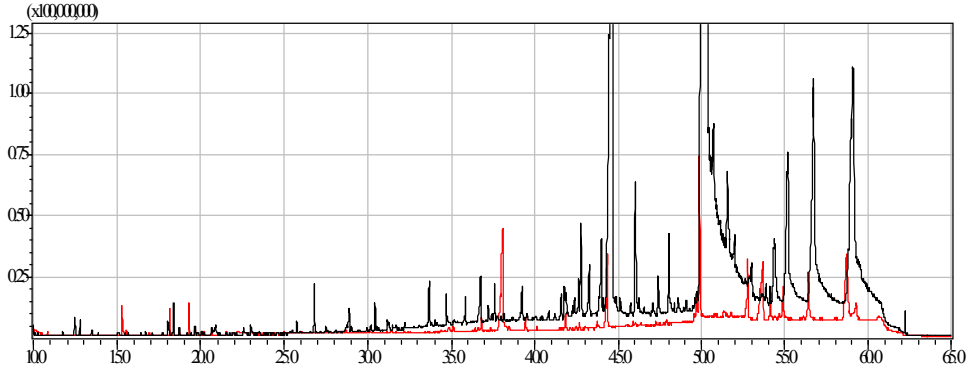

Figure 1.16 The comparison of gas chromatograph of essential oil of the leaves of *C. orbiculatus* obtained by hydro distillation and ethanol extraction.

1.3.3. Molecular identification PCR

Using the NCBI default parameters, a BLAST search of the amplified DNA barcode region of the ITS, matK and rbcL sequences identified the leaves as belonging to the species *C. orbiculatus* (Figure 1.17). The ITS gene (GenBank accession number AY864900.1) had a maximum score of 1042 and a maximum identification (maximum percentage of the identity value obtained for the matching accession) of 99% and was approximately 650 bp in length. The matK gene (GenBank accession number AB069652.1) had a maximum score of 1509 and a maximum identification of 99%. The rbcL gene (GenBank accession number HQ260774.1) had a maximum score of 985 and a maximum identification of 100%. The matK region was amplified with three different concentrations of $MgCl_2$. We intended to amplify more products, but the results showed that there were no differences in the products with 1.5 mM, 3.0 mM and 4.5 mM of $MgCl_2$. The molecular identification of plant species is beneficial, in that it does not depend on experts such as botanists to accurately identify leaves or parts of plants.

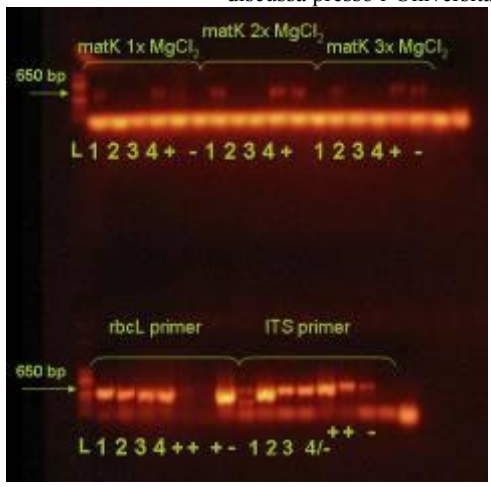

Figure 1.17 The amplified DNA of the leaves of *C. orbiculatus* from amplification of ITS, matK and rbcL region.
L: ladder; 1-2-3-4: samples; +: positive control; -: negative control

1.4. Conclusions

The complete compounds of the *Cocculus orbiculatus* leaves have been reported here for the first time. Combining the data obtained from phytochemical analysis, as well as molecular data and the bactericidal activity, is necessary to adequately profile *C. orbiculatus*.
The use of the ITS, matK and rbcL regions allowed the successful identification of the plant species.
These findings suggest that the essential oil of *C. orbiculatus* has potential as a natural antimicrobial agent. In addition, it has been reported that almost every component of the essential oil of the *C. orbiculatus* leaves possesses various biological, antimicrobial and medical activities. Additional experiments are needed for an in-depth study of the activity of the essential oil and its individual components.

2. Influence of breed on meat quality and dry-cured ham characteristics

2.1. Introduction

Pig breeding programs have been developed to meet the consumers need for hig- quality meat and for pig breeder also production system to achieve high production. The Duroc breed is known to exhibit a higher intramuscular fat contents than Large White (Latorre et al., 2003), which is contributes to the sensory quality of dry-cured ham (Cilla et al., 2006) and has been connected with improved eating quality attributes such as tenderness, juiciness and flavour. The higher eating quality of pork from the Duroc breed is associated with slow oxidative fibres and the redness of the muscle (Gil et al., 2008), but in some cases meat from this breed is dismissed because the intramuscular fat is too visible in the cuts of muscle (Bonneau and Lebret, 2010). Colour is one of the most important attributes governing the consumers' choice to purchase meat (Cheftel and Culioli, 1997; Resurreccion, 2004). As another parameter determining the eating quality of meat, tenderness (Verbeke et al., 2010) has also been studied by texture profile analysis.

Some studies have reported that breed influences the eating quality of meat (Bertol et al., 2013; Bonneau and Lebret, 2010; Suzuki et al., 2003). Therefore, this study aimed to compare the physico-chemical and microbiological characteristics of the *M. longissimus dorsi* and ham traits (texture profile, pH, Aw and colour) of meat destined for production. The meat studied included the Denomination of Protected Origin (DOP) San Daniele dry-cured ham produced from two different sires, Duroc and Goland, which were bred as Goland x Large White and Duroc x Large White.

2.2. Material and Methods

2.2.1. Animals

The 95 pigs consisted of gilts (females) and barrows (males) (see Table 1 for the distribution of gender and breed) produced as a second generation intercross between three sire lines (Goland, Duroc and Large White). The animals were slaughtered at a liveweight of 150 ± 12 kg in the slaughterhouse, after a standardised pre-slaughter treatment (12 h in lairage, electrically stunned with 350 V at 50 Hz). The musculus longissimus dorsi (MLD) was evaluated for its physico-chemical and microbiological characteristics (qualitative characteristics). A total of 100 fresh legs (hams) from gilts and barrows were used in this study. The hams were removed from the carcass by a cut parallel to the sacral vertebrae and the ischium-pubic symphysis. The foot was not removed from the leg. The hams were sent directly to a commercial meat production factory located in San Daniele del Friuli.

Table 2.1 The distribution of genders and breeds.

Genders	Breeds	
	Duroc x Large White	Goland x Large White
Females	30	19
Males	28	18

2.2.2. Dry-curing process (Ham processing)

Upon arrival, the hams were visually inspected by veterinarians and personnel of the certification staff of the Instituto Nord Est Qualità (INEQ) and marked with the DOP seal

and the date of the initial production. Ideally, the weight of the hams should be greater than 12 kg after trimming, and the meat should be a bright red colour with a pH between 5.6-5.8. The fat should be white, with a thickness of more than 1.5 cm above the head of the femur (thigh bone). The hams were processed according to the official methodology required by the San Daniele Ham Consortium. First, hams were trimmed to remove excess fat without damaging the muscles or rind, and a roller-massager was used to facilitate the discharge of residual blood from vessels and to avoid the growth of microorganisms (caused by vein defects).

The next step, salting, is the most critical phase of ham production. The hams were covered with salt (6%) and chilled for approximately 24 h at 2-3 °C in a storage room with a relative humidity (R.H.) of 90-95%. The Golden rule of the San Daniele hams' style is that the hams remain "salted" for every kilogram of weight. The second salting is performed after 4-5 days by removing the salt from the first salting step. The hams are then subjected to massage, covered with salt, and stored at 2-3 °C in a storage room. Pressing is used to facilitate the discharge of blood from the femoral artery, and this step gives the ham its typical guitar-like shape.

The hams then entered the "resting" stage, which was performed at 4-6 °C with a R.H. of 70-75% or 80-85% for an average of 21-25 days; the purpose of this step is to attain gradual dehydration and salt penetration. The next resting stage is carried out at 4-8 °C, with a R.H. of 70-75% or 80-85% for 60-70 days, during which time the salt concentration inside the hams reaches 4.0-4.5%. The hams were then "groomed," with electrical knives to remove and trim the head and to remove the humidity. Afterwards, the hams were washed for 2-3 h with water and dried with air (120 atm, 50 °C) to remove the surface slimes, called "molliga". At the pre-curing stage, hams can be stored at 12 °C for 35-40 days. The temperature can vary between 12-19 °C, depending on the size of the hams, and the R.H. is 75-90%. An inadequate R.H. can lead to the development of massive moulds on the surface (*Eurotium*, *Aspergillus*, *Penicillium*, *Mucor*, *Fusarium* and *Cladosporium*).

After seven months at the salting stage, the next step is grouting and sugnatura, in which the flat surface of the hams (coscia; the cut part of the thigh) are covered with a mixture of lard, sodium chloride, pepper and cereal flour. These mixtures soften the surface and ensure an osmotic process between this surface and the external environment.

The ripening stage, at which the hams matured and obtained good flavour, is conducted through a technological process that involves changes in humidity, temperature and air. The process takes 8 to 16 months and is carried out in naturally ventilated rooms or with air provided by air conditioning systems. At the end of the seasoning stage, the weight of the hams must not less than 7.0 – 9.0 kg.

At the end of the ripening stage, the hams can be moulded to give the guitar-like shape typical of San Daniele hams.

2.2.3. Microbiological analysis

2.2.3.1. Sampling procedure

After slaughter, part of the musculus longissimus dorsi (MLD; lombo or lonza in Italian) muscles of the carcasses were vacuum-packed and sent to the microbiology laboratories at the University of Udine to be analysed on the same day of the post-mortem. The dry-cured ham was not submitted for microbiological analysis.

2.2.3.2. Microbiological analysis

A 10 g sample was taken from each muscle and transferred to sterile full filter bags. Subsequently, 90 ml of 0.1% peptone water (8.5 g/L of NaCl from JT Baker. Deventer. Holland added to 1 g/L of peptone, Oxoid. Basingstoke. Hampshire. England) was added. The samples were homogenised in a stomacher for 1 minute. The samples were then serially diluted tenfold in 0.1% peptone water. A 100 µl aliquot of each dilution was spread onto agar plates (Table 2). Total aerobic counts were determined using Plate Count Agar (Oxoid) with an incubation at 30 °C for 24 h. The number of colony forming unit/g (cfu/g) was obtained after growth for 24-48 h to determine the viable cell counts. The detection limit was 100 cfu/g for the spread plate count analysis and 10 cfu/g for the pour plates (inclusion).

Table 2.2 The list of medium used for analysis different types of microorganisms.

Microorganisms	Media	Incubation Temperature	Incubation time
Total microbial Count	Plate Count Agar-PCA. Oxoid. Basingstoke. England	30°C	24 hour
Enterobactericeae	Violet Red Bile Agar-VRBL. Oxoid. Basingstoke. England	37°C	24 hour
Escherichia coli	Coli-ID Agar. Oxoid. England	37°C	24 hour
Coliform	Coli-ID Agar. Oxoid. Basingstoke. England	37°C	24 hour

The amount of lactic acid bacteria was determined by pour-plates on de Man-Rogosa-Sharpe agar added to delvocid in double layers. The plates were incubated in an anaerobic jar to create an anaerobic environment and supported with a tea candle. *E. coli*, coliform and Enterobacteriaceae were grown in double layers on pour plates.

2.2.4. Physicochemical analysis

2.2.4.1. Measurement pf pH

The pH value was measured at three different position in the muscles using a puncture pH-meter (Basic 20. Crison Instrument, S.A. Allela. Barcelona).

2.2.4.2. Water activity (Aw)

Water activity was measured using a AquaLab 4TE (Decagon Devices. USA) . Minced muscles and dry-cured ham samples (2.0 g) were placed in containers and allowed to equilibrate to 25°C before measurement.

2.2.4.3. Colour

Both muscles and dry-cured ham were measured for colour according to the CIE L*a*b colour system (also referred to as the CIELAB colour space), using a Minolta CR 200 colorimeter with an 11 mm-diameter aperture and D65 illuminant, calibrated by the CIELAB colour space system using a Minolta calibration plate: white tile (C: Y = 94.0; x = 0.3131; y = 0.3190).

Measurements were performed in triplicate for each sample, and the primary colour coordinates, L* (representing lightness, where L* = 0 is completely black and L* = 100 is completely white), a* (representing redness, where positive a* values mean red colours and

negative a* values mean green colours), b* (representing yellowness, where positive b* values mean yellow colours and negative b* values mean blue colours) were calculated.

2.2.4.4. Determination of drip loss from muscles musculus *longissimus dorsi*

The method used was based on Cristensen (Christensen, 2003). After 4 h of rest at approximately 7 °C, the *longissimus dorsi* was cut into 2.5-cm-thick slices. Three cylindrical cuts (25 mm in diameter) were made with a circular knife. Each was made parallel to the muscle fibres from each muscle and was immediately placed in a special EZ-DripLoss container, which was weighed before use. After sampling, each container including the meat sample was weighed and stored at 4 °C for 24 h. The muscles in the EZ-DripLoss container were weighed after 24 h, and the meat samples were then removed. The containers with the exudates/meat juices were then weighed. Drip loss measurements were expressed as a percentage of the initial weight.

Figure 2.1 The circular knife and EZ-Driploss

2.2.4.5. Texture analysis

The texture of the dry-cured ham was measured using texture profile analysis (TPA). The analyses were performed at a temperature of 25 °C with a Texture Analyser TA-XTplus (Stable Micro Systems. Surrey, UK). Three individual cylindrical slices (10 mm x 10 mm) of dry-cured ham were taken from 100 samples. Every slice was subjected to a two-cycle compression test using the 30 kg load cell; the samples were compressed to 40% of their original height with a 36-mm-diameter cylindrical probe (SMSP/36 compression plate) and cross-head speed of 1.5 mm/s.

The texture profile parameters were determined following the descriptions of Bourne (2002) and the SMS manual (Stable Micro Systems, Surrey, UK) and included hardness (N), which is the maximum force required for the initial compression of the sample, springiness (mm), which is the samples' ability to recover its original shape after the initial compression and deforming force were removed, cohesiveness (dimensionless), which means the extent to which the sample could be deformed prior to rupture, gumminess (N x mm), which is the energy required to disintegrate a semisolid food to a state of readiness for swallowing, and resilience (dimensionless), which is the ratio between the negative force input to positive force input during the first compression.

2.2.4.6. Statistical analysis

Statistical analyses of the differences were performed using one-way analysis of variance (ANOVA).

2.3. Results and Discussions

2.3.1. Muscles (meat) characteristics

The results were collected from only 85 samples, consisting of 39 barrows and 46 gilts, due to the limited time for data collection and the thesis deadline.

Neither the genetic background nor the gender affected the physico-chemical and microbiological parameters of the *M. longissimus dorsi* between the Goland x Large White and Duroc x Large White sires. The total viable counts of Enterobacteriaceae, coliform and *E. coli* (log cfu/g) on the muscles were not different between the sires (Table 2.3). The microbiological results showed no presence of *Listeria monocytogenes* and *Salmonella spp*., indicating that the hygienic/sanitary quality of the muscles met with the acceptable microbiological criteria for meat (Commission Regulation (EC) No 1441/2007 which is an amendment of Regulation (EC) No. 2073/2005).

The pH showed uniform values at 5.7. This pH value fell in the range of 5.6 – 5.8, which is considered suitable for processed meat products such as salami and ham. The meat of the Duroc x Large White had slightly less drip loss than the Goland x Large White, which agrees with the results of Latorre et al. (2003).

There were no significant differences in the CIE L*, a* and b* values ($p>0.05$) of the *M. longissimus dorsi* from different sires. The muscles of the Goland x Large White tended to have slightly higher a* values than the Duroc x Large White. The red colour (a*) of this muscle results from the oxymyoglobin concentration and oxidative status. (Gorelik and Kanner, 2001). No significant differences in the drip loss percentage were attributed to sires; however, the dip losses were higher in the Goland x Large White than in the Duroc x Large White.

Table 2.3 The qualitative characteristics of *M. longissimus dorsi* from two breed.
SD: standard deviation; TMC: total microbial counts; 1 : log cfu/g

Parameter	Goland x Large White		Duroc x Large White		General Media	
	Media	SD	Media	SD	Media	SD
L*	41.7	6.5	41.5	5.6	41.6	5.9
a*	1.6	4.3	1.1	3.6	1.3	3.9
b*	9.6	3.0	9.8	2.5	9.8	2.7
Drip-loss (%)	2.9	1.9	2.3	2.0	2.3	1.9
pH	5.7	0.2	5.7	0.3	5.7	0.2
TMC[1]	3.7	1.1	3.3	1.0	3.5	1.1
Enterobacteriaceae[1]	1.9	1.2	1.9	1.2	1.9	1.2
Coliforms[1]	1.6	0.8	1.7	0.9	1.6	0.8
E.coli[1]	1.2	0.6	1.2	0.6	1.2	0.5

2.3.2. Dry-cured ham

The dry-cured ham produced from the second generation of two breeds were not significantly different for the following observed parameters: pH ($p > 0.05$), Aw ($p > 0.05$), colour: L*, a*, b, drip loss and texture profiles (Table 2.5).

The texture profile analysis (Table 2.7, Table 2.8) indicated that there were no interactions between the texture profiles of dry-cured ham made of ham from pigs of different breeds

and genders, with respect to hardness ($p > 0.05$), springiness ($p > 0.05$), cohesiveness ($p > 0.05$), gumminess ($p > 0.05$) and resilience ($p > 0.05$). The pH, Aw, and colour of dry-cured ham were not affected by breeds.

Table 2.4 Physico-chemical and textural properties of dry-cured ham.

	Duroc		Goland	
	Mean	STD	Mean	STD
pH	5.88	0.1458	5.90	0.12
Aw	0.92	0.0080	0.9210	0.0077
L*	42.44	2.3421	41.76	2.47
a*	14.17	1.3777	14.62	1.49
b*	-4.22	26.8541	-0.26	1.87
Hardness (N)	3,826.59	1,904.81	3,641.15	1,876.32
Sringiness	0.64	0.07	0.63	0.09
Cohesiveness	0.59	0.05	0.60	0.06
Gumminess	2,351.97	1,439.61	2,257.72	1,456.60
Resilience	0.24	0.05	0.26	0.08

Table 2.5 The effects of genders and breeds on the pH, Aw and color of dry-cured ham (continue).

Genders	Breeds	pH	Aw	L *	a*	b*
F	Duroc	5.79	0.9114	42.83	13.62	-2.16
M	Duroc	5.82	0.9151	42.39	14.82	-1.99
F	Duroc	5.85	0.9134	41.23	14.77	-1.51
M	Duroc	5.94	0.9163	40.18	15.67	-1.74
M	Goland	5.80	0.9291	42.45	15.83	1.44
F	Goland	5.76	0.9310	41.23	14.16	-1.78
M	Goland	5.78	0.9267	41.27	14.34	-0.87
F	Duroc	5.83	0.9191	42.15	13.71	-186
F	Duroc	5.81	0.9249	43.65	13.55	-1.94
F	Duroc	5.75	0.9206	45.82	14.24	-0.81
F	Duroc	5.73	0.9047	45.08	12.83	-3.71
F	Duroc	5.66	0.9152	46.89	13.33	-2.82
F	Goland	5.77	0.9179	44.77	13.64	-3.75
F	Goland	5.70	0.9218	43.85	14.50	-2.91
F	Goland	5.82	0.9177	47.14	12.39	-1.76
F	Goland	5.88	0.9249	44.20	13.02	-2.47
M	Goland	5.75	0.9227	45.27	12.80	-3.41
M	Goland	5.82	0.9349	44.06	14.78	-1.97
M	Duroc	5.71	0.9153	43.00	14.68	-1.96
F	Duroc	5.75	0.9303	50.74	10.47	-4.65
M	Duroc	5.65	0.9329	48.65	11.44	-4.57
M	Duroc	5.60	0.9321	46.47	13.25	-2.94
F	Goland	5.68	0.9334	46.44	13.96	-3.77
F	Duroc	5.62	0.9255	45.56	12.90	-3.44
F	Duroc	5.62	0.9252	43.31	13.21	-1.22
F	Goland	5.69	0.9198	46.47	12.67	-2.04
F	Duroc	5.76	0.9111	44.40	13.24	-0.92
F	Duroc	5.74	0.9233	44.00	13.43	0.11
F	Duroc	5.86	0.9200	42.22	14.76	-0.31
F	Duroc	5.76	0.9178	39.83	14.55	-0.97
M	Duroc	5.81	0.9092	41.35	14.21	-0.16
F	Goland	5.88	0.9179	45.95	18.40	5.96
F	Goland	5.94	0.9237	44.23	14.08	0.31
F	Goland	6.02	0.9336	40.73	16.55	0.38
M	Goland	6.09	0.9191	39.75	14.94	0.41
M	Duroc	6.04	0.9237	41.52	16.98	1.99
F	Goland	6.04	0.9312	42.40	17.62	3.26
F	Duroc	5.98	0.9253	40.65	16.50	0.62
F	Goland	6.07	0.9252	39.28	16.88	0.22
M	Duroc	6.23	0.9365	40.81	15.61	2.03
F	Goland	6.17	0.9360	41.38	16.18	0.07

Table 2.6 (continue) The effects of genders and breeds on the pH, Aw and color of dry-cured ham.

Genders	Breeds	pH	Aw	L *	a*	b*
F	Duroc	6.06	0.9268	42.42	17.38	0.20
M	Goland	6.04	0.9266	40.94	15.80	-0.23
F	Goland	6.03	0.9214	40.92	16.86	-0.07
F	Duroc	6.01	0.9217	41.02	14.91	0.13
F	Duroc	5.88	0.9182	42.05	14.52	0.56
F	Duroc	5.93	0.9176	41.89	13.38	1.89
F	Duroc	5.86	0.9153	41.77	13.76	1.59
M	Goland	5.97	0.9190	39.93	14.53	0.76
M	Goland	5.98	0.9192	41.66	14.65	1.47
M	Duroc	5.97	0.9226	41.86	13.15	1.07
M	Duroc	5.89	0.9191	41.77	12.02	1.55
M	Duroc	5.93	0.9249	41.57	14.64	1.68
M	Goland	5.91	0.9163	37.50	13.66	1.54
F	Duroc	5.96	0.9207	42.07	14.32	0.69
F	Goland	5.90	0.9105	40.04	12.95	0.49
M	Goland	5.88	0.9184	40.75	15.12	0.72
F	Goland	5.91	0.9094	39.69	13.56	0.63
M	Goland	5.88	0.9204	40.18	14.08	-0.51
F	Goland	5.90	0.9141	42.48	13.04	-0.38
M	Goland	5.87	0.9065	42.33	13.02	0.42
M	Goland	5.86	0.9122	39.87	14.17	0.31
M	Goland	5.91	0.9055	39.79	12.53	-0.01
F	Goland	5.88	0.9125	38.47	14.22	-0.85
M	Goland	5.85	0.9184	39.15	15.29	-0.27
M	Duroc	6.03	0.9188	40.53	15.65	1.26
M	Duroc	6.01	0.9118	40.47	13.87	0.40
F	Duroc	5.96	0.9343	42.42	13.91	0.44
F	Duroc	6.09	0.9010	40.29	15.22	0.29
F	Duroc	6.03	0.9167	39.62	14.60	-0.98
M	Duroc	5.79	0.9026	38.94	13.42	2.05
F	Duroc	5.84	0.9263	41.71	14.25	0.71
M	Duroc	5.83	0.9294	41.44	13.56	2.11
M	Duroc	5.79	0.9168	39.81	12.41	2.00
M	Duroc	5.84	0.9188	42.78	13.25	-0.10
M	Goland	5.95	0.9149	40.57	15.82	0.63
M	Duroc	5.98	0.9221	42.43	15.63	0.96
M	Goland	5.93	0.9223	38.62	16.19	-0.36
M	Goland	6.01	0.9193	40.49	13.63	0.16
M	Duroc	6.00	0.9318	39.90	15.95	-0.39
F	Duroc	6.07	0.9311	40.65	13.46	-1.12
M	Duroc	6.17	0.9286	42.84	16.55	0.77
M	Duroc	5.85	0.9239	42.18	13.06	-0.55
F	Goland	6.01	0.9227	40.84	15.13	-1.55
F	Duroc	6.00	0.9194	41.77	15.53	-0.63

Table 2.7 The texture profile analysis on dry-cured ham from two different breeds (continue).

Genders	Breeds	Hardness (N)	Springiness	Cohesiveness	Gumminess	Resilience
F	Duroc	4664	0.548	0.543	2538	0.327
M	Duroc	7507	0.726	0.697	5355	0.326
F	Duroc	8817	0.727	0.728	6494	0.388
M	Duroc	9595	0.728	0.767	7373	0.425
M	Goland	5151	0.785	0.747	3525	0.61
F	Goland	12079	0.744	0.750	9110	0.461
M	Goland	7507	0.726	0.697	5355	0.326
F	Duroc	3347	0.559	0.549	1900	0.234
F	Duroc	3428	0.630	0.560	1946	0.220
F	Duroc	2723	0.571	0.544	1491	0.203
F	Duroc	3003	0.484	0.504	1521	0.180
F	Duroc	2172	0.638	0.554	1201	0.205
F	Goland	2632	0.558	0.588	1556	0.256
F	Goland	3204	0.478	0.583	1864	0.302
F	Goland	3541	0.571	0.538	1918	0.259
F	Goland	3576	0.648	0.570	2059	0.227
M	Goland	2171	0.468	0.514	1132	0.218
M	Goland	3701	0.732	0.561	2075	0.195
M	Duroc	1810	0.478	0.558	1085	0.233
F	Duroc	7381	0.659	0.632	4669	0.324
M	Duroc	2959	0.625	0.590	1778	0.220
M	Duroc	5042	0.639	0.607	3056	0.299
F	Goland	2535	0.615	0.571	1447	0.235
F	Duroc	3718	0.492	0.543	2031	0.218
F	Duroc	5246	0.619	0.564	2964	0.259
F	Goland	3153	0.594	0.585	1843	0.229
F	Duroc	2320	0.668	0.619	1443	0.255
F	Duroc	3481	0.663	0.594	2087	0.240
F	Duroc	2477	0.677	0.527	1308	0.190
F	Duroc	3198	0.598	0.555	1784	0.218
M	Duroc	1823	0.466	0.515	954	0.188
F	Goland	2344	0.570	0.546	1278	0.198
F	Goland	2734	0.613	0.550	1520	0.209
F	Goland	2718	0.644	0.607	1717	0.230
M	Goland	4222	0.595	0.589	2480	0.255
M	Duroc	2032	0.664	0.554	1127	0.185
F	Goland	3130	0.682	0.581	1820	0.210
F	Duroc	2144	0.611	0.566	1211	0.199
F	Goland	3116	0.541	0.555	1740	0.262
M	Duroc	1434	0.662	0.571	816	0.210
F	Goland	1503	0.564	0.511	772	0.190

Table 2.8 (continue) The texture profile analysis on dry-cured ham from two different breeds.

Genders	Breeds	Hardness (N)	Springiness	Cohesiveness	Gumminess	Resilience
F	Duroc	3360	0.655	0.550	1850	0.230
M	Goland	1369	0.420	0.504	690	0.192
F	Goland	1304	0.478	0.472	617	0.176
F	Duroc	3466	0.621	0.590	2048	0.230
F	Duroc	2586	0.641	0.620	1600	0.233
F	Duroc	2883	0.592	0.610	1762	0.264
F	Duroc	9319	0.684	0.677	6345	0.395
M	Goland	2362	0.581	0.614	1461	0.254
M	Goland	2490	0.649	0.624	1475	0.240
M	Duroc	2562	0.624	0.585	1506	0.240
M	Duroc	3794	0.710	0.635	2408	0.262
M	Duroc	4693	0.625	0.605	2838	0.285
M	Goland	5218	0.718	0.652	3404	0.279
F	Duroc	2547	0.620	0.566	1440	0.21
F	Goland	3846	0.661	0.628	2428	0.217
M	Goland	2313	0.701	0.590	1379	0.235
F	Goland	3760	0.669	0.620	2325	0.283
M	Goland	5052	0.655	0.622	3135	0.293
F	Goland	2946	0.615	0.627	1859	0.264
M	Goland	3807	0.746	0.633	2411	0.270
M	Goland	3755	0.720	0.643	2434	0.256
M	Goland	3615	0.606	0.615	2231	0.263
F	Goland	4034	0.607	0.620	2500	0.285
M	Goland	3251	0.627	0.602	1964	0.237
M	Duroc	2195	0.709	0.567	1243	0.186
M	Duroc	3055	0.656	0.595	1819	0.224
F	Duroc	3045	0.666	0.570	1732	0.234
F	Duroc	3419	0.710	0.603	2065	0.223
F	Duroc	3712	0.656	0.567	2102	0.209
M	Duroc	3604	0.774	0.659	2374	0.251
F	Duroc	3422	0.683	0.619	2117	0.233
M	Duroc	2251	0.589	0.543	1275	0.198
M	Duroc	3544	0.621	0.601	2135	0.233
M	Duroc	3259	0.633	0.636	2067	0.220
M	Goland	3137	0.687	0.602	1892	0.217
M	Duroc	4083	0.673	0.579	2357	0.211
M	Goland	5302	0.717	0.592	3141	0.223
M	Goland	3538	0.614	0.634	2246	0.257
M	Duroc	4754	0.685	0.629	2991	0.269
F	Duroc	4921	0.665	0.574	2853	0.211
M	Duroc	2671	0.635	0.591	1588	0.218
M	Duroc	4425	0.616	0.617	2713	0.235
F	Goland	4605	0.708	0.595	2731	0.228
F	Duroc	5783	0.745	0.613	3537	0.254

2.4. Conclusions

Analysis of the qualitative characteristics of muscles and the psychochemical properties of dry-cured ham produced from the Goland x Large White and Duroc x Large White showed have no significant differences.

3. Improving the quality of sliced cooked ham in modified atmosphere packaging.

3.1. Introduction

The shelf-life of sliced cooked-ham packed in modified atmosphere packaging (MAP) depends on the growth of lactic acid bacteria (LAB) and the remaining oxygen residue in the packaging. Lactic acid bacteria (LAB) are a group of gram-positive bacteria, some of which are beneficial to the production of fermented foods and can protect food products against pathogenic bacteria by producing bacteriocins. However, under conditions of low oxygen (microaerophilic), acidity (pH 5.5) and low temperature (refrigeration), LAB can cause spoilage and are responsible for the deterioration of processed meat in MAP and fresh meat products in chill storage (Arvanitoyannis and Stratakos, 2012; Chen et al., 2012; McMillin, 2008; Samelis et al., 2000). LAB produce lactic acid as a result of carbohydrate fermentation, causing white patina, discolouration, and unpleasant odours and flavours (Audenaert et al., 2010; Cayré et al., 2003; Metaxopoulos et al., 2002; Vasilopoulos et al., 2008; Vermeiren et al., 2006). Remaining oxygen inside packaging can change the colour of ham from red to brown, sometimes leading to discolouration. The negative effects of LAB and residual oxygen are significant problems for the cold-cut industry. Thus, a different approach is needed to overcome these challenges. There are many new food packaging systems, such as those that combine different gas mixtures, laminating films which prevent photo-oxidation, oxygen absorbers, and the use of high pasteurisation temperatures. However, the problem has not been solved completely, and the shelf-life of cooked meat is limited to 20-23 days. This has prompted the suggestion of various post-cooking or post-slicing technologies, such as high pressure, antimicrobial agents, organic acids and protective cultures for biopreservation (Metaxopoulos et al., 2002; Vermeiren et al., 2004). Recently, the use of bioprotective cultures have been recommended to eliminate oxygen residues. Furthermore, the aim of this work was to study the use of different microbial cultures such as *Lactobacillus curvatus*, *Lactobacillus sakei* and a mixture of *Lactobacillus curvatus*/*Lactobacillus sakei* (50/50) to improve the quality of sliced cooked ham packaged in MAP.

3.2. Materials and Methods

3.2.1. Meat products

Cooked hams used for this experiment were produced and manufactured at the Salumificio Carlo Meroni S.r.l company (Muggio, Province of Monza and Brianza, Italy).
Sliced cooked hams (1 mm thickness) packaged in M.A.P were stored at 4 ºC up to the expiration date (30 days). The qualitative formulation of additives used for producing them included salt, sodium nitrite, potassium nitrate and sugars. For the packaging experiment, sliced cooked ham (100 g) was placed in a rectangular PE/EVOH/PE/PEEL/PET R-tray, with a thickness of 200 μm and overall dimensions of 15 x 10 x 3 cm. The laminated film used for the packaging cover consisted of APET12/EVOH/PE95. The hams were packaged using a Multivac T200 tray sealer, in modified atmosphere packaging (MAP) under conditions of < 5% O_2. The oxygen concentration was measured with an Analyser Check Mate 9900. The packaged hams were stored at 4 ºC for 30 days below artificial light and were analysed on days 0, 10, 20, and 30. On the sampling day, samples were taken at three different locations on the packaging (top, middle and bottom) to determine the total bacteria counts, lactic acid bacteria counts, pH and colour. The entire sampling experiment was performed three times at all stages investigated.

3.2.2. Bacterial strains, preparation and inoculation of hams

Lactobacillus curvatus and *Lactobacillus sakei* used in this experiment were obtained from the Italy branch supplier of Chr. Hansen, Denmark.
Culture of lactic acid bacteria was rehydrated in Peptone physiologic salt solution (9 g/L NaCl, 1 g/L bacteriological peptone, Oxoid. Milan. Italy) at 40 °C for 1 h. Suspension was serially diluted in peptone physiologic salt solution, and the appropriate decimal dilutions were spread-plated on MRS agar plate to determine the actual concentration of the culture. A stock culture (mother solution) was prepared from an appropriate dilution to obtain a final concentration of 10^7 cfu/ml. The mother solution was used directly for inoculation of the hamburger and the final bacterial cell concentration was of 10^5 cfu/g of ham.

3.2.3. Microbiological analyses

For microbiological analysis, a 10 g sample of ham was taken aseptically, diluted tenfold in 90 ml of Peptone Physiologic Salt, (0.85 % NaCl-J.T. Baker, Mallinckrodt Baker BV, The Netherlands with 0.10% peptone-Oxoid. Basingstoke. England), and homogenised in a Stomacher (Lab-Blender 400, pbi International, Milan, Italy) for 1 min. Serial decimal dilutions of the homogenates were made using Peptone Physiologic Salt. Aliquots (0.1 ml) of the appropriate dilution were prepared for spread plating to allow the enumeration of total plate counts in PCA (Plate Count Agar-CM 0463. Oxoid, Ltd., Basingstoke, Hampshire, England) and were incubated at 30ºC for 24 h. Lactic acid bacterial counts were determined by pour plating of two layers in de Man-Rogosa-Sharpe agar (Oxoid) modified by adding 1 ml/l of Delvocid (25 mg/ml) (DSM Food specialities. B.V., Delft, The Netherlands) and incubation at 42 ºC for 48 h in a candle jar to create microaerophilic conditions. A candle jar is a container in which a lit candle is introduced before the jar lid is closed. The candle's flame burns until it is extinguished by oxygen deprivation, and a carbon dioxide-rich and oxygen-poor atmosphere is created within the jar.

3.2.4. Measurement of the pH value

The pH value was measured at 10 different points in each package using a pH meter (Basic 20, Crison Instruments, S.A, Allela, Barcelona, Spain). The pH value was measured directly in the product by inserting a pH meter probe into the sample.

3.2.5. Measurement of color

Colour measurements were performed on the surface of the hams using the CIE L*a*b colour system, an international standard for colour measurement proposed by the Commission Internationale d'Eclairrage (CIE) in 1976. The measurements were assessed in terms of lightness, redness and yellowness, with a Konica Minolta CR200/CR-231 chromameter in ten different positions in triplicate. The L* (spectral intensity) is the luminance component associated with the lightness, also called brightness or whiteness, and the values range from 0 (black) to 100 (white). The a* is associated with changes from red to green, with a positive a* indicative of red and a negative a* indicative of green. The b* is associated with changes from yellow to blue, with positive b* indicative of yellow and negative b* indicative of blue. Both the a* and b* values range from -120 to 120) (Allen and Cornforth, 2010; Garcia-Navas et al., 2012; Kane et al., 2003; King et al., 2011). Before measurement, the colorimeter was calibrated with a standard white tile.

3.2.6. Sensory analysis

The ham slices used in this sensory analysis included control slices (non-inoculated with lactic acid bacteria), as well as those inoculated with *L. curvatus* those inoculated with a mix of *L. curvatus* and *L. sakei*, and those inoculated with a mix of *L. sakei* and *L. sakei*. The hams were assessed for 9 attributes by 12 untrained panellists. The attributes included the following: fermented and rancid odour (odour characteristics); sourness, sweetness, freshness/pungency, meat-taste (taste); acidness, bitterness (flavour characteristics); and slimy appearance (Baublits et al., 2006; Válková et al., 2007). The same sample was given to each panellist at the same time. The samples were placed on polystyrene plates and served to each panellist. The nine parameters of the sensory characteristics of the ham were evaluated on a 4-point scale, ranging from excellent (1) to poor/unacceptable (4). The panellists were given one glass of mineral drinking water, unsalted crackers and a ballot and were first asked to take a bite of cracker and a mouthful of water to cleanse their palate before starting and between each sample.

3.2.7. Statistical Analysis

All statistical analyses of the differences between each parameter was carried out using a one-way analysis of variance (ANOVA). The differences in the mean values within the parameter were compared with Tukey's honest significant test using the StatGraphics software package (Statistical Graphics, Statistical Graphics Corp., Rockville, Maryland).

3.3. Results and Discussion

The results including the microbiological data, oxygen concentrations, pH and colour analysis at different sampling days (0, 10, 20, 30) are summarised in Table 3.1 - Table 3.4. The data showed that there were small differences between the hams treated with bioprotective cultures and the control. There were significant differences in terms of the pH, oxygen concentration, total microbial and lactic acid bacterial counts, and colour, but the results were strictly dependent on the examined samples and the various storage times.

Table 3.1 The values of different parameters observed on sliced cooked ham packaged in MAP at 4 °C day 0.

Days	Starter	TPC	LAB	O_2	pH	L*	a*	b*
0	Control	4.13a	4.50a	0.05a	6.31	57.65b	8.56a	8.19a
0	*L. curvatus*	6.30c	6.80a	0.23b	6.32	53.73ab	8.78a	9.67ab
0	*L. curvatus /sakei*	5.20b	6.90a	0.20b	6.29	57.49b	9.60a	11.52b
0	*L. sakei*	4.87b	6.43a	0.22b	6.31	51.82a	8.60a	8.35a

TPC: Total Plate Counts; LAB: Lactic Acid Bacteria; O_2: oxygen concentrations; L*: lightness; a*: redness; b*: yellowness

abc: media values followed by different letter are significantly different ($P < 0.05$)

Table 3.2 The values of different parameters observed on slice cooked ham packaged in MAP at 4 °C day 10.

Days	Starter	TPC	LAB	O_2	pH	L*	a*	b*
10	Control	4.50a	6.93a	0.17a	6.21ab	55.68a	18.15a	2.81a
10	*L. curvatus*	5.40ab	8.47a	0.18b	6.29b	56.51a	15.78a	1.96a
10	*L. curvatus* /*sakei*	5.33ab	8.77a	0.23a	6.13	58.43a	15.72a	3.28a
10	*L. sakei*	6.00b	8.53a	0.19a	6.2ab	55.90a	16.14a	3.38a

Table 3.3 The values of different parameters observed on slice cooked ham packaged in MAP at 4 °C day 20.

Days	Starter	TPC	LAB	O_2	pH	L*	a*	b*
20	Control	2.20a	8.33a	0.17b	5.61	61.86b	14.68a	2.17a
20	*L. curvatus*	2.43a	9.17a	0.20c	5.81b	55.40ab	17.52a	2.37a
20	*L. curvatus* /*sakei*	2.33a	8.73a	0.13a	5.81b	58.78ab	16.04a	2.29a
20	*L. sakei*	2.33a	8.60a	0.17b	5.78ab	58.55ab	15.80a	3.46a

Table 3.4 The values of different parameters observed on slice cooked ham packaged in MAP at 4 °C day 30.

Days	Starter	TPC	LAB	O_2	pH	L*	a*	b*
30	Controll	6.20c	8.50a	0.14a	5.95	57.65b	8.56a	8.19a
30	*L. curvatus*	3.57b	8.33a	0.12a	5.69	53.73ab	8.78a	9.67ab
30	*L. curvatus* /*sakei*	3.30b	8.13a	0.05a	5.67	57.49b	9.60a	11.52b
30	*L. sakei*	2.53a	8.23a	0.36a	5.76	51.82a	8.60a	8.35a

Table 3.5 presents the results of the scores obtained for each parameters of the sensory profile. As shown, the bioprotective cultures have improved the sensory characteristics of sliced cooked-ham. In fact, the sliced cooked ham inoculated with bioprotective cultures did not present odours or tastes indicative of deterioration, and white-viscous slime was absent. Conversely, a white-viscous slime was present in the untreated sliced cooked ham. The panellists particularly appreciated the slices of cooked ham treated with the *L. curvatus* biocultures. During 30 days of storage, the bioprotective cultures had a significant ($p < 0.05$) effect on the colour of the hams. The colour characteristics of the bioprotective culture-treated hams had L* (lightness) values ranging from 51.82 to 58.78, a* (redness) values between 8.60 and 17.52, and b* (yellowness) values between 1.73 and 11.52. In the control, the colour characteristics of the L* values ranged from 55.68 to 61.86, while the a* values fell between 8.56 and 18.15, and the b* values were between 1.73 and 8.19. Therefore, the hams treated with bioprotective cultures had more stable and attractive colours, compared with untreated hams.

Table 3.5 The sensory results of different attributes of sliced cooked ham treated with bioprotective culture and packaged in MAP.

Parameters	**Control**	***L. curvatus***	***L. curvatus/sakei***	***L. sakei***
Fermented	12/12	2/12	3/12	5/12
Rancid	7/12	2/12	3/12	3/12
Sour	7/12	2/12	3/12	3/12
Sweetness	2/12	8/12	6/12	5/12
Pungent	8/12	4/12	4/12	6/12
Meat-taste	5/12	8/12	7/12	6/12
Acidness	9/12	8/12	7/12	6/12
Bitterness	9/12	3/12	5/12	5/12
Slimes	12/12	3/12	5/12	5/12
Final Value	4	1	2	3

The values expressed by the ratio of perceive of the attribute and the total numbers of panellists.

* Evaluation: panelist classified hams based on scale 1: excellent to poor/unacceptable (4).

3.4. Conclusions

L. curvatus and *L. sakei* are microorganisms used for as starters in the production of salami. Both species have an outstanding bioprotective capacity against food-borne pathogens such as *L. monocytogenes* and *Salmonella spp* and also have a positive influence on the chemical-physical and sensorial characteristics of meat products. In fact, the data demonstrated that *L. curvatus* and *L. sakei* could extend the shelf-life of sliced cooked ham to 30 days and affect the taste, odour and colour stability. In addition, these strains inhibited the growth of indigenous bacteria and LAB and consequently, the typical white-viscous slime was not observed on the treated products. These cultures also improved the flavour and microbial quality of sliced cooked ham and reduced the concentration of oxygen in the packages. Consequently, the red colour of the ham remained stable until the end of the shelf life. Reducing the concentration of oxygen in the packages also limited the oxidation of lipids. In conclusion, our data confirm that *L. curvatus* and *L. sakei* are fully capable of inhibiting the growth of indigenous lactic acid bacteria in sliced cooked ham.

4. Use of ozone to decontaminate equipment used in food production

4.1. Introduction

Ozone (coming from the Greek word ozein, meaning "to smell") is a highly reactive molecule consisting of three oxygen atoms bound together and is a powerful oxidant. Two types of ozone are formed in nature, stratospheric and tropospheric ozone. Stratospheric ozone is formed by the photolysis reaction of normal oxygen ultraviolet solar radiation at wavelengths below 242.5 nm in the stratosphere, at a concentration of approximately 0.04 ppm. Approximately 90% of atmospheric ozone is formed in the stratosphere, and this ozone has a highly valuable role in the absorption of excess ultraviolet radiation (UV-b), which is harmful to life on earth and to the biosphere. For humans, excess UV exposure can lead to skin cancer, cataracts, impaired immune systems, and melanoma. Excess UV can also cause damage to sensitive crops such as soybeans and increased stress to marine phytoplankton, leading to direct consequences for human food supplies from oceans (Amann et al., 2008; EPA, 2003; Hocking et al., 2007; Spencer et al., 2002).

Ozone is also formed in the troposphere by the photochemical reactions of precursor pollutants such as NOx (from road transportation, combustion in power plants and industries, and international marine shipping), VOCs (from fossil fuels, solvent use, agricultural activities and biomass burning), methane (from coal mining, the gas and oil industries, landfills, ruminant animals, rice cultivation, and biomass burning) and carbon monoxide (from deforestation and the burning of savannah and agricultural waste) in the presence of sunlight. Tropospheric ozone has highly oxidative properties and is harmful to humans (with adverse health effects on the skin and respiratory tract), vegetation and materials. The lowest annual average tropospheric ozone concentration in remote background areas in Europe have ranged between 40 and 90 μg/m3 (Amann et al., 2008).

This study focused on the benefit of ozone as an innovative antibacterial and antifungal agent for cleaning and sanitising stainless steel surfaces.

Numerous studies have reported that ozone inactivates bacteria and reduces microbial populations of gram negative, gram positive bacteria and spoilage yeast, presence in vegetative cell or spore: *Escherichia coli*, *Salmonella enteritidis, Salmonella typhimurium, Pseudomonas aeruginosa, Yersinia enterocolitica, Listeria monocytogenes, Staphylococcus aureus, Enteroccoccus faecalis, Bacillus cereus, Bacillus megaterium*, *Candida parapsilosis, C. albicans, Zygosaccharomicyes bailii, Hansemula anomala, Saccharomyces rosei, Pichia farinosa, Kluyveromyces marxianus, Debaryomyces hansenii* and *Asperigillus niger* (Broadwater et al., 1973; Dave, 1999; Ewell, 1946; Farooq and Akhlaque, 1983; Finch et al., 1988; Ingram and Haines, 1949; Naitoh, 1992; Ortega et al., 2007) and fungi (Antony-Babu and Singleton, 2009; Vijayanandraj et al., 2006; Zotti et al., 2008) in foods (Coll Cárdenas et al., 2011; Dondo et al., 1992; Horvath et al., 1985; Kaess and Weidemann, 1968; Kim et al., 1999; Kolodyaznaya and Suponina, 1975; Zagon et al., 1992), fruits (Achen and Yousef, 2001; Baranovskaya et al., 1979; Bazarova, 1982; Öztekin et al., 2006), drinking water (O'Donnell et al., 2012) and wastewater treatment (Loeb, 2011), food handling and processing equipment (Greene et al., 1999; Guzel-Seydim et al., 2004), crop protection (Venta et al., 2010), sanitizing water for washing food and packaging (Costanzo, 2007) and medical and dental applications (Arita et al., 2005). It was also studied the effect of ozone to prevent the development of *Aspergillus ochraceus* on the surface of Milano-type sausages (Iacumin et al., 2012). The efficacy of ozone inactivates microorganisms also influenced by pH, temperature and humidity (Edwell, 1938). The mortality rate of microorganisms is increased at pH 5.7 to 10.1 (Fair et al., 1968).

Outbreaks involving foodbourne pathogens have been frequently traced to a contamination of food by food processing equipment, most of which is made of stainless steel (Andre et al.,

2012; Farrell et al., 1998; Greene et al., 1993; Khadre and Yousef, 2001a; Kusumaningrum et al., 2003). Improper hygienic practices during food preparation and processing lead to the attachment of bacteria to food contact surfaces and to the development of biofilms, which can then contaminate food (Araújo et al., 2010; Beresford et al., 2001; Bernbom et al., 2011; Hjelm et al., 2002; Hood and Zottola, 1995; 1997).
The low concentration of ozone studied here was maintained below the WHO guidelines for air quality (daily maximum of 8 hours at 100 $\mu g/m^3$) and the EU air quality directive (target value: daily maximum of 8 hours at 120 $\mu g/m^3$, not to be exceeded on more than 25 days per calendar year). The potentially adverse health effects of ozone to the workers must be taken into consideration when low concentrations of ozone are used as industrial disinfectants (Hoof, 1982; Thorp, 1950).
Ultraviolet (UV) radiation was also studied here for the removal of spoilage microorganisms as an alternative for bactericidal agents. The main concern with UV radiation is chemical residues and toxicity, as well as unpredictable changes in the physical, chemical, and organoleptic properties of foods. The bactericidal and inactivation effects of UV radiation has been studied on the surface of fresh produce associated with common foodbourne pathogens such as Salmonella, *Listeria monocytogenes*, *E. coli* O157:7. The effects of UV radiation on the survival of *Chronobacter* spp (*Enterobacter sakazakii*) and on the increased UV resistance in DNA repair-proficient and -deficient strains of *E. coli* have also been studied (Cantwell and Hofmann, 2008; Cheigh et al., 2012; Goodson and Rowbury, 1990; Kuo et al., 2013; Maktabi et al., 2011; Sommer et al., 2000; Yaun et al., 2004).
The purpose of this study was to examine the potential of ozone and ultraviolet radiation at low concentration to inactivate and/or reduce gram-negative and gram-positive bacteria and fungi on stainless steel surfaces.

4.2. Materials and Methods

Several bacterial and fungal species were employed in this study. The microorganisms studied were environmental contaminants (bioaerosols), faecal indicators and potential pathogens from both gram-positive and gram-negative bacteria.

4.2.1. Microorganisms

The antibacterial activity of the ozone was evaluated against the following gram-positive bacteria: *Bacillus subtilis* var *globigii*, *Lactobacillus casei*, *Lactobacillus plantarum* *Lactobacillus curvatus*, *Listeria innocua*, and *Staphylococcus equorum* and against the following gram-negative bacteria: *Shewanella putrefaciens*, *Escherichia coli*, *Pseudomonas putida*, and *Salmonella enteritidis*. The antifungal activity was also evaluated against *Aspergillus niger* and *Penicillium nalgiovense*.

4.2.2. Preparation of bacteria cultures

Stock cultures were obtained from the culture collection of the Department of Food Microbiology at the University of Udine and included *B. subtilis* var *globigii*, *L. innocua*, *S. equorum*, *Shewanella putrefaciens*, *E. coli*, *P. putida*, and *S. enteritidis*. The cultures were stored at -80ºC in Brain Heart Infusion broth containing glycerol (10% v/v). The stock cultures of *L. casei*, *L. plantarum*, and *L. sakei* were stored at -80 ºC in MRS broth.
Prior to each experiment, stock cultures of *B. subtilis* var *globigii*, *Shewanella putrefaciens* and *P. putida* were grown on plate count agar plates and incubated at 30 ºC for 24 h.
The *L. casei*, *L. plantarum*, and *L. sakei* cultures were grown in MRS agar modified by the addition of 1 ml/L of Delvocid (25 mg/ml) and were incubated at 42 ºC for 48 h in a candle jar to create microaerophilic conditions. A candle jar is a container where a lit candle is introduced before closing the jar's lid. The candle's flame burns until it is extinguished by

oxygen deprivation, which creates a carbon dioxide-rich and oxygen-poor atmosphere in the jar.
L. innocua, *S. equorum*, *E. coli*, and *S. enteritidis* cultures were grown on plates made from Brain Heart Infusion broth containing 15 g/L of agar for 24 h at 37 ºC.
The cultivated cultures were harvested, transferred into 3 ml of Brain Heart Infusion broth, and incubated at 30 ºC for 24 h for *B. subtilis* var *globigii* and *S. putrefaciens*, at 30 ºC for 48-52 h for *P. putida*, and at 37 ºC for 24 h for *L. innocua*, *S. equorum*, *E. coli* and *S. enteritidis*.

4.2.3. Preparation of fungi cultures

Aspergillus niger and *Penicillium nalgiovense* were also obtained from the Department of Food Microbiology at the University of Udine, were cultured in malt extract broth containing 15 g/l of agar in sterile glass tubes of 16 mm diameter, and maintained at 4 ºC. Before use in the experiments, one loop of the fungi cultures were taken from the maintained stocks, and cultures were grown at 30 ºC for 5 days on plates made from malt extract broth containing 15 g/l of agar.

4.2.4. Preparation of inoculum

The bacterial cultures were centrifuged to obtained pellets, which were then transferred into 9 ml of sterile Peptone Physiologic Salt (8.5 g/L NaCl with 0.1 g/L peptone). The fungal inoculum was prepared by harvesting all of the fungal colonies and transferring them into 9 ml of sterile Peptone Physiologic Salt The optical density (OD) of the bacterial and fungal suspensions were determined using a spectrophotometer. An OD of 0.1 at 600 nm corresponded to10^7 cfu/ml or 7.0 log cfu/ml.
A suspension of 10^7 cfu/ml was used to inoculate spatulas, and the final bacterial cell concentration was approximately 10^5 cfu/spatulas.
The actual concentration of each bacterial and fungal suspension was verified by the spread-plate method in the appropriate medium for each species.

4.2.5. Experimental design

The equipment consisted of flat stainless-steel spatulas which were 220 mm in length and 50 mm in width. Only the lower part of each spatula, consisting of a rectangle with a length of 110 mm and a width of 55 mm (perimeter of 55 cm^2), was inoculated. Before and after use, the spatulas were washed, wrapped with aluminium foil and sterilised at 121 ºC for 15 minutes.

Figure 4.1 The stainless steel spatula.

4.2.6. Inoculation of Spatula

The spatula surfaces were inoculated with 10 µl of the suspension (OD600 = 0.1). Three to five replicates and one negative control (inoculated without treatment) per microorganism were subjected to ozonation. The experiments were performed by the following two methods:
a) "Wet" method: 10 µl of the suspension was dropped in the centre of the spatula. This method was performed directly in the Martik Srl plant.
b) "Dry" method: 10 µl of the suspension was distributed at the surface of the spatula using an L-shaped spreader, and the spatulas were dried in an incubator at 40 ºC for 15 minutes. Subsequently, the spatulas were wrapped again in aluminium foil and carried to the Martik Srl plant.

The effect of ozone was also evaluated using spread-plates with 10 µl of each bacterial and fungal suspension. The suspensions were serially dilution from 10^2 to 10^7 and plated in triplicate on agar suitable for each organism. One series of three untreated plates was used as a control.

4.2.7. Ozone treatments

4.2.7.1. Gaseous ozone treatment

The inoculated plates and spatulas from the wet and dry methods were treated in a chamber made of AISI 304 stainless steel with a volume of 0.3888 m^3 at the Martik Srl plant located in Coseano, Udine, Italy. The plates and spatulas were exposed to gaseous ozone at a range of 2-4 ppm for 12 hours (Figure 4.2). A generator with a single 600x400x1620 mm compartment, which was sealed and positioned outside of the chamber, was used to produce ozone through electrical discharges (GAT500 12Vdc, Microfox Srl, Italy). An ozone sensor (Analyzer 4480, Interscan Corporation, USA) was used to maintain a constant concentration of ozone. The 18L and 18M reagent vials (Gastec Corporation, Japan) were pumped through the system, and an analyser was used to check the calibration of the instrument.

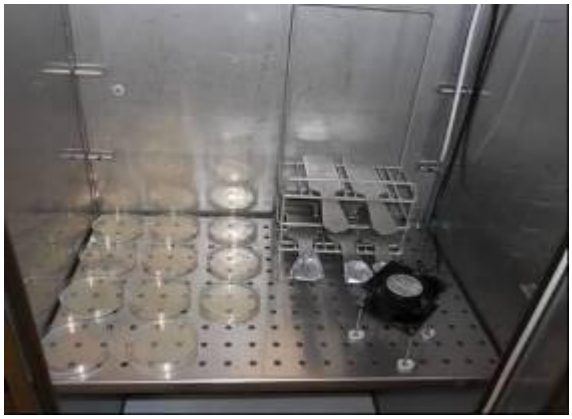

Figure 4.2 The internal chamber for gaseous ozone treatment.

4.2.7.2. Aqueous ozone treatment

Spatulas inoculated by the dry method were placed in a tank of water connected to a tank exchange, where the water was enriched with ozone. The enrichment was performed by injecting air containing ozone into the inlet water tube located at the base of the tank. The air introduced into the tank was expelled from the top of the tank through a suitable vent valve. The circuit was a closed cycle, with the water from the tank passing through the enrichment tank and then returning to the tank where it was in contact with the inoculated spatulas (Figure 4.3).

Figure 4.3 The plant for aqueous ozone treatment.

An ozone level sensor was inserted into the circuit to allow the ozone concentration of the water to be monitored and adjusted. To improve the efficiency of the plant, the exchange tank was placed in a room (20 ºC) so that the water temperature could be reduced.
A Prominent OZVa1080E generator was used to test ozone concentrations up to 2 ppm. The ozone concentration of the water was evaluated with a Prominent DULCOTEST OZE 3-mA-2 ppm ozone sensor, which was located in the hydraulic circuit at the tank exchange outlet. The electrochemical sensor was calibrated using a multiparameter photometer (HI 83226-02. Hanna Instrument. Rhode Island. USA). A multiparameter photometer was used to calibrate the photometric ozone and temperature. Because there was no automatic regulation to test ozone concentrations up to 4 ppm, the ozone generator was adjusted once at the beginning of every test, and the ozone concentration during the test was verified retrospectively with the photometric sensor (ACP 4001-2/O3. Chemitec. Firenze. Italy).

4.2.7.3. Humid-Gaseous ozone treatment

The procedures described above for the gaseous ozone treatment were also used for the humid-gaseous treatment, but the tests were conducted in an aerosolised chamber sprayed with water to create humidity levels between 97-100%. The humidity was assessed with a digital thermo-hygrometer (PROTOS TH060H. Errepi. Italy).

4.2.8. Ultraviolet treatment (UV-treatment)

Spatulas and plates inoculated by the dry method were placed in a UV steriliser (ABATIK 832 A2A. Martik Srl. Italy) (Figure 4.4).

Figure 4.4 The UV steriliser (ABATIK 832 A2A).

The UV structure was made of AISI 304 stainless steel, with a mirrored inner surface. A removable grill, also made of stainless steel, was placed inside to hold the tools. The bottom side was covered with plastic material to avoid damaging sharp tools. A fluorescent lamp (Philips TUV 8 W, Holland) with an ultraviolet emission power of 2.1 W was placed under the lockable lid and was automatically turned off whenever the lid was lifted.

4.2.9. Microbiological analysis

At the end of the treatments, the Petri dishes were incubated at various temperatures based on the bacterial or fungal species for 24-48 h. The enumeration of the bacteria and fungi were recorded and expressed as log cfu/ml.

Each inoculated spatula was transferred into a sterile bag, rinsed with 10 ml of peptone physiologic salt, and rubbed to remove the cells. The solution was serially diluted in peptone physiologic salt, and the appropriate dilutions were spread-plated on agar medium suitable for each bacterial and fungal species. The plates were incubated for 24-48 h. The enumeration of the bacteria and fungi were recorded and expressed as log cfu/ml.

Microorganisms	Media	Incubation Temperature	Incubation time
Bacillus subtilis var globogii , Shewanella putrefaciens, Pseudomonas putida	Plate Count Agar -PCA. Oxoid. Basingstoke. England	30°C	24 hour
Lactobacill us casei, L. plantarum, L. sakei	De Man, Rogosa, Sharpe -MRS (Oxoid. Basingstoke. England) broth with agar and Delvocid	30°C	48 -52 hours
Listeria innocua, Staphylococcus equorum, Escherichia coli , Salmonella enteritidis	Brain Heart Infusion (Oxoid. Basings toke. England) broth with agar.	30°C	24 hour
Aspergillus niger, Penicillium nalgiovense	Malt Extract -AM (Oxoid. Basingstoke. England) broth with agar	30°C	48-120 hour

Table 4.1 The list of medium used for analysis microorganisms and the time of incubation.

4.3. Results and Discussion

4.3.1. Gaseous ozone treatment

Gaseous ozone was applied to the inactivated microorganism at two ozone concentrations, 2.00 and 4.00 ppm. The initial experiments were performed only by dry method, and the ozone concentrations were increased gradually from 0.5 to 4.0 ppm for 12 h to evaluate the effect of different concentrations on the survival of *S. putrefaciens*, as shown in Table 4.2.

Table 4.2 The log reduction of *S. putrefaciens* in various concentration of ozone in dry method. SD: Standard deviation

[Ozone] (ppm)	[Initial load] log cfu/55 cm2	[Retrieved] log cfu/55 cm2	Log reduction	SD
0.50	6.00	5.71	0.29	0.26
1.00	6.48	5.32	1.16	0.38
2.00	5.51	3.87	1.64	0.31
2.00	6.04	5.68	0.36	0.11
2.00	3.15	2.66	0.49	0.21
4.00	5.67	5.10	0.57	0.27
4.00	3.51	2.86	0.65	0.22

The dry method did not greatly reduce the microbial load. As the bacterial cells were hydrated and more sensitive to ozonation (Kim et al., 1999) in the dry method, the wet method was then assessed. Table 4.3 shows a comparison of the wet and dry methods. There was variation in the log reduction of the 12 tested microorganisms following exposure to 4 ppm of gaseous ozone. The log reduction by the dry method was 0.9–3.09 log cfu/spatula and that by the wet method was 1.14-4.24 log cfu/spatula. These findings confirmed that hydrated bacterial cells (dry method) were more sensitive to ozonation. Humidity also plays a role in the efficiency of inactivation by ozone, as the efficiency of ozone inactivation increased as the relative humidity increased (Li and Wang, 2003; Zoutman et al., 2011). The log reduction of *E. coli* and *P. nalgiovense* was greater by the dry method than by the wet method. The log reductions of *B. subtilis* were different between the 2 methods, with higher standard deviations resulting from the dry method compared to the wet method. Similar to our results, Foegeding (1985) also reported variability in the ozone resistance of spores from different microorganism and *Bacillus* species. This variability could result from the presence of the spore form of *B. subtilis*, which is more resistant to ozonation than non-spore forms. *B. subtilis* spores have demonstrated ozone resistance up to 45-75 ppm (observed by Li, C.-S) (Li and Wang, 2003).

Table 4.3 The log reduction of 12 microorganisms in dry and wet method exposed with 4 ppm gaseous ozone.

Microorganisms	**Log Reduction (cfu/spatula)**			
	Dry	**SD**	**Wet**	**SD**
Shewanella putrefaciens	0.65	0.22	2.69	0.23
Bacillus subtilis	2.07	1.78	2.14	1.91
Escherichia coli	2.11	0.00	1.84	0.89
Lactobacillus plantarum	1.32	0.06	1.56	0.78
Lactobacillus casei	1.96	0.64	2.05	1.05
Lactobacillus sakei	1.37	0.74	1.74	0.36
Listeria innocua	3.09	0.41	3.99	1.39
Pseudomonas putida	1.02	0.03	1.65	0.07
Salmonella enteriditis	0.90	0.14	2.60	0.84
Staphylococcus equorum	3.05	0.91	4.24	0.75
Aspergillus niger	1.00	0.00	1.14	1.01
Penicilium nalgiovense	1.30	0.00	1.23	0.57

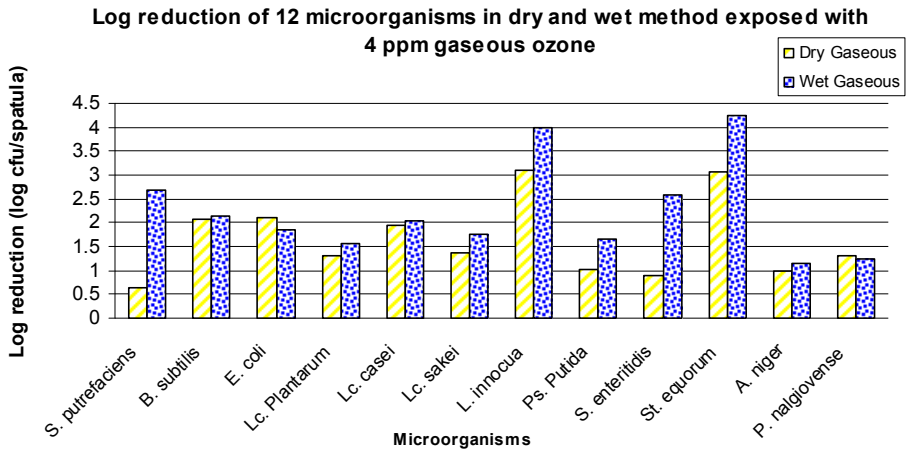

Figure 4.5 The difference in log reduction of 12 microorganisms as affected by application of dry and wet method in 4 ppm gaseous ozone.

E. coli inactivation by the dry method seems to be more effective than by the wet method, although the difference was only 1 log cfu. In contrast, Pirani (2010) demonstrated that the log reduction of *E. coli* was higher with the wet method, with a reduction of 6 log cfu following treatment with 1 ppm for 1 hour. In comparison, another study (Coll Cárdenas et al., 2011) reported an *E. coli* reduction of 0.6 to 1.0 log cfu/g following the treatment of meat samples with 72 ppm of gaseous ozone for 3 and 24 h.
The inactivation of *Lactobacillus* species (*L. plantarum*, *L. casei*, *L. sakei*) was less effective by the dry method compared to the wet method, although the differences were less than 1 log cfu.
The log reduction values obtained from spatulas inoculated with *L. innocua* agreed with the results of Previdi et al., (2009), where treatment with 35.2 ppm gaseous ozone for 1, 5 and 10 minutes resulted in reductions of 4.0, 4.6, 5.1 log cfu, respectively.
The log reduction recorded in Petri dishes confirmed the importance of humidity for the efficiency of gaseous ozonation at 4 ppm. The log reduction of 3.78 until 6.70 log cfu/ml of main solution were achieved (initial contamination solution) in the Petri dishes, related to the humidity of the medium agar. The sensitivity of microorganisms to ozone also may depend on the organic nature of the medium, which could affect the resistant to treatments. The medium provides physical protection, and organic nutrients and water in the medium may reduce the levels of ozone exposure (Ingram and Haines, 1949; Restaino et al., 1995).

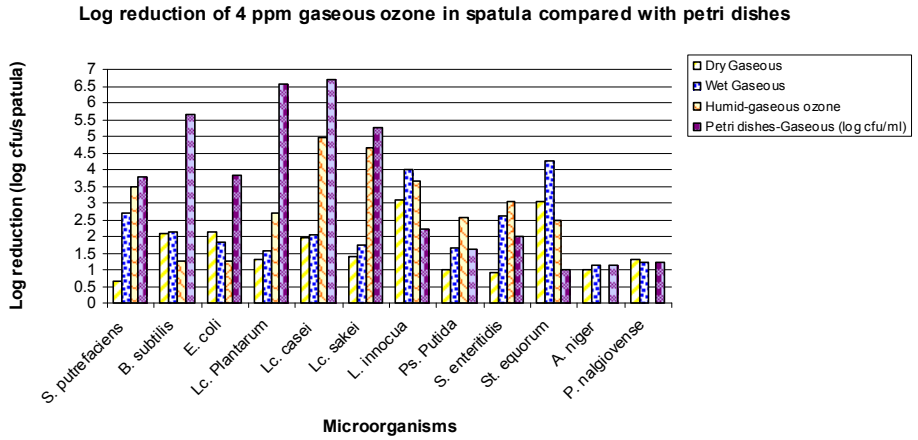

Figure 4.6 The log reduction of 4 ppm gaseous ozone in spatula compared with in the petri dishes.

Here, higher log reductions were observed following treatment of Petri dishes with 4 ppm (Figure 4.6), compared to treatment of inoculated spatulas. These results contrast with those of another study, which compared the log reductions on spatulas inoculated with different species of bacteria (*S. putrefaciens*, *B. subtilis*, *E. coli*, *L. plantarum*, *L. casei*, *L. sakei*). The log reduction of *L. innocua* in Petri dishes reached 3.60 log cfu/ml at 2 ppm of gaseous ozone, most likely due to physical protection afforded by the medium.

Table 4.4 The log reduction of 2 ppm gaseous ozone in dry and wet method.

Microorganisms	Log reduction (log cfu/spatula)			
	Dry	SD	Wet	SD
B. subtilis	0.88	0.42	2.28	1.43
L. innocua	1.84	0.00	1.44	0.12
Ps. Putida	1.30	0.00	4.11	0.00
A. niger	1.00	0.00	1.48	0.00

The inactivation of 4 microorganisms by 2 ppm of gaseous ozone was more efficient by the wet method than by the dry method, except for *E. coli* (Table 4.3). The higher log reduction of *A. niger* by the wet method confirmed that ozonation is more efficient in conditions of greater moisture. This finding was also reported by Raila at al., (2006), who studied the efficacy of ozonation to reduce *A. niger* mycomycetes on grains and demonstrated a 3-fold reduction (initial contamination: 1.1 x 10^4 cfu/g; after treatment: 3.5 x 10^3 cfu/g) at 22% moisture and a 2.2-fold reduction (initial contamination: 1.1 x 10^4 cfu/g; after treatment: 3.2 x 10^3 cfu/g) at 15.2% moisture. In other words, treatment in 22% moisture led to a reduction of 0.50 log cfu and that in 15.2% moisture led to a reduction of 0.35 log cfu.

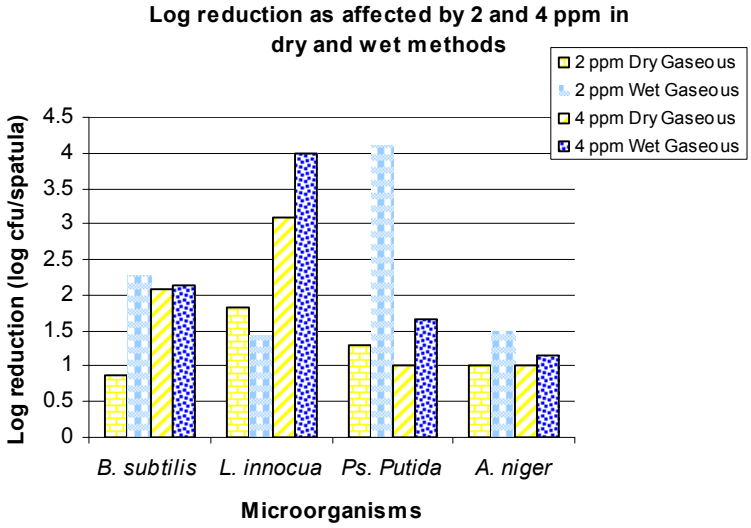

Figure 4.7 Log reduction by 2 and 4 ppm ozone in dry and wet methods.

Figure 4.7 shows the log reduction related to the inactivation of bacteria and fungi in response to 2 and 4 ppm of gaseous ozone by the wet and dry methods. The highest log reduction occurred in *L. innocua* following treatment with 4 ppm of gaseous ozone by the wet method, further confirming that the wet method yielded higher log reductions than the dry method. The results of the treatment of *P. putida* and *A. niger* were unexpected, as 2 ppm of gaseous ozone caused a greater log reduction than 4 ppm, a finding which may have been due to unrecovered surviving microorganisms.

4.3.2. Aqueous ozone treatment

The inactivation of 12 microorganisms by aqueous ozone was conducted only at 4 ppm. Microbiological analysis of the water used to immerse the spatulas was also performed to verify the reduction in microorganisms following treatment. However, microorganisms could not be recovered before or after treatment.

The inactivation results shown Table 4.5 demonstrate that the log reductions vary between 1.04 and 4.87 log cfu/spatula for different organisms. There were significantly greater levels of reduction following the gaseous treatment compared to the aqueous treatment, confirming the high solubility of ozone in water and the fact that ozone retains its antimicrobial properties when dissolved, even if it is not completely soluble (Ishizaki et al., 1986; Previdi et al., 2009; Sadatullah et al., 2012).

Table 4.5 The exposures of 4 ppm aqueous ozone in the log reduction of microorganisms.

Microorganisms	Log reduction (log cfu/spatula)	SD
Shewanella putrefaciens	2.09	0.64
Bacillus subtilis	4.87	0.41
Escherichia coli	3.65	0.00
Lactobacillus plantarum	1.56	0.55
Lactobacillus casei	1.56	0.55
Lactobacillus sakei	1.56	0.55
Listeria innocua	2.52	0.36
Pseudomonas putida	1.41	0.59
Salmonella enteriditis	4.15	0.00
Staphylococcus equorum	2.18	0.00
Aspergillus niger	1.04	0.47
Penicillium nalgiovense	1.22	0.29

In a previous study, Previdi (Previdi et al., 2009) observed reductions of more than 5.5 log cfu of *L. innocua* (standard deviation = 0.36) following treatment at 2.37 ppm of aqueous ozone for 10 minutes, reductions of 3.7 log cfu at 1.28 ppm for 10 minutes and reductions of almost 7.0 log cfu at 0.72 ppm. In contrast, the current study employed a higher ozone concentration (4 ppm) and a longer exposure time but found reductions of only 2.52 log cfu.

The solubility and stability of ozone in water are both affected by numerous parameters including high temperatures, which reduce the solubility and stability of ozone. When a solution is prepared by the injection of bubble ozone, the solubility of the ozone increases. Ozone decomposes at high pH. The purity of water also affects the rate of solubility, as ozone gas dissolves faster in de-ionised water than in tap water. In addition, the presence of organic substances such as cells and cell debris may alter the inactivation of microorganisms. For example, the presence of 1% locust bean gum, high levels of protein, 1% sodium caseinate, and 1% fat (whipping cream) conferred protection to *Bacillus stearothermophilus* spores and to vegetative *E. coli* and *S. aureus* cells (Guzel-Seydim et al., 2004; Khadre et al., 2001). These parameters may differentially affect the antimicrobial efficacy of ozone.

4.3.3. Comparison gaseous and aqueous ozone

Figure 4.8 shows the log reduction induced by 4 ppm of gaseous (by the wet and dry methods) and aqueous ozonation. Although the results demonstrated a high degree of variability for tested microorganisms, the greatest log reductions generally occurred in *S. putrefaciens*, *L. casei*, *L. sakei*, *L. innocua*, *P. putida*, *S. equorum* and *A. niger* via the wet method of gaseous ozonation.
Considerable log reductions of *B. subtilis*, *E. coli* and *S. enteritidis* were also observed following aqueous ozonation.

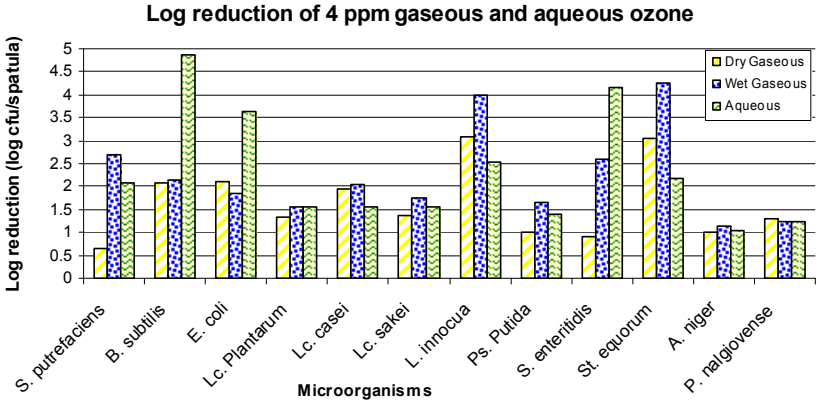

Figure 4.8 The log reduction by 4 ppm gaseous in dry and wet method, compared with 4 ppm aqueous ozonation.

Khadre and Yousef (2001) found that aqueous ozone treatment disrupted the outer coat of *B. subtilis* spores, which may explain why aqueous ozone treatment led to higher log reductions of *B. subtilis* than gaseous ozone treatment. Various log reductions (1.3-6.1) of *Bacillus* species have also been recorded following different exposures to gaseous and aqueous ozone (1-11 ppm) (Khadre and Yousef, 2001a, b). Komanapali and Lau (1996), found that short exposure times to high concentrations of gaseous ozone (600 ppm for 1-5 minutes) compromised the membrane permeability of *E. coli* K-12 but did not affect its viability. This result may explain why aqueous ozone is more effective than gaseous ozone against *E. coli*. Previdi et al., (2009) obtained much greater reductions (7 log cfu) than those observed in the current study, even with low concentrations (0.55, 0.79 and 2.59 ppm) of aqueous ozone for 10 minutes. The finding that the log reduction of *S. enteritidis* was greater in aqueous than gaseous ozone was also supported by the study of Dave in Khadre et al., (2001), in which the cell membrane was disrupted upon treatment with aqueous ozone. Thus, humidity appears to contribute to the efficiency of ozone in the reduction of microorganisms(Guerin, 1963). A study by Previdi et al., (2009) demonstrated log reductions of 4.0 and 3.9 log cfu in *B. subtilis* subjected to ozonation at 1.0 and 0.55 ppm, respectively, for 20 minutes.
In a study performed by Greene et al., (1993), stainless steel was inoculated with *Ps. fluorescens* and subjected to 0.5 ppm of aqueous ozone for 10 minutes, resulting in reductions of 5.6 log cfu. The reductions in the current study were 1.41 log cfu.
The results for *L. plantarum* and *L. casei* were obtained in a different way. Unlike the other microorganisms, the microbial loads were recovered from 10 ml out of total volume of 42 l of water in the tank, and 1.56 log cfu/spatula were measured after treatment. The ozonation

of *A. niger* and *P. nalgiovense* resulted in low levels of reduction, 1.04 and 1.22 log cfu/spatula, respectively, confirming the resistance of fungal spores demonstrated by Previdi et al., (2009).

The log reductions of microorganisms reported by other studies were not always comparable to our results, due to differences in the types and concentrations of ozone as well as the duration of the treatments and the microorganism species. Some studies concluded that bacteria within the same group (gram-positive or gram-negative) could be killed by the same treatment (Khadre et al., 2001; Restaino et al., 1995). In contrast, this study demonstrated that different treatment conditions achieved the same reduction of gram-positive bacteria. For example, 4 ppm of aqueous ozone resulted in the high reduction of the gram-positive bacteria *B. subtilis*, while other gram-positive bacteria, including *L. casei*, *L. sakei*, *L. innocua*, and *S. equorum*, were effectively reduced with 4 ppm of humid-gaseous ozone. The greater amounts of peptidoglycan in the cell walls of gram-positive bacteria resulted in a higher resistance compared to the gram-negative bacteria. However, 4 ppm of aqueous ozone was able to reduce the gram-positive *B. subtilis* as well as the gram-negative bacteria *E. coli* and *S. enteritidis*.

4.3.4. Humid-Gaseous ozone treatment

The different susceptibility of the microorganisms towards 4 ppm of humid-gaseous ozone resulted in variations in the log reductions (Table 4.6). The combination of humidity and gaseous ozone had a strong effect and led to significant differences in the reduction of *S. putrefaciens*, *L. plantarum*, *L. casei*, *L. sakei*, *Ps. putida*, and *S. enteritidis*. The application of humid-gaseous ozone treatment appears to be more effective than gaseous ozone treatment alone for these microorganisms. The humid-gaseous ozone was less effective than gaseous ozone treatment for *B. subtilis*, *S. equorum* and *E. coli*, with both the dry and wet methods (Figure 4.9). Therefore, humid-gaseous ozone treatment is appropriate for the reduction of *B. subtilis*, *S. equorum* and *E. coli*.

Table 4.6 The log reduction of 4 ppm humid-gaseous ozone against bacteria.

Microorganisms	Log reduction (log cfu/spatula)	SD
Shewanella putrefaciens	3.48	0.28
Bacillus subtilis	1.27	0.23
Escherichia coli	1.27	0.13
Lactobacillus plantarum	2.68	0.43
Lactobacillus casei	4.97	0.71
Lactobacillus sakei	4.65	0.21
Listeria innocua	3.66	0.35
Pseudomonas putida	2.56	0.41
Salmonella enteriditis	3.03	0.21
Staphylococcus equorum	2.47	0.36

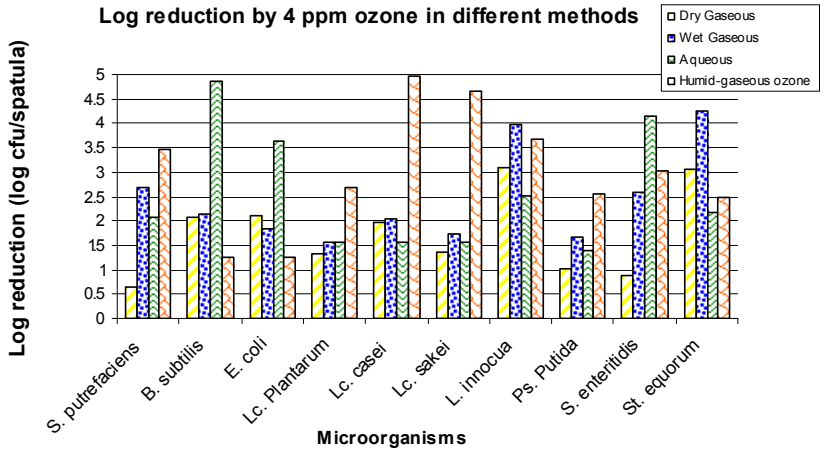

Figure 4.9 The log reduction by 4 ppm of gaseous (dry and wet methods), aqueous and humid-gaseous ozone.

4.3.5. Ultraviolet treatment (UV-treatment)

Humidity (the wet method) also appeared to reduce the load of microorganisms treated with UV more efficiently than dry method (Figure 4.10).

Although the agar plates may not completely represent the results of UV inactivation, higher log reductions were observed with the plates than with the spatulas for *Ps. putida*, *L. innocua* and *A. niger*, but not for *B. subtilis*. The agar appeared to create a barrier, protecting *B. subtilis* against the UV radiation. The dosage of UV irradiation should be measured to identify doses which effectively eliminate microorganisms.

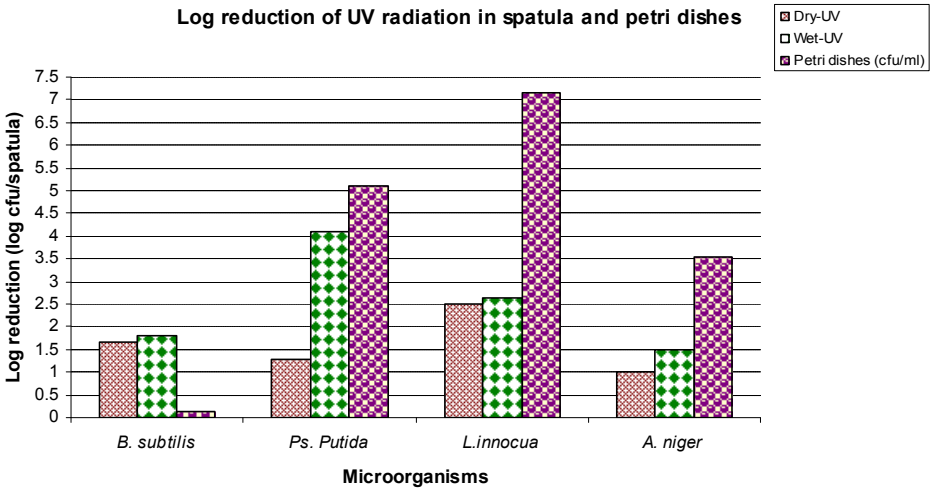

Figure 4.10 Log reduction by UV radiation in spatula with dry, wet method and petri dishes.

4.4. Conclusions

The present study shows the potential antibacterial and antimycotic uses of ozone, which could be in the gaseous, aqueous (ozonated water) and humid gaseous forms. The microorganism reduction varied for each type of treatment. This study proved the importance of moisture and the presence of water in the efficacy of ozone treatment.

Treatment with 4 ppm of gaseous ozone by the wet method may effectively kill *S. putrefaciens*, *L. casei*, *L. sakei*, *L. innocua*, *S. equorum* and *A. niger*. Treatment with 2 ppm of the gaseous ozone by the wet method effectively kills *Ps. putida*, while 4 ppm of aqueous ozone is suitable for the reduction of *B. subtilis*, *E. coli* and *S. enteritidis*.

Humid-gaseous ozone constitutes an appropriate method for the reduction of *S. putrefaciens*, *L. sakei*, *L. casei*, *L. plantarum*, *Ps. putida*, and *S. enteritidis*.

Further research is needed to clarify the effectiveness of ozone as an antibacterial and sanitising agent against different bacterial and fungal species on stainless steel surfaces.

5. Use of bioprotective cultures to improve the hygienic- sanitary quality and sensory attributes of hamburger

5.1. Introduction

The hamburger (also called svizzera or pressata in Italian) is a patty of pressed ground meat, usually composed of beef and/or chicken, cooked mainly on plates (Cucina, 2012). The entry of fast food chains into the international market led to the development of this sandwich, which consists of bread, minced meat (hamburger), sausage, and seasonings (Rai, 2012). The word "hamburger" is derived from Hamburg, the second largest city in Germany. In German, "hamburger" could be a noun describing an individual from Hamburg or an adjective describing something from Hamburg (Cooking, 2012).

In the late eighteenth century, Germany possessed the largest ports in Europe. During the first half of the nineteenth century, Hamburg established itself as one of the largest transatlantic ports in Europe. Most of the immigrants from German-speaking countries and Europe embarked to the United States from this port. The immigrants brought some of their favourites recipes with them, including the hamburger, and introduced their culinary customs to their host countries.

The origins of the hamburger remain unclear. The oldest recorded document referring to hamburger steak was in a menu from the Delmonico restaurant in New York, where hamburger steak was served for $0.11 (Wikipedia, 2012).

Over time, hamburgers started to be commercialised and introduced to the world as fast food by America (Food, 2010). Many different recipes for hamburger have been created over the years, and these are not only made of beef but also of chicken, pork, turkey, fish and vegetables.

In Italy, the nutritional values for the same amount and type of commercialised hamburgers are categorised as "light," with the following ingredients: energy: 164 kcal/100 g; protein: 16.8 g; soy protein: 3.9 g; carbohydrate: 4.1 g, carbohydrates from sugar: 1.7 g; total fat: 8.9 g; saturated fat: 4.4 g; unsaturated fat: 4.1 g; polysaturated fat: 0.3 g; sodium: 0.4 g; phosphor: 148 mg to 18.5% RDA; dietary fibre: 0.7 g (Menu, 2012).

There are several machines used to form hamburgers. These machines produce hamburgers of identical form and homogenous composition, permitting high yields, reduced production time, and maximum hygienic conditions without any manipulation. The automatic hamburger forming machine (Nilma. Parma. Italy) allows the operator to choose the desired thickness of each piece of hamburger, up to 22 mm. The machine is fully automatic with a productivity that can reach 2,200 pieces per hour. There are models with a single head, double head (suitable for circular, oval, square or other preferred shapes of hamburgers), and triple head that can produce any shape or dimension at a rate of 150 patties per minute.

Figure 5.1 The MS-automatic moulding machine for hamburgers (Nilma, 2012).

The muscle meat from healthy animals is usually free of germs but is susceptible to microbial contamination by both pathogenic and spoilage bacteria, even up to the moment of cooking and consumption. The potential source of contamination depends on the condition of the animals before, during and after slaughter and the transportation, marketing and consumer handling of the meat. (Beumer and Kusumaningrum, 2003; Luning et al., 2011; McDonald and Sun, 1999). Microorganisms such as *Pseudomonas* spp, *Brochothrix thermosphacta*, *Shewanella putrefaciens*, and cocci-coagulase-negative Enterobacteriaceae can cause spoilage. Contamination can also be caused by psychrotropic and pathogenic species such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium perfringens*, *Campylobacter jejuni* and *Yersinia enterocolitica* or by Enteropathogenic species such as *Escherichia coli* and *Salmonella spp*. During the transport of meat from the farm to the customer, the shelf-life can be reduced (Borch et al., 1996; Raab et al., 2011) and the risk of microbiological contamination exists (Desmarchelier et al., 2007; Pin et al., 2011); therefore, it is necessary to maintain the hygienic-sanitary quality of the meat. The use of modified atmosphere packaging (MAP), which contains oxygen and carbon dioxide, is intended to preserve and increase the shelf life of hamburgers. During refrigerated storage, the presence of high concentrations of oxygen (40-80%) causes the transformation of myoglobin into oxymyoglobin, a process that results in the bright red colour of meat (Lambert et al., 1991). Carbon dioxide (20-30%) in MAP inhibits the growth of aerobic spoilage and pathogenic bacteria (Zakrys et al., 2009). The presence of oxygen maintains the attractive appearance of the burgers for a few days (Djenane et al., 2003; Paleari et al., 2004; Scanga et al., 2000), but the burger colour eventually darkens due to the growth of aerobic bacteria (Zee et al., 1984; Zhao et al., 1994). At the beginning, carbon dioxide in the MAP inhibits the growth of aerobic microorganisms, but higher concentrations of carbon dioxide are needed to prevent the growth of aerobic spoilage bacteria. However, carbon dioxide concentrations above 30% are unlikely to be used because oxygen concentrations lower than 60% lead to the loss of colour and brightness of the meat (Paleari et al., 2004). Carbon dioxide appears to cause its maximum inhibitory activity at concentrations above 25% (Gill, 1996; Gill and Badoni, 2002; Gill and DeLacy, 1991). Commercial hamburgers packaged in MAP and stored at refrigeration temperature have a shelf-life of 7-9 days, based on the expiration date written on the packaging. Recently, combinations of hygienic quality control and protective technology have been used to improve and extend the shelf life of meat and meat products by limiting the growth of spoilage and pathogenic bacteria. One of the protective technologies is the use of bioprotective cultures(Comi et al., 2011; Devlieghere et al., 2004; Gill and DeLacy, 1991; Proller, 1997; Vasilopoulos, De Maere, et al., 2010; Vasilopoulos, De Mey, et al., 2010; Vasilopoulos et al., 2008; Vermeiren et al., 2004). In a study by Comi et al., (2011), bioprotective cultures of *L. curvatus* and *L. sakei* improved the physical-chemical and sensory properties of sliced cooked ham packed in MAP. The authors observed that both of the bioprotective cultures could extend the shelf-life, give an attractive colour, and inhibit the alteration and presence of white sticky slime produced by indigenous LAB in sliced cooked ham stored at 4 ºC. Therefore, the objective of this study was to use bioprotective cultures to improve the hygienic and sanitary quality and sensory attributes of hamburger and to extend its shelf-life.

5.2. Materials and Methods

5.2.1. Sample preparation, storage condition and sampling methods

Meat cut from different anatomical parts of adult cattle were ground, mixed, divided into 4 batches of 50 kg each, and then formed into patties. The first lot was packaged directly and used as the control, while the other lots were spiked with a mixture of lactic acid bacteria (LAB) at a final concentration of 10^5 cfu/g of product. Lot 2 was spiked with a mix of *Lactobacillus carnosus*/*Lactobacillus sakei* + *Staphylococcus xylosus* at a ratio of 1/1. Lot 3 was spiked with *Lactococcus lactis* subsp. *lactis*/*Lactobacillus sakei* + *Staphylococcus xylosus* at a ratio of 1/1. Lot 4 was spiked with *Lactobacillus carnosus*/*Lactobacillus sakei* + *Staphylococcus xylosus* at a ratio of 1/2. The burgers were packed in modified atmosphere packaging (MAP) consisting of 70% O_2 and 30% CO_2 and placed inside 15x10x3 cm rectangular trays of 200 μm in thickness, made of PET/PE/EVOH/PE ANTIFOG - EVOH. The trays were laminated with a top film consisting of APET/PE/EVOH/PE. The packaged burgers were stored at 4±2 ºC for 12 days in artificial light. A 0, 6, 9 and 12 days, 10 boxes were taken for microbiological and physico-chemical analysis.

5.2.2. Bacterial strains, preparation and inoculation of hamburgers

Lactobacillus carnosus, Lactobacillus sakei, Lactococcus lactis subsp. *lactis* and *Staphylococcus xylosus* used in this experiment were obtained from the Italy branch supplier of Chr. Hansen, Denmark.

The cultures were rehydrated in Peptone physiologic salt solution (9 g/L NaCl, 1 g/L bacteriological peptone, Oxoid. Milan. Italy) at 40 °C for 1h. Suspension was serially diluted in peptone physiologic salt solution, and the appropriate decimal dilutions were spread-plated on MRS agar plate to determine the actual concentration of the culture. A stock culture (mother solution) was prepared from an appropriate dilution to obtain a final concentration of 10^7 cfu/ml. The mother solution was used directly for inoculation of the hamburger and the final bacterial cell concentration was of 10^5 cfu/g of hamburger.

5.2.3. Microbiological analysis

For microbiological analysis, a 10 g sample of ham was taken aseptically, diluted tenfold in 90 ml of Peptone Physiologic Salt, and homogenised in a Stomacher for 1 minute. Serial decimal dilutions of the homogenates were made using Peptone Physiologic Salt. Aliquots (0.1 ml) of the appropriate dilution were prepared for spread plating to allow the enumeration of the total bacterial counts and were measured in Plate Count Agar incubated at 30 ºC for 48 h.

The enumeration of lactic acid bacteria was performed using de Man-Rogosa-Sharpe media (pH 5.4) with 15 g/l agar, which was modified with 1 ml/l Delvocid (25 mg/ml). The plates were incubated in microaerophilic conditions at 42 ºC for 48 h prior to the bacterial enumeration. Yeasts and moulds were enumerated in malt agar incubated at 30 ºC for 72 h. *Escherichia coli* was quantified in Violet Red Bile Agar (Oxoid, Ltd., Basingstoke, Hampshire, England) following incubation at 44 ºC for 24 h. *Staphylococcus aureus* were enumerated in Baird-Parker agar medium supplemented with egg yolk tellurite emulsion and incubated at 35 ºC for 24 h, after confirmation with a coagulase test. *Brochothrix thermosphacta* were enumerated in streptomycin-sulfate-thallous acetate-cycloheximide agar with selective supplements following incubation at 22 ºC for 48-96 h. Sulphite-reducing clostridia were quantified in DRCM agar (Merck. Darmstadt. Germany) following incubation at 37 ºC for 24-48 h in an anaerobic jar with an AnaeroGen sachet (Oxoid. Basingstoke. England). *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella sp*p. (ISO

6579), *Listeria monocytogenes* (ISO 11290-1) and *Yersinia enterocolitica* were detected according to the recommended methods for the microbiological analysis of foods (Lombardy Region – Official Bulletin of the Lombardy Region, 4th Suppl. Extraordinary No. 24, June 17th 1995 and methods OM 7/12/93).

5.2.4. pH measurements

The pH value was measured at 10 different points in each package using a pH meter (Basic 20, Crison Instruments, S.A. Allela. Barcelona. Spain). The pH value was measured directly in the product by inserting a pH meter probe into the sample.

5.2.5. Total Volatile Basic Nitrogen (TVB-N) measurements

The total volatile basic nitrogen (TVB-N) was evaluated by the method proposed by Pearson (Pearson, 1973).

5.2.6. Color measurements

The colour was measured using a Minolta Chromameter CR-200 and the CIE Lab system. After calibration with standard white tiles, the chromameter was positioned perpendicular to the patty surface, and 10 different positions were evaluated for each sample immediately after the package was opened. The evaluated parameters were L*, a*, and b*. L* describes the white intensity or brightness, with values ranging from 0 (black) to 100 (white). The a* values describe the redness (a*> 0) and b* describes the yellowness (b*> 0). The final value was expressed as the respective average of ten measurements.

5.2.7. Sensory analysis

Sensory analysis was assessed by 12 non-professional panellists. Cooked burgers from 4 lots, with each lot containing 10 packages, were evaluated. The panellists were asked to identify the products in descending order from the best to the worst, taking into account the following parameters: odour (fermented, rancid), taste (sweet, sour, fresh, pungent, meat-taste, rancid) and flavour (ammonia, sweet, sour, bitter) (Baublits et al., 2006; Válková et al., 2007).

5.2.8. Statistical analysis

The values of the various parameters were compared by one-way analysis of variance. The averages were compared with the Tukey's honest significant test through the Statistical Graphics software package.

5.3. Results and Discussion

The performances of the different microbial populations and the pH values of the different batches of analysed hamburgers are shown in Figure 5.2 - Figure 5.5 and Table 5.1. According to the trend of the total microbial count (TMC) (Figure 5.2), a difference in the level of contamination was already observed at time 0. There was difference of approximately 0.5 log or 1 log cfu/g. Lots 1 and 2 presented an initial TMC level of 6.0 log cfu/g, while Lots 3 and 4 presented values of 7.0 and 6.5 log cfu/g, respectively. These TMC values exceeded the limits proposed by the EEC Regulation 2073/2005, which gave maximum TMC values 5.7 log cfu/g (m, 3 U.C) and 6.7 log cfu/g (M, 2 UC) at the end of production. In lots 2, 3, 4, the high TMC is not due to the starter inoculation, as this was added to the meat at 5 log cfu/g. These TMC values are different from those obtained by other authors for minced meat marketed in Italy (Marino et al., 1995; Paleari et al., 2004;

Santoro et al., 1994). Paleari et. al, (2004) observed TMC values less than or equal to 5 log cfu/g in ground meat.
During refrigerated storage, the TMC values of 4 lots increased up to 8 and 9 log cfu/g, despite the presence of CO_2 in MAP. This increase might be due to the activity of the psychrotropic microorganisms *Brochothrix thermosphacta*.

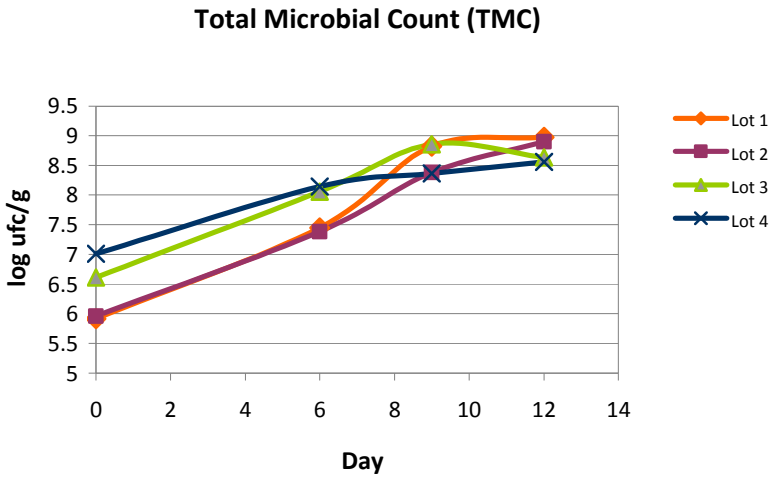

Figure 5.2 The evolution of total microbial count of hamburger packaged in MAP and stored at 4±2 ºC.

Figure 5.3 shows the performance of yeast. In this case, the initial concentration differed depending on the lot, with values between 1.7 and 2.5 log cfu/g. Lot 3 appeared to be the least contaminated by yeast initially but increased by 1 log cfu/g by day 12, making it the most contaminated lot. The yeast counts for Lots 1, 2, and 4 remained constant over 12 days.

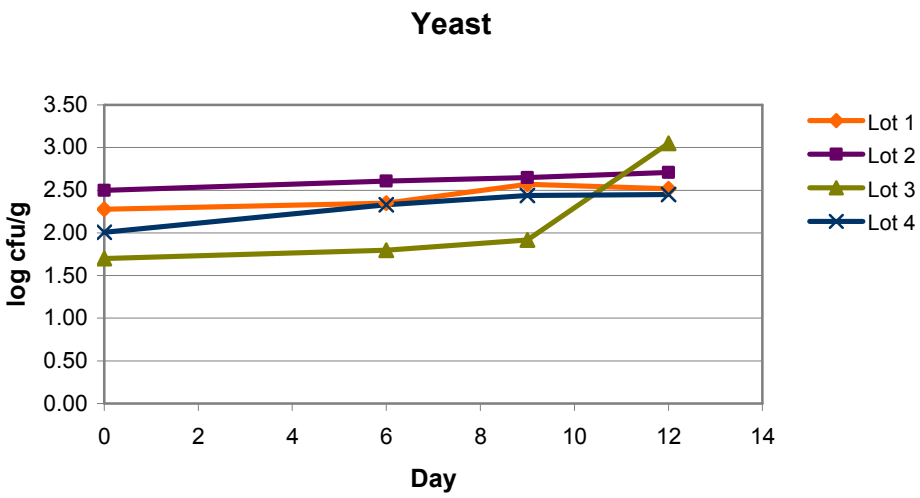

Figure 5.3 The count of yeast in hamburger packed in MAP and stored in 4±2 ºC.

Figure 5.4 shows the growth of *Brochothrix thermosphacta*, a typical psychrotropic microorganism, which is responsible for the alteration of meat products and refrigerated-meat products (Comi et al., 2011; Paleari et al., 2004). In Lots 2, 3, and 4, where bioprotective cultures were added to the hamburgers, barely any growth of *B. thermosphacta* was observed, with an increase of approximately 1 log cfu/g by day 12. Conversely, there were *B. thermosphacta* increases of 2 log cfu/g in the control (Lot 1). Clearly, the bioprotective cultures slowed down and inhibited the growth of *B. thermosphacta*. Similar results have also been demonstrated in different products by other authors. Santoro et al., (1994) have observed increases of *B. thermosphacta* up to 8 and 9 log cfu/g in minced meat packaged in MAP and stored in refrigeration up to 12 days.

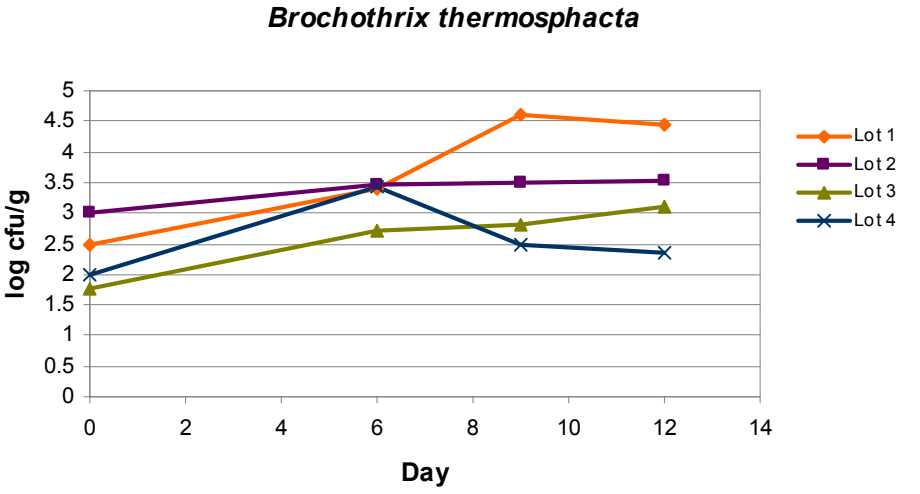

Figure 5.4 The performance of *B. thermosphacta* in hamburger packed in MAP and stored in 4±2 ºC.

Figure 5.5 shows the growth of the lactic acid bacteria (LAB). Without the addition of bioprotective cultures, the initial LAB contamination of Lot 1 was almost 4 log cfu/g. Other authors (Marino et al., 1995; Paleari et al., 2004; Santoro et al., 1994) have reported LAB concentrations similar to those observed in Lot 1.
In Lots 2, 3, and 4, which were purposely inoculated with bioprotective cultures, the LAB concentration was 5 log cfu/g, representing the concentration of the initial inoculum. However, the concentration of the LAB increased regardless of the presence of 70% O_2 in the MAP. As microaerophilic microorganisms, the LAB grew well in conditions of less than 3-5% oxygen. As shown at day 12 of hamburger observation, the LAB concentration of Lot 1 was slightly higher than 7 log cfu/g, whereas in Lots 2, 3, and 4, the concentrations were between 8 and 9 log cfu/g. However, these growth trends of LAB could not be compared with the findings of other authors who did not intentionally inoculate meat with bioprotective cultures.

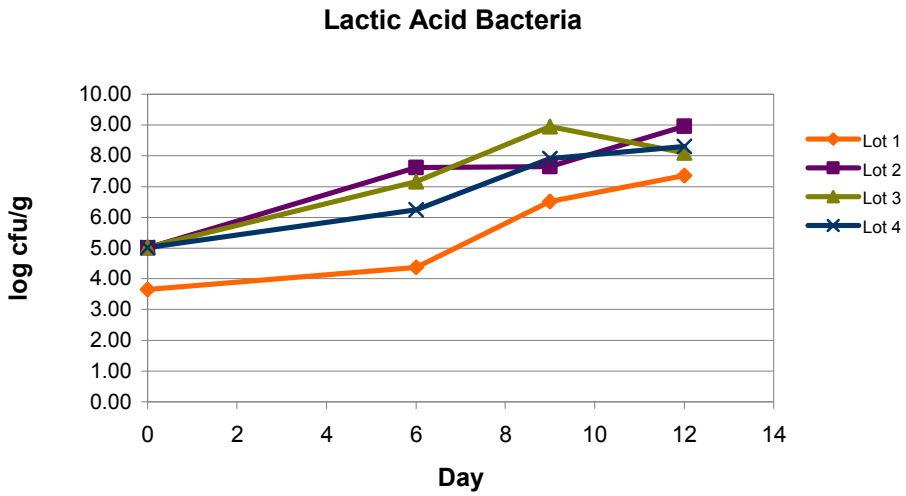

Figure 5.5 The growth of Lactic acid bacteria in hamburger packed in MAP and stored in 4±2 ºC.

Table 5.1 The dinamic microbial populations in hamburgers.
Absence: absence in 25 g

Time	**0**	**6**	**9**	**12**
Microorganisms				
E. coli	< 1.0	< 1.0	< 1.0	< 1.0
Clostridia H_2S+	< 1.0	< 1.0	< 1.0	< 1.0
S. aureus	< 1.0	< 1.0	< 1.0	< 1.0
Salmonella spp.	absence	absence	absence	absence
L. monocytogenes	absence	absence	absence	absence
Campylobacter spp.	absence	absence	absence	absence
Y. enterocolitica	absence	absence	absence	absence

Table 5.1 shows the dynamic microbial populations in the presence of pathogenic or potential indicators of faecal and environmental contaminants. The presence of *Clostridium* H_2S+ producers, *Staphylococcus aureus* and *Escherichia coli*, were below the threshold limit of the method (1 log cfu/g), while the classical meat pathogens such as *Salmonella spp*., *Listeria monocytogenes*, *Campylobacter spp*. and *Yersinia enterocolitica* were absent in 25 g of meat, as confirmed at each sampling time. The absence of *L. monocytogenes* and *Salmonella sp*p. seems unexpected because it is estimated that at least 20% of fresh meat is contaminated with *L. monocytogenes* and approximately 30% of meat is contaminated with *Salmonella* spp (Cantoni, personal communication), despite the application of strict microbiological hygienic controls. In Marino et al.(1995), on the previous work highlighted the presence of *Escherichia coli* exceed the limit imposed by the current Commission Regulation (EC) 2073/2005 in some samples of hamburgers. The work of Marino et al. highlighted the presence of *Escherichia coli* exceeding the limits imposed by the current Commission Regulation (EC) 2073/2005 in some samples of hamburgers. However, the presence of *St. aureus* and Clostridium H_2S+ producers correlated with the findings by Marino et al. (1995). The absence of *L. monocytogenes* and *Salmonella* spp. was attributed to the quality of the meat and to the randomness in sampling.
These authors always isolated *L. monocytogenes* and *Salmonella spp* from meat and/or meat products (Juven et al., 1998). In previous microbiological hygienic controls performed by the same factory on lots of fresh meat (data not shown), *Salmonella sp*p and *L. monocytogenes* were isolated from 40% and 30% of the analysed samples, respectively.

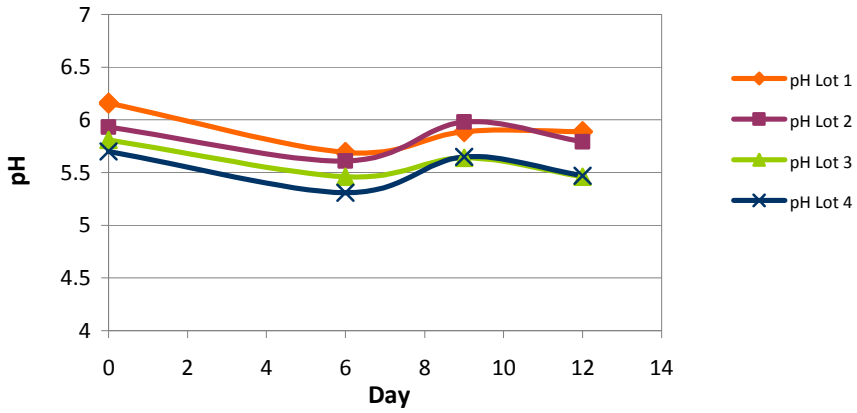

Figure 5.6 The pH of hamburger packaged in MAP and stored at 4±2 ºC.

The LAB activity in the lots inoculated with the bioprotective cultures is also shown in Figure 5.5. The trends in pH and total volatile basic nitrogen (TVB-N) are presented in Figure 5.6 and Table 5.2. Lots inoculated with bioprotective cultures demonstrated lower pH levels. The pH of Lot 1 remained higher than those of Lots 2, 3 and 4 up to day 12; however, Lot 1 had a higher pH even at day 0 (Figure 5.6). The TVBN of Lot 1 increased significantly over time and showed higher levels than the other lots at day 12. According to the TVBN limit of 30 mg of nitrogen/100 g (as described for fishery products by the Commission Decision 95/149/EC of 8 March 1995), the hamburgers of Lot 1 were inedible within 9 days of storage, while Lots 2, 3, and 4 were edible up to 12 days of storage. It is plausible that competition caused by the bioprotective starters slowed and/or inhibited the alteration and consequently reduced the production of TVBN, as shown in Table 5.2.

Table 5.2 The TVBN of hamburgers packaged in MAP and stored at 4±2 ºC.

Lot	0	6	10	12
1	16 ± 1 a	24 ± 3 a	35 ± 4 a	43 ± 5 a
2	15 ± 2 a	21 ± 2 a	25 ± 3 b	28 ± 4 b
3	15 ± 1 a	19 ± 3 a	21 ± 2 b	24 ± 6 b
4	15 ± 3 a	20 ± 3 a	22 ± 3 b	25 ± 6 b

ab: media values of samples mg N/100 g. Different letter indicated significant difference $p < 0.05$.

Table 5.3 shows the results of the colour evaluation using the L*, a* and b* parameters at days 0 and 12. No significant differences were observed between Lot 1 and the other lots. Until day 12, the L*, a* and b* parameters were similar between Lots 1, 2, 3 and 4. As expected, there were significant colour changes in the hamburgers of all lots after 12 days of storage. During this time, the hamburger colour changed from bright red to dark red (darkening), due to the oxidation of myoglobin, which is caused by the presence of oxygen in the MAP. Visual analysis determined that the colour of the hamburgers in Lots 3 and 4 were more attractive than those of Lots 1 and 2 at day 12 (Figure 5.7 – 5.10).

Figure 5.7 The hamburgers of Lot 1, 2, 3, 4 at T0.

Figure 5.8 The hamburgers of Lot 1, 2, 3, 4 at T6.

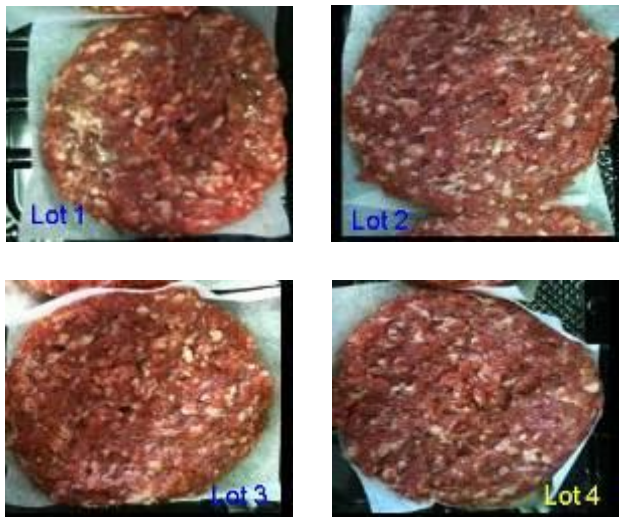

Figure 5.9 The hamburgers of Lot 1, 2, 3, 4 at T9.

Figure 5.10 The hamburgers of Lot 1, 2, 3, 4 at T12.

Table 5.3 The colour of hamburgers treated with bioprotective culture.
a: the letter indicated significant difference $p < 0.05$ (mean ± standard deviation values)

	1			2			3			4		
Day	L*	a*	b*	L*	a*	b*	L*	a*	b*	L*	a*	b*
0	98.91±1.20	4.12 ±1.74	-7.13±2.97	104.32±4.63	5.46±1.62	-7.74±3.35	100[a]±5.19	5.19±3.33	-9.07±2.34	105.83±0.70	3.11±0.81	-10.6±1.23
6	101.81±3.06	2.31±0.51	-6.46±0.57	105.15±2.44	3.87±0.56	-4.25±0.12	106.38[a]±0.88	2.76±0.48	-6.09±0.47	103.91±2.24	2.44±0.44	-7.73±0.58
9	105.84±7.54	-1.08±4.86	-5.21±0.70	106±6.48	2.06±3.54	-2.96±0.77	110.52[a]±2.43	-2.37±2.89	-1.51±4.39	103.03±1.73	1.59±2.65	-5.17±2.54
12	42.78±2.83	18.4±2.50	5.8±1.30	41.82±0.95	18.22±1.41	6.08±1.42	40.41[a]±4.04	17.37±1.51	6.40±0.19	43.57±4.02	16.67±1.19	5.58±0.83

Therefore, the extended shelf-life observed in hamburgers supports the use of bioprotective cultures. Hamburgers were acceptable up to 9 days of storage at 4±2 ºC, in terms of the low microbial loads and the TVBN values, which were below the acceptable limits (30 mg of nitrogen/100 g).

The sensory analysis supported this same conclusion. Table 5.4 shows that the bioprotective cultures improved the sensory attributes of the hamburgers. Hamburgers with bioprotective cultures did not present odours, flavours or sticky white slime indicative of deterioration. In contrast, a sticky-white slime was observed in some hamburgers from Lots 1 and 2. In Lot 2, this may have been due to the excessive growth of the bioprotective cultures (see Figure 5.5), as the concentrations of the lactic acid bacteria (9 log cfu/g) were significantly higher than in the other lots. The panellists preferred the taste of the hamburgers from Lots 3 and 4, which contained the bioprotective *Lactococcus lactis* subsp. *lactis*/*Lactobacillus sakei* + *Staphylococcus xylosus* (ratio of 1/1) and *Lactobacillus carnosus*/*Lactobacillus sakei* + *Staphylococcus xylosus* (ratio of 1/2), respectively. These microorganisms seem to have improved the sensory quality of the hamburgers and inhibited the growth of autochthonous bacteria (Figure 5.2).
Oxidation was not observed in the four lots of hamburgers here, regardless of the presence or absence of bioprotective cultures. However, these findings are beneficial for the elimination of slimes, discolouration and browning caused by autochthonous LAB.

Table 5.4 The sensory panel scores of cooked hamburgers.

Sensory attributes	**Lot 1**	**Lot 2**	**Lot 3**	**Lot 4**
Fermentation	10/12	3/12	4/12	4/12
Rancid	5/12	4/12	3/12	3/12
Sweet	2/12	5/12	5/12	4/12
Pungent	10/12	5/12	5/12	5/12
Meat	3/12	6/12	6/12	9/12
Sour	6/12	6/12	7/12	7/12
Bitter	9/12	6/12	3/12	5/12
Ammonia	12/12	6/12	3/12	4/12
Presence of slime	7/12	7/12	4/12	5/12
Finale scores*	**4**	**3**	**1**	**2**

*Final scores: the panellists requested to ranked the products within the scale from 1 (excellence) to 4 (worst).

5.4. Conclusions

Bioprotective cultures, used as mixed cultures of *Lactobacillus carnosus*/*Lactobacillus sakei* + *Staphylococcus xylosus* (1/1 ratio), *Lactococcus lactis* subsp. *lactis*/*Lactobacillus sakei* + *Staphylococcus xylosus* (1/1 ratio), and *Lactobacillus carnosus*/*Lactobacillus sakei* + *Staphylococcus xylosus* (1/2 ratio), could be employed as bioprotective cultures in fresh meat, minced meat and hamburger. These cultures inhibit pathogenic bacteria and consequently improve the hygienic-sanitary and organoleptic qualities of the meat. This work demonstrated that bioprotective cultures inhibited *Brochothrix thermosphacta,* although typical meat pathogens such as *L. monocytogenes* and *Salmonella sp*p. were not detected. Bioprotective cultures were also able to reduce the TVBN to below 30 mg of nitrogen/100 g; although these standards are not enforced by law, they indicate a high quality product. The sensory traits of hamburgers were affected by the presence of the bioprotective cultures, as the odours, flavours, and sticky white slime indicative of deterioration were not observed. Therefore, the bioprotective cultures evaluated in this study can potentially extend the shelf life and improve the sensory properties of hamburger.

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