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MICROBIAL CULTURES AND LEAF EXTRACTS OF MYRTUS COMMUNIS L., STRATEGIES DESIGNED TO ENSURE QUALITY, SAFETY AND HEALTHY PROPERTIES OF FOOD PRODUCTS

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English abstract
Food quality is a wide topic, made up of several attributes such as health and hygiene safety, the correct nutrient intake and the sensory satisfaction. In their totality these properties correspond to the ability to meet the health expectations and answer hedonistic needs. As result of this awareness was the born of a new binomial, often inseparable, well defined by the term "*safety-health food*", which emphasizes the role of safety as an intrinsic part of the quality from which it cannot be separated due to the great impact on human health. Therefore, it can be affirmed that food security represent a precondition of food quality. This correspond to the absence of any danger to the consumer who uses food properly prepared, stored and handled.
Despite the advances in technology, food safety is still an open question, not only for developing countries, but also for the industrialized world.
Food represent given their physical and chemical properties a supportive environment for the growth and the proliferation of spoilage and pathogenic microbial species which are able to enter the body through the gastrointestinal tract where the often occur first symptoms of infection. The severity of the food diseases in humans varies, moving from mild symptoms to life-threatening conditions and the hazard intensity depends by several key factors moving from the health state, the immunity system conditions of the host and the antimicrobial resistance of bacteria, corresponding to the ability of microbes to resist the effects of drugs. The risks of contamination are ever present for this reason requires prevention and monitoring throughout the food chain.
The food industry increasingly tends to produce food with high organoleptic standards, with a long shelf life and with suitable nutritional values. At the same time the consumer requires intensified food security, food products minimally processed and that improve their state of health, in combination with a reduction of the use of chemical additives.
The increasing interest in the substitution of synthetic additives by natural ones and also the awareness that the prolonged use of antibiotics increase the resistance of many microorganisms to drugs, have encouraged research on preservatives coming from natural sources such as secondary metabolites of bacteria and plants. Therefore aim of this PhD thesis was to investigate the antimicrobial properties *in vitro* and *in situ* of an hydro-alcoholic extract obtained from leaves of Myrtus communis L. and of *Lb. curvatus* strain producing-bacteriocin(s).
Myrtus communis leaves extract obtained through maceration at ambient temperature for 24 hours was tested against 105 indicators strains tested among which food borne pathogens and spoilage bacteria belonging to different species, evaluating the chance to use it in food system through a development of an active packaging. The in vitro results showed a wide spectrum against both Gram positive (*Br. thermosphacta, L. monocytogenes, L. innocua, S. aureus*) and Gram negative bacteria (C. jejuni, C. coli and P. fragi). Among the pathogenic Gram positive bacteria *Listeria monocytogenes OH* was found more sensitive to the myrtle effect with MIC and MLC values respectively of 12.5 and 25 mg DM/ml, recording a diameter of inhibition zone of 26 ± 0.05 mm and a titer of 25600 AU ml-1. Results obtained through in vitro assays have been also confirmed by a challenge study carried out, inoculating L. monocytogenes OH in gorgonzola cheese added of myrtle leaves extract in concentration of 12.5 mg/g and stored at refrigerating temperature for 2 weeks, where the initial concentration of the pathogenic strain gradually decreased during 9 days until to disappear after 15 days.
Among Gram negative bacteria, *P. fragi* and *Campylobacter spp.* seems to appear equally sensitive to the myrtle leaves hydroalcoholic extract. Surprisingly, in literature there are no previous findings about the effect of myrtle on *Campylobacter*, in spite of its importance as a foodborne pathogen. Therefore, positive data obtained against this specie result very remarkable and interesting. Polyphenols characterized by mass spectrometry are the main molecules responsible of the antimicrobial activity of this hydroalcoholic myrtle leaves extract. One of their mode of action is the cellular membrane damage which was determined by spectrofluorimetric assay of cell viability assay.
Another pertinent objective of this thesis was to study the antagonistic activity *Lb. curvatus* 54M16 isolated from fermented sausage of Vallo di Diano *in vitro* and *in situ* against a pathogen strain of great food interest which is *L. monocytogenes*. Moreover, the technological characteristics were determined in order to investigate its suitability to be used as starter and protective culture for sausage fermentation.
The strain was found to possess the gene that encodes the production of different bacteriocins, including the sakTα, the sakTβ, the curvacina A, the sakX and the SAKP, identified by MALDI-TOF-MS analysis. The production of bacteriocins was monitored in MRS broth and evaluated for different temperature conditions (10, 15, 20, 25 and 30 ° C) and times (0, 24, 48 and 72 hours). The strain was able to produce the bacteriocin(s) and in all the conditions listed above, except when incubated at a temperature of 10 ° C, recording the maximum activity 6400 (AU ml -1) after 24 hours at 20, 25 and 30 ° C .
The antilisterial activity has also been investigated *in situ*, adding the strain to the fermented sausage samples which were intentionally inoculated with the strain of L. monocytogenes ATCC 7644. The results show the end of the maturation period, a reduction of the microbial load of 0.5 Log compared to the sample without the 54M16 strain. Finally, to evaluate the potential use of this strain as a starter culture in the sausage fermentations were also determined its technological characteristics, resulting that *L. curvatus* 54M16 strain has a good acidifying and proteolytic activities being able to hydrolyze the sarcoplasmic proteins.

Italian Abstract
La qualità alimentare è un vasto argomento, definito e determinato da diversi fattori quali la salubrità, la sicurezza igienico-sanitaria, il corretto apporto di nutrienti e la qualità sensoriale. Nel loro insieme queste proprietà corrispondono alla capacità di soddisfare le aspettative del consumatore sia da un punto di vista salutare oltre che puramente edonistico. Da questa consapevolezza deriva la nascita di un nuovo binomio, spesso inscindibile, ben definito con il termine di "sicurezza alimentare-sanitaria", che sottolinea il ruolo della sicurezza come parte intrinseca della qualità da cui non può essere separato a causa del grande impatto sulla salute umana. Pertanto, è possibile affermare che la sicurezza alimentare rappresenta un presupposto della qualità alimentare, corrispondente all'assenza di qualsiasi pericolo per il consumatore che consuma alimenti adeguatamente preparati, conservati e manipolati.
Nonostante i progressi della tecnologia, la sicurezza alimentare risulta essere ancora una questione aperta, non solo per i paesi in via di sviluppo, ma anche per quelli industrializzati.
Date le loro proprietà fisiche e chimiche, gli alimenti rappresentano un ambiente favorevole alla crescita e alla proliferazione di specie microbiche patogene ed alterative che sono in grado di entrare nell'organismo umano attraverso il tratto gastrointestinale dove spesso si verificano i primi sintomi di infezione. La gravità delle malattie di origine alimentare negli esseri umani è variabile, passando da sintomi lievi a condizioni di pericolo di vita. L'intensità del rischio dipende da diversi fattori come lo stato di salute e le condizioni del sistema immunitario dell'ospite, e la resistenza batterica, intesa come resistenza ai farmaci. Poiché il rischio di contaminazione è onnipresente, risulta necessario prevenirlo e controllarlo lungo tutta la catena alimentare. L'industria alimentare tende sempre più a produrre alimenti con elevati standard organolettici, con una shelf life prolungata e con valori nutrizionali appropriati. Allo stesso tempo, il consumatore richiede alimenti più sicuri, minimamente trasformati e salubri, che non presentino additivi chimici.
Dunque, il crescente interesse per la sostituzione di additivi sintetici con quelli naturali e la consapevolezza che l'uso prolungato di antibiotici aumenta la resistenza di molti microrganismi ai farmaci, hanno incoraggiato la ricerca di conservanti provenienti da fonti naturali, come i metaboliti secondari di batteri e piante. Pertanto, lo scopo di questa tesi di dottorato è stato quello di studiare le proprietà antimicrobiche *in vitro* ed *in situ* di un estratto idroalcolico ottenuto da foglie di *Myrtus communis L.* e di un ceppo di *Lb. curvatus* produttore di batteriocine per un loro potenziale impiego in sistemi alimentari.
L'estratto idroalcolico di foglie di mirto (WEME70) ottenuto mediante 24 ore di macerazione a temperatura ambiente, è stato testato contro 105 ceppi indicatori tra i quali erano presenti patogeni ed alterativi di origine alimentare appartenenti a specie diverse, ed è stato utilizzato per lo sviluppo di un packaging attivo al fine di impiegarlo in matrici alimentari. I risultati *in vitro* hanno mostrato un ampio spettro d'azione nei confronti sia di batteri Gram-positivi (*Br. thermosphacta, L. monocytogenes, L. innocua, S. aureus*) che Gram-negativi (*C. jejuni, C. coli e P. fragi*). *L. monocytogenes* OH è risultato essere il patogeno più sensibile tra i Gram-positivi mostrando valori di MIC e MBC rispettivamente pari a 12,5 e 25 mg SS/ml, un diametro di inibizione di 26 mm e un titolo di 25600 AU ml $^{-1}$. I risultati ottenuti *in vitro* sono stati confermati da uno studio *in situ*, dove un formaggio Gorgonzola, inoculato con il ceppo di *L. monocytogenes OH* ed addizionato dell'estratto in concentrazione finale di 12,5 mg SS/g è stato conservato a temperatura di refrigerazione per un periodo complessivo di 2 settimane, durante le quali è stata determinata la carica microbica del patogeno che mostrava una riduzione graduale fino a risultare totalmente assente al termine dell'esperimento.
Tra i batteri Gram-negativi, invece, non sono state rilevate differenze significative tra i ceppi di *P. fragi* e *Campylobacter spp*. in termini di sensibilità all'estratto. Sorprendentemente, ad oggi in letteratura non sono presenti dati che dimostrino l'effetto antimicrobico di estratti di mirto nei confronti di *Campylobacter spp*, nonostante l'indiscussa importanza di tali patogeni. Pertanto, i soddisfacenti risultati ottenuti nei confronti di tale specie risultano interessanti e promettenti. Diversi composti polifenolici caratterizzati mediante spettrometria di massa sono risultati essere le molecole principalmente responsabili dell'attività antimicrobica di questo estratto idroalcolico di foglie di mirto. Mediante saggio fluorimetrico della vitalità cellulare è stato inoltre determinato uno dei meccanismi d'azione di tali molecole che consiste nel danneggiamento della membrana cellulare.
Altro obiettivo di questa tesi è stato lo studio e la valutazione dell'attività antimicrobica *in vitro* e *in situ* di un ceppo di *Lb. curvatus* 54M16 isolato da salsiccia fermentata di Vallo di Diano, in particolare contro un ceppo di *L. monocytogenes* ATCC 7644 risultato particolarmente sensibile. Il ceppo risulta possedere il gene che codifica la produzione di diverse batteriocine, tra cui la sakT$_\alpha$, la sakTβ, la curvacina A, la sakX e la sakP, identificate mediante analisi MALDI-TOF-MS. La produzione delle batteriocine da parte del ceppo in MRS brodo è stata monitorata e valutata in diverse condizioni di temperatura (10, 15, 20, 25 e 30°C) e tempi (0, 24, 48 e 72 ore). Il ceppo era in grado di produrre la batteriocina/e in tutte le condizioni sopra elencate, tranne quando incubato alla temperatura di 10°C, registrando la massima attività 6400 (UA ml $^{-1}$) dopo 24 ore a 20, 25 e 30°C. L'attività antimicrobica nei confronti di Listeria è stata inoltre investigata *in situ* , addizionando il ceppo ad insaccato carneo inoculato intenzionalmente con il ceppo di *L. monocytogenes* ATCC 7644. I risultati mostrano al termine del periodo di stagionatura una riduzione della carica microbica di 0.5 Log rispetto al campione non addizionato del lattobacillo. Infine, per valutare il potenziale impiego di questo ceppo come coltura starter nella preparazione di fermentazioni carnee sono state determinate le sue caratteristiche tecnologiche, da cui è emerso che il ceppo di *L. curvatus* 54M16 possiede una buona capacità acidificante, e proteolitica risultando in grado di idrolizzare le proteine sarcoplasmatiche.

Preface

Food quality and safety have been of great interest in the last 15 years in the public discussion, in the food industry and in research. Motivations can be traced inthe great changes in the world food system, which have introduced new critical points to resolve, increasing the sensitivity in terms of safety and quality. Despite of modern improvements in slaughter hygiene and food production techniques, the food safety is an increasingly important public health issue **(WHO, 2002)**. Modern technologies, good manufacturing practices, quality control in food processing and hygiene and safety concepts such as risk assessment and HACCP have reduced but not eliminated the likelihood of food-related illness and product spoilage in industrialized countries. The problem of foodborne illness is still prevalent, with serious consequences for public health and socio-economic conditions of national and international **(FAO, 2003).** In fact, several food scares and health emergencies occurred between the old and the new millennium, have generated international agreements on the regulation of agricultural and agro-food products, amplifying the legislative activity of the European Union, especially view to improving standards of health and hygiene in the food chain ("from farm to fork"). Therefore in addition tothe existing hurdle technologies, new methods are needed to ensure the microbiological safety of food and improve technological processes, without giving up to the quality and healthiness of the product. These goals are to be achieved, taking into account the current needs and demands of the consumers. Over the past decades, important changes in our food system, food environments and consumption have been driven by technological advances which have influenced and changed the eating habits of the people. In response to these changes, the food market is due to evolve. In fact, simultaneously to a strong expansion of health food sector, was registered a change in the variety of products available on the market **(Grevink et al. 2002).** This trend can be explained by the widespread awareness that health is closely related to food. Therefore, the food is not considered only as a nutritional source but also as a preventive tool of health. One aspect that is attracting particular attention from the experts is the consumers interest not only for the product but also for the manufacturing process used to develop the specific product**(de Barcellos et al., 2010).**Especially the Western population showed a trend toward consumerism called "green", which translates into the predilection by consumers of food products with a reduced content of chemical additives and a low impact on the environment **(Smid and Gorris, 1999).** A possible alternative between different technologies already available is represented by the bio-preservation, able to exploit the potential antimicrobial activity of natural compounds which play an important role in the natural defence or competition systems of living organisms (ranging from microorganism to insect, animals and plants). The environment is widely populated from many types of natural antimicrobials mainly represented by the secondary metabolites of plants and microorganisms, which represent a promising alternative to chemical preservatives.
The problems related to the application of conventional antibiotics, including antimicrobial resistance, environmental problems, carcinogenity, side effects and high costs, have contributed to the development of novel technologies for food preservation employing natural antimicrobials such as bacteriocins and plant extracts to inhibit the microbial growth in many food matrices (**Mauriello et al. 2004; Gálvez et al. 2007; Ercolini et al. 2010; La Storia et al. 2012; Mauriello and Villani, 2012**).
Among the main innovative technologies in food industry to preserve products appear the use of active packaging and the use of natural antimicrobials.
Based on this trend, the aim of this thesis work has been focused on the study of natural substances in order to develop potential new systems to improve the quality and to ensure the microbiological safety of foods.

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1. State of art

1.1 Myrtus Communis L.

The chemical and physical properties of food allow colonization and development of a great number and variety of pathogenic and spoilage microorganisms as well as of microorganisms involved in desired fermentation processes. Food safety can be ensured through the creation of a hostile environment for unwanted microorganisms, capable at the same time to support the growing and the metabolic activities of the useful ones.

The current food industry trend follows the production of food with high organoleptic standards, with a long shelf life and with appropriate nutritional values. Meanwhile consumer demand consists of safer food, minimally processed and healthy, often identified in foods free of chemical preservatives..

The most common additives to appear on food labels are antioxidants (to prevent deterioration caused by oxidation), colours, emulsifiers, stabilisers, gelling agents and thickeners, preservatives and sweeteners.

The increasing interest in the substitution of synthetic additives by natural ones and also the awareness that the prolonged use of antibiotics increase the resistance of many microorganisms to drugs, have encouraged research on vegetable and fruit sources**(Reena Mohanka and Priyanka 2014)**.Natural products such as plant extracts and essential oils, taken from different parts of the plant (fruits, leaves, stems, flowers) are among the most alternative agents examined and studied at present to replace the conventional antibiotics, as they are able to produce a great variety of secondary metabolites, widely used as precursors or as compounds in the pharmaceutical industry. Medicinal plants, such as *Myrtus communis L.* are a source of new compounds which can be used in both the food industry and for medical purposes, primarily as antimicrobial agents.Recently, many researchers have focused their attention on extracts from herbs, such as oregano, rosemary, thyme and myrtle.

For its interesting beneficial properties, especially for the antimicrobial activity against several food pathogens, myrtle has been subject of study in this thesis work. Myrtus communis L. is one of the important aromatic and medicinal species belonging to the Myrtaceae family, comprising at least 133 genera in more than 3800 species distributed in temperate and tropical regions, is an evergreen shrubtypical of the Mediterranean flora but also present in some areas of the Middle East and Asia (**Twaij et al. 1988**). In Italy it grows along the coast and in the inner hills, and it is spread especially in the islands, where it is one of the most characteristic species. It is an evergreen sclerophyll shrub or small treehighly branched forming a close full head, compact, 1.8–2.4 m in height, covered with ovate or lanceolate evergreen leaves; it has solitary axillary white or rosy flowers, followed by black berry which is spherical in shape with dark red to violet in color. Its leaves are very fragrant, which is the reason for the extensive use of the plant in the perfume and cosmetic industries.Flowering occurs in the month of June. In the food industry, different parts of this plant haveseveral uses such as flavoring meat and sauces. In the industrial formulation myrtle leavesare also used in sweet liquors with advertised digestive properties and most commonly foods flavoured with the smoke of myrtle are common in rural areas of Italy or Sardinia (**Gortzi et al. 2008**).

Myrtus communis L. is a plant traditionally used as an antiseptic and disinfectant drug **(Bonjar 2004)**, hypoglycemic agent **(Sepici et al. 2004)**, moreover used to treat candidiasis, heal wounds, and in the therapy of urinary tract diseases. The plant contains many biologically active compounds andconcerning to the chemical study of the plant, several compounds have been isolated from the leaves, the essential oil and the fruits. According to the literature, myrtle essential oils are endowed with antibacterial, antifungal, antioxidant and antimutagenic properties (**Aleksic and Knezevic, 2014; Tumen et al. 2012**). The antimicrobial activity is usually ascribed also to polyphenols and hydrolyzable tannins compounds which represent main secondary metabolites of this plant.

In fact, like other schlerophyllous shrubs typically evolved under similar environments, *M. communis L.* present an interesting spectrum of leaf polyphenolic compoundswhich appears interesting from both a biological and ecophysiological point of view (**Romani et al., 1999**).Polyphenols are a group of highly hydroxylated phenolic compounds which exist in the extractive fraction of several plant components, include flavanols, flavonols, flavanones, flavones, anthocyanins, proanthocyanidins (tannins), hydroxystilbenes, and aurones (**Dicko et al., 2005**). Polyphenols are proved to have bactericide activities against a huge number of pathogenic bacteria. Among the phenolic class in myrtle leaveswere found hydrolysable tannins (gallotannins, galloyl-glucosides, ellagitannins, galloyl-quinic acids), flavonoids such as quercetin, catechin and myricetin derivatives and volatile oils, coumarins, myrtucommulone (MC) A and B, semimyrtucommulone (S-MC) (which are unique oligomeric, nonprenylated acylphloroglucinol compounds), caffeic, gallic and ellagic acids, (**Romani et al., 2004; Appendino et al., 2006; Wannes et al., 2010; Brada et al., 2012**).Due to several useful properties, among which antimicrobial effects, plants compounds result now subject of medical studies (**Cushnie et al., 2005)**.

Myrtle essential oils (MEOs) obtained through distillation and organic solvent extracts of leaves, yellow or greenish yellow with a refreshing odour, was used in the past for the treatment of lung disorders and have been widely investigatedfor their antimicrobial activities against clinical pathogens, food-borne and food spoilage bacteria, including *Escherichia coli*, *Klebsiella aerogenes*, *Proteus vulgaris*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *P. vulgaris*, *P. mirabilis*, *Campylobacter jejuni*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus pneumoniae*, *S. pyogenes*, *S. agalactiae*, *Listeria monocytogenes*, *Bacillus subtilis* and some molds and yeasts **(Sumbul et al., 2011)**. Has been alsoinvestigated and documented the inhibitory activities of MEOs on some oral pathogens including *S. pyogenes* (β-hemolytic streptococci group A), *S. mutans* , *A. actinomycetemcomitans* , *P. gingivalis* and *C. albicans*

associated with pharyngitis, dental caries, periodontal diseases, oral abscesses **(Mehdi et al., 2014).** As resultby **Shiri et al. (2014)** and **Yadegarinia et al. (2006)**studies*M. communis* has shown a potent antimicrobial activity against both Gram-positive and Gram-negative bacteria. Data collected from numerous studiesgive us accurated info on the composition of the various myrtle essential oils which can be slightly different.The oil composition is quite variable and influencedfrom several factors like: the geographic area of origin, the season of harvest and the length of distillation **(Tuberoso et al., 2006; Ghnaya et al., 2013).** The chemical composition of the MEOs, analysed by Gas/Cromatography (G/C), in literature results as rich in monoterpenes hydrocarbons (53-58%), particularly α-pinene, (11-35%); 1,8-cineolealso known as eucalyptol (13-19%); linalool, (7-12%); α-terpineol, 1-7%; and limonene, (5-14%)**(Ozek et al., 2000).**

Cox et al. 2001 reported to impart for 1,8-cineole, which represent togheter with α-pinene one of the major compound, a microbicidal effect on *E. coli*, *S.aureus* and *C. albicans.*

The phenol compounds with a hydroxyl group (-OH) were found to posses the major antimicrobial activity among the EO constituents. It seems that the presence of the hydroxyl group is related to the inactivation of the enzymes. In fact, they interact with the membrane causing leakage of cellular components, change fatty acid and phospholipid constituents, impair energy metabolism and influence genetic material synthesis (Ceylan and Fung, 2004). Likewise to the phenol compounds, the site of action of the terpenes is the cell membrane. They permeate through the membranes causing them to swell, thus inhibiting respiratory enzymes and causing partial dissipation of the pH gradient and electrical potential (Sikkema *et al.*, 2004).

The phenolic components are chiefly responsible for the antibacterial properties of the EOs, nevertheless some evidence indicate that minor components have a critical part to play in antibacterial activity, possibly by producing a synergistic effect with the other active components**(Marino et al., 2001).**

Numerous investigations have suggested that the difference in the chemical composition of myrtle essential oil greatly depends from method used for the extraction (hydrodistillation, steam extraction, solvent extraction) and this may also influence antimicrobial properties (**Berka- Zougali et al., 2012**).

1.1.2. Historical and current use of Myrtus communis

The myrtle word comes from the Latin "*Myrtus*" which comes from the greek "*Myrtos*", but probably the origin of the word is Semitic while "*communis*" means common plant growing in groups.The first reference of Myrtle in the Bible is in Nehemiah 8:15 in regard to the celebration of the feast of Tabernacles **(Sumbul et al. 2011).**
The *Myrtos* noun is linked to the greek myth of Myrsine, an invinciblegirl for the athletic competitions who was transformed from Pallade in a Myrtle tree, due to a passing a young man in a gymnastics competition. Since ancient times myrtle, for its unquestionable beauty, it was consecrated to the gods of love and dedicated to Venus, considered for this as a symbol of eternal love , of conjugal fidelity and poetic glory.This shrub was much loved by the Greeks and Romans which used myrtle as ornamental and aromatic plant: a wreath of myrtle crowned the winners of the competitions and the poets.
In Greek mythology and ritual the myrtle was sacred to the goddesses Aphrodite and also Demeter. Therefore, linked to the goddess of love, sex and beauty, myrtle appeared in several legends and took place in Aphrodite rituals **(figure 1).**
Myrtle is a venerated tree a branch of which was used as an essential accompaniment in all religious functions. It was considered by Jews as a symbol of peace and as an emblem of justice **(Abrahams, 2004).**Its flowers, traditionally considered good luck, were present in the bridal bouquet.
Plinio told us that many myrtle trees growing where Rome was founded, thus alluding to the everlasting fame of the city. According to the legend, the Romans and the Sabines were reconciled, after the famous rape, they were purified with myrtle branches and that's why at the foot of the Capitol they were planted two myrtle trees.
The ancient Egyptians adorned hair and clothes, in addition they used to crush the leaves and add them to wine to treat fever and infection. Dioscoride used the same recipe for stomach, bladder and pulmonary infections.
The cosmetic properties, therapeutic and culinary myrtle are known since ancient times. The use phytocosmetic Myrtle dates back to the Middle Ages:in medieval perfumers the "water angels" lotion, with astringent and tonificant properties, was obtained by the distillation of the flowers and leaves. *Myrtus communis Linn.* represents one of the important drugs being used in the Unani System of Medicine, which isbased on the fundamentals of Hippocratic medicine,since ancient Greece period (**Kirtikar and Basu**, **1988)**In past times, ripe fruits (berries) were used as food integrators because of their high vitamin contents and the fruit decoction was used to bath newborns with reddened skin, while the leaves decoction and fruit was helpful for sore washing.In Iranian traditional medicine (ITM), where it is recognized as "*Aas*" (associate in applied science) and its berries are known by the name of *Habb-ul-Aas*, it is used to treat dermal diseases like wart and one method of therapy is using myrtle topically (**Ghadami Yazdi et al. 2013).**

The ancient popular use of Myrtus communis is still practiced in the whole Mediterranean area, especially in the Italian regions of Calabria and Sardinia where leaves and berries of myrtle are widely used for flavouring foods like roasts and game, to spice up the brandy or grappa, to make a typical myrtle liqueur and to produce a balsamic drink called myrtle tea **(Ziyyat et al. 1997).**Because of the high oil content of their leaf glands that makes it strongly aromatic it is also used in cosmetic and perfumery to produce a variety of soaps, perfumes and colognes with feature myrtle note. In aromatherapy the essence of myrtle finds employment especially in respiratory diseases due to its mucolytic, expectorant, antispasmodic properties probablylinked to blockade of voltage dependent calcium channels (**Janbaz et al. 2013)**;The decoction of the leaves used in the past, as said before, is still used for vaginal lavage, enemas in addition to the treatment of respiratory diseases **(Marcini & Maccioni 1998).**
It is also used to treat gingivitis and some ulcerative disorder of the oral cavity: **Babaee et al. 2010** have confirmedthis property validatingthe efficacy of a paste containing Myrtus communis as treatment for mouth ulcers, in particular in the treatment of recurrent aphthous stomatitis reducing discomfort and pain of patients.
Several data collected from ethnobotanical scientific literature of the second half of the 20th Century illustrate its use in the folk veterinary phytotherapy in Italy for gastrointenstinal complaints (diarrhea and intestinal infections in humans and animals and the difficult digestion) and problems of the skin and wounds (tones up the joints, fights perspiration and excessive oiliness of the skin)**(Viegi et al. 2003).**The astringent- antidiarrheal and antiseptic properties are due to the tannins, while the digestive qualities are related to the essence**(Atzei et al. 1991).**

1.1.3. Composition of Myrtus communis

The main secondary metabolites of *Myrtus communis L*. are represented by polyphenols, essential oils, volatile substances, acids, flavonoids, tannins, anthocyanins and fatty acids. Several studies carried out on the composition of the aerial parts of the plant myrtle also revealed the presence of several specific chemical compounds. It is well known that the dried leaves contain 1,8-cineole, linalool, linalyl acetate, terpineol, terpinolene, which contribute to the characteristic odor of myrtle, in addition to tannin and flavonoid compounds.
Their content is quite influenced by many variables (the region of origin, climate, exposure to the sun, the season of ripening and harvesting, the analytical procedure employed) but independently from this,they were found in noticeable

amounts as predominant compounds, following the order listed above, as confirmed by the majority of studies of interest(**Asllani U. 2000; Farah et al., 2006; Jerkovic et al., 2002; Ozek et al., 2000; Tuberoso et al., 2006**). Leaves and flowers contain high amounts of polyphenols compounds among which essential oils, phenolic acids, myricetin glycosides, flavonoids and tannins. The volatile fraction is characterized by a fatty acid ethyl esters fraction responsible of the characteristic fruity note of myrtle, and by the terpenes fraction also detected in the flower and fruit oils in different proportions, among which the following compounds are the main representatives: myrtenil acetate, 1,8-cineole, limonene, linalool and α-pinene. It is widely demonstrated thatthe fruit and flower oil contents is always less than leaf oils, this is in line with the big difference of the percentage observed for some compounds such as linalool.

Berries contain tannins, anthocyanins, fats and organic acids, their contents depends on the ripening period and the type of solvent used for the extraction (**Messaoud et al ., 2012**).

In general is possible to affirm that myrtle extracts profile constitutes polyphenolic compounds, which are grouped in three major chemical classes – phenolic acids, tannins and flavonoids.

Among phenolic acids found there are garlic, ellagic, caffeic, syringic, vanillic and ferulic acid, while tannins components of *M. communis*as another essential oil component comprise hydrolysable tannins (gallotannins) and proanthocianidins (condensed tannins). Finally, main flavonoid compounds found in myrtle extracts are myricetin, quercetin, catechin, and their derivates. In particular, some of the myricetin and quercetin derivatives (flavonols) found in myrtle extracts are myricetin-3-d-galactoside, myricetin-3-d-rahmnoside, quercetin-3-rutinoside, quercetin-3-d-rahmnoside, and catechin derivatives (flavanols) (**Hayder et al., 2008; Messaoud C and Boussaid M. 2011;Yoshimura et al., 2008)**.

All myrtle extracts were very rich in polyphenols, but it is evident that the content of these compounds is quite variable depending on the plant part used. Leaf extracts contain significantly higher amount of total phenolic compounds than berry extracts, and as flowers as well are very rich in tannins compared to the myrtle stem which instead seems to be moderately rich in flavonoids like catechin. Moreover, myrtle leaves were found also rich in flavonols and flavanols (**Aidi Wannes et al., 2010**; **Romani et al., 2004**).

Overall, the major compounds present in myrtle leaves, stems and flowersisα-pinene (10 -60%),immediately followed by the 1,8-cineol (10-40%) (**Brada et al., 2012**; **Ghasemi et al., 2011;**).

α-Pinene is an organic compound of terpene which seems to be a broad spectrum antibiotic, found in the oils of many species like as coniferous trees, also found in the essential oil of rosemary and present in some oils such as eucalyptus oil and orange peel oil (**Nissen et al., 2010)**.

1.1.4 Antimicrobial activity of Myrtus communis

As mentioned previously, the antimicrobial properties of myrtle extracts obtained from plant areal parts like leaf and stem have been investigated against spoilage and most common human pathogenic bacteria showing good results **(Akin et al., 2010; Alem et al., 2008; Amensour et al., 2010;)**Several studies have been conducted on MEOs to evaluate their antibacterial effect on Gram-positive and Gram- negative strains, with different growth properties and often cause food borne diseases. Theefficacy has been confirmed in literature for the following bacteria: *Bacillus cereus, Candida albicans, E. coli, Klebssiela, Mycobacterium spp.,L. monocytogenes , P. aeruginosa, P. mirabilis, P. vulgaris, S. aureus, S. typhi, Shigella,*(**Bouzouita et al., 2003; Celikel and Kavas 2008;Rasooli et al., 2002;Zanetti et al., 2010)** and overall the inhibitory effect is reported and classified as strong, medium or weak, closed related to the microorganism resistance.In fact, itshould be noted that the antimicrobial activity depends on several factors, which includes: i) methodology of extraction, ii) composition and concentration of antimicrobial compounds, iii) microbial specie and its occurrence level, iv) substrate composition and v) processing conditions and storage. So the strong variability of these factors create a difficulty to compare results from different studies. Regarding this last aspect, a strong variability among microorganisms belonging to different species was observed due to a species-specific effect in the myrtle extract related to its chemical composition.
Generally, gram-negative bacteria have been reported to be more resistant than Gram-positive to myrtle extracts antimicrobial effect, because cell wall lipopolyssaccharide may prevent that essential oils active compounds reach the cytoplasmic membrane**(Senatore et al., 2006; Chanegriha et al., 1994).** Although the considerable presence of studies using dried herbs or spices or their extracts **(Aktug and Karapinar 1986; Al-Jedah et al., 2000; Leuchner and Zamparini 2002; Sakandamis et al., 2002)**a small number of trials have been carried out using myrtle extracts or MEOs in food matrices (**Gündüz et al, 2009).** However well EOs perform in antibacterial assays in vitro, it has generally been found that a greater concentration of EO is needed to achieve the same effect in foods **(Smid and Gorris, 1999)**.
Laboratory media not always are representative of food systems as the greater availability of nutrients in foods, the intrinsic properties (fat/protein/water content, antioxidants, preservatives, pH, salt and other additives) may enable bacteria to repair damaged cells faster. Moreover, the extrinsic determinants (temperature, packaging in vacuum/gas/air, characteristics of microorganisms) can also influence bacterial sensitivity.
As consequence of these factors, a considerable variation for resistanceof the same microorganism to the same spice, essential oil and extract has been observed among tests *in vitro* and *in situ*.
Djenane et al., 2011 describe a good sensitivity of *S.aureus* to the EOs extracted from myrtle leaves, showing a minimal inhibitory concentrations (MIC) in a range of 0.05-0.22% (v/v). While, a lower activity was observed against *E.coli* probably attributable to the Gram negative nature.Spite of this, also the gram positive pathogen showed reduced sensitivity when inoculated in food matrix. In fact, substantial differences were determined by adding an amount of twice the MIC concentration (0.10 – 0.44%) of the essential oil of myrtle in the minced beef experimentally contaminated with the strain of S. aureus and stored at 5°C.
Besides the variables closely related to the food system that can affect the final outcome of experimental data, it must be added a crucial factor represented by the complex chemical composition of myrtle which makes hard to define causes leading to the loss of antimicrobial activity. Some scientists have focused their studies on the isolationof bioactive compoundsfrom myrtle leaves, discovering a phloroglucinol with antibiotic properties, known as myrtocommulone A, and a phloroglucinol more polar and abundant, identified as semi-myrtucommulone. These molecules have proved to have wide antimicrobial activity against several strains of Staphylococcus aureus, showing MIC values between 0.5-2 g/ml and 32-64 µg/ml for myrtocommulone A and semi-myrtucommulone, respectively**(Appendino et al., 2002).** These low values of MIC motivate the application of the pure compounds, and a mixture with other molecules enhance the antimicrobial activity of the essential oils **(Aleksic and Knezevic, 2014)**.Spite of this, till now the exact mechanism of antibacterial action of spices like myrtle and their derivatives is not yet clear, but it might depend on a single compound or could be due by the synergic action of several substances **(Lanciotti et al., 2004)**.

1.1.5 In vitro test of antibacterial activity

Accurate determination of bacterial susceptibility to antibiotics is essential to the successful management of bacterial infections and to the comparative analysis of antimicrobial agents. This can be done by a number of techniques, which include the disc diffusion method, the broth dilution assay and the Etests. The effectiveness of antibiotics can be assessed by their ability to suppress bacterial growth, described by the MIC, or by their ability to kill bacteria, characterized by the minimal lethal concentration (MLC). MIC correspond to the lowest concentration of the compound capable of inhibiting the growth of the challenging organism **(Mann and. Markham, 1998)** while MLC is the lowest concentration able to kill the 100% of microorganisms tested.
MIC is usually derived by means of tests in solid media, whereas both MIC and MLC can be determined in broth dilution assays.

The agar diffusion assay is an important technique for assessing microbial susceptibility to antibiotics, which has found application worldwide over the past 50 years. It has a number of variation, which include the cup method, the paper disc method, the standardized single disc method, as well as related approaches like the Etest. The diffusion of active substance into the agarose medium leads to inhibition of bacterial growth in the vicinity of the source and to the formation of clear 'zones' without bacterial lawn. The diameter of these zones increases with the antimicrobial substance concentration. Determination of MIC using these approaches, as well as using the microdilution technique, has been shown to produce comparable results. A number of factors affect the accuracy and reproducibility of the agar diffusion method, including thickness and uniformityof the gel, the choice of cut-off size for the inhibition zones and breakpoints, temperature etc. When these factors are controlled or taken into consideration, analysis of data from the agar diffusion assays relies on theoretical models, which allow to define the validity of each model.In any case, has to be taken in account that the ability to compare data from different studies is limited due to differences in test methodologies and in the criteria selected for the determination of the end-point.

The diffusion assays result to be unsuited to nonpolar substances testing (e.g. essential oil) because the components are partitioned through the agar according to their affinity with water.

When testing non-water-soluble antimicrobials such as essential oils, it is necessary to incorporate an emulsifier or solvent into the test medium to ensure contact between the test organism and the agent for the duration of the experiment. The agents most commonly used are Tween 80 (polysorbate 80) and Tween 20 (polyoxyethylene (2) sorbitan mono-laurate) (Beylier 1979; Walsh and Longstaff 1987; Patkar etal. 1993; Chand etal. 1994; Carson etal. 1995b), ethanol (Biondietal. 1993; Deansetal. 1994) and DMSO (Scortichini and Rossi 1991)The suitability of ethanol has also been questioned as it has been reported to have a marked potentiating effect on the activity of some antimicrobial agents at concentrations of ¼5% (Van Doorne 1990).The objective of this study was to develop a broth microdilution assay suitable for the assessment of antimicrobial activity of myrtle leaves extract using a chemically and microbially inert stabilizer, to define the MIC and MLC values.

1.1.6 Food active packaging

Many refrigerated foods represent a favorable substrate for the growth of undesirable microorganisms which contribute to the fast deterioration of product compromising the microbiological and organoleptic quality.
One way to control microbial growth in these food products, thereby improving safety and delaying spoilage, is the application of antimicrobial dips or sprays on the surface of the product (**Kerry et al., 2006**).
Unfortunately, in most cases the efficacy of the antimicrobial compounds is restricted due to their inability to migrate into food matrices or because partially inactivated by the interaction with food components.
One new approach to overcome these limitations is the use of antimicrobial packaging, where antimicrobial substances can be incorporated directly into the packaging material for example by coating of the surface of the packaging film. This is defined as active packaging which represent an innovative concept where the package, the product, and the environment interact to prolong the shelf life, enhance safety, or improve sensory properties, while maintaining the stability and quality of the product (**Khalil and others 2013**).The main objective of this study is to evaluate the antibacterial activity of myrtle leaves extract incorporated into a polyethylene film against S. aureus, L. monocytogenes, in plate overlay assays (in vitro) and when associated with ready-to-eat foods, like cheese and sliced cured meat, but was also tested in highly perishable food like raw beef (in situ), during long-term refrigerated storage (4 °C). The information from this study may be of use to develop new and/or novel ways to control pathogenic microorganisms in meat and dairy products.

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1.2 Lb. curvatus 54M16 strain producing bacteriocin(s)

The bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity produced by different groups of bacteria (**Jack et al., 1995**). The first bacteriocin was discovered in 1925 by Gratia, and in the past 20 years there has been an increasing interest in such compounds as possible preservative agents for food, and as potential supplements or replacements for currently used antibiotics (**Yang et al., 2014**). The lactic acid bacteria (LAB) produce an several antimicrobial substances among which: organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides, and bacteriocins as well **(El-Ziney et al., 2000; Holtzel et al., 2000; Magnusson and Schnürer, 2001)**. The majority of the bacteriocins produced from LAB can be grouped in one of the classes proposed by **Klaenhammer (1993)**. Recently, the structure, biosynthesis, genetics and food application of LAB bacteriocins have been reviewed **(O'Sullivan et al., 2002; Chen and Hoover, 2003; Cotter et al., 2005; Fimland et al., 2005)**. Between various of species belonging to the genus Lactobacillus isolated from meat, often are found strains of *Lactobacillus curvatus* and *L. sakei* able to produce several bacteriocins (**Hequet et al., 2007**).

Four bacteriocins produced by *L. curvatus* strains have been found: curvacin A (**Tichaczek et al., 1993**), curvaticin 13 (**Sudirman et al., 1993**), curvaticin FS47 (**Garver and Muriana, 1994**) and sakacin X (**Hequet et al., 2007**). Curvacin A produced by *L. curvatus* LTH 1174 is identical to sakacin K produced by *L. sakei* CTC 494 and to sakacin A from *L. sakei* Lb 706 (**Axelsson and Holck, 1995**; **Aymerich et al., 2000**; **Leroy and De Vuyst, 2005**).

L. sakei produces several bacteriocins: sakacin A **(Schillinger and Lucke, 1989)**, sakacin B (**Samelis et al., 1994**), sakacin G (**Simon et al., 2002**), sakacin M (**Sobrino et al., 1992**), sakacin K (**Hugas et al., 1995**), sakacin P (**Tichaczeket al., 1994**), sakacin T (**Aymerich et al., 2000**), sakacin Q (**Mathiesen et al., 2005**), sakacin X (**Vaughan et al., 2003**) sakacin 674 (**Holck et al., 1994**), lactocin S (**Skaugen et al., 1997**) and bavaricin MN (**Kaiser and Montville, 1996**). All sakacins possess strong antilisterial activity, so recent approaches in the preservation of meat products are increasingly directed toward biocontrol using bacteriocinogenic Lactobacillus species as protective microflora to inhibit growth of *L. monocytogenes* and many others undesired microorganisms (**Castellano et al., 2004**; **Hugas et al., 1995**; **Mataragas et al., 2003**; **Vermeiren et al., 2004**).

The characteristic properties of bacteriocins produced by LAB make them suitable for food preservation: (i) are generally recognised as safe substances, (ii) are nontoxicon eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) are generally pH and heat-tolerant, (v) they have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (**Galvez et al., 2007**).

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2. Aim of the study

Main purpose of this thesis was to investigate the antimicrobial activity of natural substances obtained from plant and bacterial sources in order to evaluate their potential use as natural preservatives in food products as alternative to widespread chemical additives, currently undesired from industry and consumers.

2.1 Antimicrobial activity of Myrtus Communis L. hydro-alcoholic leaves extract on food spoilage and pathogenic bacteria

Recently, there has been increasing interest in discovering new natural antimicrobials driven by and increasing concern of the consumers about foods free or with lower level of chemical preservatives.

Contextually, plant products, spices and herbs have been investigated and documented for their antimicrobial properties for several centuries (**Sagdiç 2003**).

Spices are becoming popular as natural antimicrobial agents to be used for a wide variety of purposes, including food preservation. Their active compounds have been included in class of naturally occurring food preservatives and have their inclusion in foods allowed by food production regulator offices **(Brull and Coote, 1999)**.Spices are the common dietary adjuncts that contribute to the taste and flavour of foods as well as are recognized to stabilize the foods from the microbial deterioration.

The inhibitory effect of spices on a variety of microorganisms is well known, although considerable differences for resistance of different microorganisms to a given spice and of the same microorganisms to different spices has been observed (**Arora and Kaur, 1999**).

Commonly, plants are able to synthesize secondary metabolites involved in their defense mechanism, the main representative are phenolic compounds, most of which are simple phenols or their oxygen-substituted derivatives, phenolic acids, quinones, flavonoids, flavones, flavonols, tannins, terpenoids and essential oils. Many of these phytochemicals have been found to exert antimicrobial properties **(Cowan, 1999).**

Recently, several vegetable extracts containing antimicrobial compounds have been studied demonstrating a great capacity of inhibition against several undesirable and harmful bacteria.

The present work therefore, attempts to determine the chemical composition and evaluate antibacterial activity of an hydroalcoholic extract obtained from leaves of Mediterranean *Myrtus communis L*. to evaluate its potential use in food preparation to replace synthetic food preservatives. After the 24 hours maceration period, dry matter, pH, toxicity, total phenolic and flavonoids contents, antimicrobial activity against foodborne pathogens and spoilage bacteria were analyzed *in vitro* and *in situ*. Furthermore, this study characterizes the chemical components considered mainly responsibles of the antibacterial effects of leaves extract from *M. communisL.,* investigating the mode of action of these molecules on bacteria.

2.2. Bacteriocin production by Lb. curvatus and its potential use as starter culture in fermented sausage products

In this study a strain of *Lb. curvatus* 54M16, previously isolated from fermented sausage, was examined for production of bacteriocin(s) and the antimicrobial activity was investigated against some food borne pathogens and spoilage bacteria. In particular, were carried out the molecular mass analysis, N-terminal amino acid sequencing and detection of bacteriocin genes. Therefore, was evaluated the ability of the strain to inhibit the growth of *L.monocytogenes* in food matrix. The main technological properties of the strain, among which the ability to reduce nitrates and to hydrolyze proteins, the superoxide dismutase (SOD), aminopeptidase and catalase activities, were determined in order to investigate its suitability to be used as starter culture for sausage fermented preparations.

3. Result and discussion

3.1 Myrtus Communis L. Hydroalcoholic leaves extract: chemical composition, antibacterial properties and potential application as natural preservatives

The antimicrobial activity of an hydroalcolic leaves extract of *Myrtus communis L*. was evaluated *in vitro* and *in situ*, while the chemical characterization of the main bioactive compounds was carried on through mass spectrometry analysis.

3.2 Materials and Methods

3.2.1 Myrtle Leaves water-ethanol (WEME) and aqueous (AME) extracts preparation

Fresh myrtle leaves were harvested from plants growing in Battipaglia (Salerno, Italy) in September 2012. Leaves were dried at low temperature (30°C) in an incubator for 5 days and then finely-powdered using a mixing grinder.

Three different extracts were prepared, using different solvents: ethanol in concentration of 40% and 70% and distilled sterile water.

The extraction protocol was the following: an amount of 10 g of leaves powder was mixed respectively with 100 mL of each solvent indicated above and left to shake for 24, 48 and 120 hours at room temperatures, using a rotary shaker. Extracts was filtered through Whatman no. 42 filter paper in a Buchner funnel for removing plant particles, centrifuged at 9000 *g* for 10 min at 20°C, recovering the supernatant. After filtration the volume of myrtle leaves extracts recovered corresponded to a yield to approximately 75-80% of the initial volume; the remaining liquid is not recovered since was still associated to the retentate. Then, the extracts containing ethanol were concentrated under reduced pressure at 40°C, in order to eliminate up to 90% of the solvent using a rotary evaporator (Buchi Heating Bath B-490, Buchi Rotavapor R-200). Tree extraction replicates of each solvent were prepared. Therefore, water-ethanol myrtle extracts (WEME40 and WEME70) and the aqueous myrtle extract (AME) at concentration of 100 mg of dry matter (DM)/ml were membrane filtered (0.22 µm, Acrodisc filter, Millipore) and an aliquot was stored at 4°C until use, whereas the other was freeze dried (FDM) and stored under vacuum at 4°C. In order to determine residual ethanol in the WEME, HPLC analysis was performed. The samples were diluted in ultrapure water and micro-filtered (0.22 µm). HPLC system (Gilson 307 Series HPLC system) was fitted with column MetCarb68Hcolumn (Varian) in an oven thermostated at 65°C. Columns were eluted at 0.4 ml/min by 0.25 N H_2SO_4 in ultrapure water. A refractometer (RID 133, Gilson) was used as detector. External standards (ethanol at different concentration) were used for quantification.

Dry matter content was assessed by drying 1 ml of the hydroalcoholic extract for at least 2 h in a thermostatic oven at 105 ± 1 °C and as soon as it reached a constant temperature the weight was calculated using the following formula:

$$DM\ (\%\ w/w) = [\ (W_{105°C} - W_l) /\ W_e] \times 100$$

DM: dry matter (%w/w)

W_l: weight of empty lens (mg)

$W_{105°C}$: weight of dried myrtle extract (mg) after 2h at 105°C

W_e : weight of myrtle extract before drying(mg)

While,pH measurements were carried out with a Mettler Toledo GmbH pH meter (mod. MP200)**(table 2a, 2b and 2c)**

3.2.2 Total phenolic and flavonoid contents

The concentration of total phenols (TP) in WEME70 extract was assayed using the Folin Ciocalteu method, based on colorimetric oxidation/reduction reaction, using the gallic acid as a standard phenolic compound. The total phenolic content were estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g of dry matter. The total volume of reaction mixture was miniaturized to 1 ml: 600µlof water and 10 µl of sample were mixed, and then 50 µl of undiluted Folin–Ciocalteu reagent were subsequently added to them. After 1 min, 150 µl of 2% (w/v) Na2CO3 were added and the volume was made up to 1 ml with water. After 2 hours of incubation at 25 °C, 300 µl of the mixture were transferred into a well of the microplate, the absorbance was measured at 760 nm in a microplate spectrophotometer reader (BioTek) and compared to a gallic acid calibration curve (0.05–3.5 mMol) elaborated in the same manner. The data were presented as the average of triplicate analyses.

Furthermore, was also determined the total flavonoid content (TFC) using a method described by **Dewanto et al. (2002)**, using rutin as standard flavonoid compound.0.25 ml of the WEME70 appropriately diluted or rutin standard solution was mixed with 1.25 ml of distilled water in a test tube, followed by addition of 75 ml of a 5% (w/v) sodium nitrite solution. After 6 min, 150 ml of a 10% (w/v) aluminium chloride solution was added and the mixture was allowed to stand for a further 5 min before 0.5 ml of 1M NaOH was added. The mixture was made up to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm. The mean (± SD) results of triplicate analyses were expressed

as rutin equivalents (RE) in milligrams per gram of dry-material. The calibration curve range was 0.01-0.7 mMol (R^2= 0,9966).

3.2.3 Chemical composition of water-ethanol myrtle extract

WEME70 extract was purified and concentrated by a solid phase extraction using the zip-Tip® tips which are simple tips for micropipette containing a small amount of cationic resin; they allow to desalinate, concentrate and purify the analyte of interest. Their use is very simple and the procedure to be performed is very fast. Afterwards, a preliminary screening to determine the molecular weight of main components of WEME70 it has been performed by MALDI-TOF/MS experiments carried on by a Voyager DE-PRO time-of-flight mass spectrometer (PerSeptive Biosystem, Framingham, MA, USA) equipped with a N_2 laser (337 nm, 3 ns pulse width). The 2,5-dihydroxybenzoic acid (DHB) was purchased from Sigma (St. Louis, MO, USA) and was used as matrix. The sample (2 µl) was loaded on the target and dried, then 1 µl of a mixture of 10 µg /ml DHB in 0.5% ACN was added. Mass spectrum acquisition was performed in the positive and linear reflector mode accumulating 200 laser pulses. Data of collected spectra, were investigated for measurement of the molecular weights and get an overiew of the mix structure. As a result the mixture was fractionated by LC MS/MS and of each identified component has been obtained the structure.
Further to the partition chromatography of the whole extract detailed below, two phases with different polarity have been obtained and analysed to better characterize the components with biological interest.

3.2.4 Separation and characterization of the water-ethanol myrtle extract after re-extraction with hexane

The myrtle extract at 11.5 % of ethanol was subjected to partition chromatography (liquid-liquid extraction) with n-hexane (1:1), generating two phases: the n-hexane fraction and the water ethanol fraction. The n-hexane fraction (300 mg) was then processed to column chromatography (silica gel for column chromatography, 70 – 230 mesh, purchased from Merck) eluting with different solvent mixtures (30ml) with increasing polarity gradient. Column fractions were assayed and combined according TLC separation: silica gel plates (Merck, $60F_{254}$, 20 x 20 cm x 0.25 mm), eluent n-hexane/ethyl ether (7:3; 6:4; 5:5), ethyl ether, acetone and methanol to yield 23 fractions detected by UV light (254nm) and H_2O/H_2SO_4 (1:1) spray reagent. Antimicrobial effect of collected fractions, in concentration of 2mg DM/ml, was evaluated by agar well diffusion assay against a group of selected indicators belonging to the followings species: *L.monocytogenes*, *Br. thermosphacta*, *S. aureus*, *P. fragi*, *C. jejuni* and *C. coli*. Finally, the structures of fractions showing activity was confirmed by mass spectrometry.

3.2.5 Characterization of hexane fraction (LPP)

In order to characterize the bioactive compounds responsible of antibacterial activity for the hexane fraction, MALDI-ToF/MS experiments were carried out using DHB as matrix.
Each spectrum was taken by the following procedure: 2 µL aliquot of each hexan fractions from myrtle extract was loaded on a stainless steel plate together with 1 µL of DHB (10 mg in 1 mL aqueous 50% ACN). Mass spectrum acquisition was performed in both positive/negative linear and reflectron mode by accumulating 200 laser pulses. The accelerating voltage was 20 kV.
Spectral data were collected in the range of 149 – 1500 m/z using positive/negative ionization with the acquisition in the reflector mode.
LC/ESIQTOF-MS/MS analyses were performed by a quadrupoletime-of-flight (QTOF) Ultima hybrid mass spectrometer (Waters), equipped with an electrospray ion (ESI) source operating in the positive ion mode, and a nanoflow highpressure pump system model CapLC (Waters). Samples (1 ml) were loaded on a 5 mm 9 100 mm i.d. ZorbaxTM 300 SB C18 trap column (Agilent Technologies), and analytes were separated on a 15 cm 9 100 mm i.d. Atlantis C18 capillary column at 1 ml/min flow rate, using aqueous 0.1 % trifluoroacetic acid (mobile phase A) and 0.1 % trifluoroacetic acid in 84 % aqueous acetonitrile (HPLC grade) (mobile phase B). Chromatography was carried out by linear gradient at room temperature, according to the following program: from 0 % B at time 0 to 60 % B in 40 min, then to 90 % B in 5 min, at last to0 % B in 5 min. The equilibrium time between consecutive analyses was 5 min. LC–MS was performed operating in both (continuum) MS mode and in MS/MS mode for data dependent acquisition of fragmentation spectra. The spectra were acquired at the speed of 1 scan/s. The source conditions were the following: capillary voltage, 3,000 V; cone voltage, 100 V; extractor, 0 V; RF lens, 60. Raw data were processed by MassLynxTM version 3.5 software (Waters). Mass spectrometer calibration was carried out on the basis of the multiple charged ions from fibrinopeptideGlu (m 1,570.57 Da, Sigma) introduced separately. Singly charged ions specific of each compound were used for peptide monitoring. A resolution C20,000 and a mass accuracy of ±10 ppm were calculated in the working mass range.

3.2.6 Characterization of ethanol fraction (PP)

The polar fraction was preliminarily analyzed by MALDI TOF MS in DHB matrix using both positive and negative ionization following the procedure mentioned above and already used for the hexane fraction. Subsequently the PP was then separated by RP-HPLC for the structural characterization and for the study of the antimicrobial activity.
The HPLC apparatus was composed of an Agilent 1100 HP Series (Agilent Technologies, Palo Alto, CA, USA) modular system equipped with a UV–visible detector to monitor the column effluent. The system was fitted with a 250 × 4.6 mm i.d. C-18 reversed-phase column, PN 884 999.901, Zorbax (DuPont, Wilmington, DE, USA).The eluent was HPLC-grade water containing 0.1% (v/v) TFA (solvent A) and acetonitrile–0.1% (v/v) TFA (solvent B).The sample was loaded at an initial injection volume of 100 µL and was separated at a constant flow-rate of 0.8 ml min−1, with a linear gradient of solvent B in the following proportions (v/v): 0 – 5 min, 5% B; 5 - 65 min; 70% B; and 65–70 min, 80–100% B. The total run time was 70 min and UV detection was carried out at .. nm. HPCORE ChemStation software was used to monitor and integrate the eluted peaks. Before analysis the PP of myrtle extract sample was filtered through a 45 µm filter (Millipore). For analytical runs 100 µl sample were used (20 ran x 1mg) . The column effluent was monitored at 220, 254 and 280 nm. All collected fractions were dried by nitrogen gas and stored at – 20°C protected from light, in order to identify ones with antimicrobial activity by in vitro tests. (20 ran x 1 mg). 29 fractions were collected by HPLC and then submitted to *agar well diffusion assay* as previously mentioned (paragraph 3.2.4).
The fractions which showed antimicrobial activity against the indicators selected were analyzed by mass spectrometry MALDI-TOF and LC-ESIQ-TOF MS/MS with the same methodologies mentioned for the LPP fraction.

3.2.7 Cytotoxic activity of WEME70

3.2.7.1 Cell culture

Human colon adenocarcinoma Caco-2 cells (American Type Culture Collection,Rockville, MD, USA) were grown at 37°C in h-glucose MEM containing: 1% (by vol) non-essential amino acids and supplemented with 10% (by vol) de-complemented fetal bovine serum (FBS) (Flow, McLean, VA, USA), 100 U·mL-1penicillin, 100 mg·ml^{-1} streptomycin, 1% L-glutamine and 1% sodium pyruvate. Cells were grown (17–21 passages) in a humidified atmosphere of 95% air/5% CO_2at 37°C. After incubation for 24 hours in Minimum essential medium (MEM) with 10%FBS, the cells were incubated with different concentrations 1 - 2.5 - 5 - 10 - 20 - 40 - 80 – 100 and 200µg - of WEME70. All experiments were performed in triplicate.

3.2.7.2 Sensitivity of the cell lines to WEME70

We assessed the sensitivity of the cell lines tested to WEME70 using a microplate colorimetric assay that measures the ability of viable cells to transform a soluble tetrazolium salt (MTT) to an insoluble purple formazan precipitate. Cells were plated at the appropriate density (5×10^3 undifferentiated Caco-2 cells per well and 10×10^3 differentiated Caco-2 cells per well) in 96-well microtitre plates. After 24 hours, cells were exposed to various concentrations of WEME70 for 24 hours. Then, 50 µL of MTT (1 mg·mL-1) and 200 µL of medium were added to the cells in each well. After 4 hours incubation at 37°C, the medium was removed, then the formazan crystals were solubilized by adding 100 µL of isopropanol - HCL and by mixing it in an orbital shaker for 20 min. Absorbance at 570 nm was measured using a plate reader. Experiments were performed in triplicate. As a control, DMSO was added to untreated cells

3.2.8 Antibacterial activity of myrtle extracts in vitro

3.2.8.1 Bacteria cultures and growth conditions

A collection of 105 bacterial strains were used in this study as indicator organisms. The strains *Brochothrix thermosphacta* ATCC 11509, *Pseudomonas fragi* ATCC 3456, *Listeria monocytogenes* ATCC 7644, *Listeria monocytogenes* ATCC 932, *L. innocua* ATCC 1770, *Staphylococcus aureus* ATCC 20231, *S. aureus* ATCC 25993,*S. aureus* ATCC 19095,*S. aureus* ATCC 14458 and *E.coli* ATCC 25992 were from the American Type Culture Collection (ATCC); *S. aureus* RIMD 31092 was from the Centre Nationale des Toxemies a Staphylococques, (CNTS, France); *S. aureus* NCTC 9393,*Campylobacter jejuni* NCTC 11351 and *C. jejuni* NCTC 11168 were from the National Collection of Type Cultures (NCTC, London, UK); *C. coli* CETC 7571 and *S.aureus* CETC 239 were from the Spanish Type Culture Collection; *L. innocua* IFI1was isolated in the Institute of Industrial Fermentations (IFI), Madrid, Spain; 2 biotypes of *C. coli* and 9 of *C. jejuni* were from the Hospital la Paz (Madrid), *C. jejuni* 118 was also a clinical isolate supplied by the Microbiology Department of the Health Institute Carlos III (Madrid, Spain); 6 strains of *C. jejuni* and 5 of *C. coli* were isolated from the chicken food chain which is the main reservoir related with the infection in humans in the Institute of Food Science Research (CIAL), Madrid; *L. monocytogenes* OH, *L. monocytogenes* SA, *L. monocytogenes* CAL, *S. enteritidis*, *H. alvei* 53M and *E. coli* O157:H7 were taken from the collection of the Department of Agriculture, Division of Microbiology - University of Naples as well as 29 biotypes of *P. fragi* and 32 of *B. thermosphacta* isolated from meat products.

All bacterial cultures, except for Campylobacter strains, were grown in Tryptone Soya Broth (TSB, Oxoid, Milan, Italy) supplemented with 0.5% yeast extract and incubated for 24 hours to the optimal growth temperatures (20°C for psychrotrophic strains like *P.fragi* and *Br. thermosphacta* and 37°C for all the others mesophilic microorganism).

The working cultures were maintained in TSB with 25% glycerol at –20°C and stock culture was kept at 4°C on Tryptone Soya Agar (TSA) slants.

Cultures of *Campylobacter* were regularly distributed on Mueller Hinton agar supplemented with 5% defibrinated sheep blood (MHB) (Biomedics, Madrid, Spain) and subsequently incubated at 38°C on a shaking platform at 150 rpm for 48 hours microaerobic conditions (85% (v/v) of nitrogen, 10% (v/v) carbon dioxide, and 5% (v/v) oxygen) maintained with a variable Atmosphere Incubator (in vain) (MACS-VA500, Don Whitley scientific, Shipley, UK). After the incubation period the isolated colonies were inoculated in Brucella broth (BB) (Becton, Dickinson and Company, Le Pont de Claix, France) and grown for 18 hours in the same incubation conditions indicated above. The working cultures were maintained in BB 25% glycerol at -20°C.

3.2.8.2 Preliminary screening of antimicrobial activity of WEMEs and AME to select the working extract

Antimicrobial activity of the three myrtle extracts prepared (WEME40, WEME70 and AME) was evaluated by the direct assay of the agar well diffusion against selected food spoilage and pathogenic strains in order to define the best extraction method and for the selection of extract to study. The agar-well diffusion method was applied as previously described (Villani et al.1994) to determine the inhibition zone of growth. Inoculums were prepared with freshcultures of microbial strains, cultured on TSB for 18hoursat relative optimum temperature. One milliliter of inoculum was mixed with 19 mL of TS soft Agar medium plates (TSA containing 0.7% agar) and the bacteria final concentration was of 10^6CFU/mL. After 10 min, wells(d = 6 mm) were made in the Petri plates using a sterile cork borer. Fifty microliter of each filter-sterilized myrtle extracts were dropped into the well. Ethanol at 11.5% and the extracts neutralized with NaOH (1 N) to pH 7.0, were used as controls. In order to accelerate the diffusion in agar, plates were stored for 30 min at 4°C, and then incubated at relative optimum temperature (20°C for *B. thermosphacta* and *P. fragi* strains and at 37°C for all the others indicator strains).After 24 hours of incubation the antimicrobial activity was observed measuring the diameter of inhibition zones around the wells using a caliper. Results were calculated as the mean of 3 experiments ± SD **(table 1b)** and based on these, a selection of the extract which showed the highest antibacterial activity was done in order to use it for microbiological experiments and studied for the chemical characterization.

3.2.8.3 Antimicrobial activity of WEME70

The results came out from preliminary screening on myrtle extracts determining their antibacterial activity led to conduct following experiments exclusively on the WEME70. For further analysis were prepared three batches of WEME70, from myrtle leaves collected in September 2012 as well, and used for statistical analysis.

The spectrum of activity of WEME 70 has been completed towards all the strains not previously tested and mentioned in section 3.2.8.1 using the agar well diffusion assay used in preliminary screening as described above. The results were calculated as the mean of 3 experiments.

The myrtle extract titer was determined by the serial two-fold dilution method previously described by **Mayr-Harting et al. (1972)** commonly used for bacteriocin sample.

Ethanol at 11.5% was used as solvent dilution and was simultaneously assayed as the negative control.

Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units (AU) per millilitre. The units were determined in each experiment against the respective indicator strain.

Determinations were performed in triplicate.

3.2.8.4 Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of WEME

Bacteriostatic and bactericidal concentrations of the WEME70 were determined only against the microorganisms that exhibited sensitivity in the previous assay. Each strain was grown over-night at optimal temperature in liquid media added of increasing concentrations of WEME70 ranging from 0to 100 mg of dry matter for ml, and microbial growth was checked by viable count.

MIC was defined as the lowest concentration of myrtle extract at which bacteria failed to growth in liquid media, but yet viable when 100 µl of culture broth were plated on agar media. While, MLC was defined as the lowest concentration of myrtle extract at which bacteria failed to growth in liquid media, but were not cultured after 100 µl samples were plated on agar media.

MIC and MLC of WEME were determined in TSB for *Listeria* and *Staphylococcus* strains, in mPTSB (TSB added with 2.8 ml/L of bromocresol purple and 1% of glucose) for *P. fragi* strains and mBTSB (TSB added with 2.8 ml/L of bromocresol purple and 15 g/L of glycerol) for *B. thermosphacta* strains by using a serial two-fold broth dilution method (CLSI, 2006). After over-night growth in TSB to the growth temperature of each strain, the bacterial cells of indicator strains were harvested by centrifugation at 4°C, washed and resuspended in Ringer's solution (Oxoid, Milan, Italy) to obtain a 0.5 McFarland standard of the selected bacteria. The final concentration of bacteria in 2.5 ml of each broth (mPTSB and mBTSB) was about $1x10^5$ CFU/ml (confirmed by viable counts on TSA plates). A serial two-fold dilutions of filter-sterilized WEME70, containing from 0 to 100 mg of dry matter (DM)/ml were used. Two-fold dilutions of water-ethanol at concentration of 11.5% (v/v) were used as control. The tubes were incubated under agitation tothe optimum temperature of each indicators (20°C for *P.fragi* and *B. thermosphacta* , 30°C for *Listeria* and *Staphylococcus* strains). 100 µl of cell suspensions from tubes showing no growth (no colour change of bromocresol purple) were sub-cultured on TSA plates to determine the MIC and MBC values.

3.2.8.5 Exposure of resting cells to WEME

Three strains of *P. fragi* (A1, H83 and T81) and three strains of *B. thermosphacta* (B02, EC71 and Gc202) were chosen to evaluate bactericidal kinetics of myrtle extract. The strains were grown at 20°C up to log-phase in TSB broth. The cells were harvested by centrifugation (8000 *g*, 10 min) and resuspended in sterile 50 mM potassium phosphate buffer, pH 7.0, to yield about 10^4-10^5 CFU/ml. WEME was added to give a final concentration of 100, 50, 25 and 0 mg DM/ml. The cell suspensions were incubated at 20°C. Samples were withdrawn at 0, 2, 10, 20, 60 and 120 min and viable counts were determined on TSA agar (20°C for 24 h). Cell suspensions without WEME were used as controls. The experiments were performed three times and mean total number of viable cells from bactericidal kinetics experiments at each time interval was converted in Log viable cells. For each species two-way ANOVA was carried out for results at 5 and 10 minutes in order to evaluate the significance of the effect of strain, log (dose) and their interaction.

3.2.8.6 Antimicrobial activity of hexane and ethanol fractions of WEME70

The antimicrobial activity of the two phases previously separated by liquid-liquid extraction (3.2.4) renamed as less polar phase (LPP) and polar phase (PP) was determined by *spot on lawn method* and defined as AU ml^{-1}, following the protocol illustrated in the paragraph 3.2.8.3 already used to determine the antimicrobial activity of WEME70.

The concentrations tested of the two different solutions obtained from the partition chromatography were respectively of 86,47 mg of DM/ml for the PP and 15.8 mg of DM/ml for the LPP. Main indicator strains employed were represented by *L. monocytogenes, S. aureus, Br. thermosphacta* and *P. fragi spp*.

For pathogenic strains mentioned above, were also defined the MIC and MLC values by broth dilution method described in the section 3.2.8.4 , testing different concentration (0.05, 1 and 2 mg/ml) of both different myrtle fractions, respectively against the microorganisms which proved to be susceptible from the agar well assay. Experiments were performed in triplicate and values were reported as means ± standard deviation.

Moreover, was also determined the antimicrobial activity of all collected fractions obtained by column chromatography of n-hexane myrtle phase (LPP) and by RP-HPLC of ethanol phase (PP) by agar well diffusion assay against main indicators strains in order to narrow the field of investigation and to characterize the main active compounds.

3.2.8.7 Effect of hexane and ethanol fractions on membrane integrity of pathogenic bacteria

3.2.8.7.1 Fluorescent cell viability assay

Control cells of *L. monocytogenes, S. aureus and C. coli* (not exposed to LPP and PP) and cells exposed for 24 hours to PP and LPP of WEME70 alone (PP: 0.05 mg/mL; LPP: 0.05 mg/mL) in TSB broth were stained using a LIVE/DEAD BacLight bacterial viability kit for microscopy (Molecular Probes, Eugene, OR, USA) as described by the manufacturer.

This assay uses mixtures of SYTO® 9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide (PI).

SYTO® 9 stain has a high affinity for DNA and exhibits enhanced fluorescence upon binding with an excitation maximum at 483 nm and fluorescence emission maximum at 503 nm, is particularly used for staining live and dead Gram-positive and Gram-negative bacteria. While PI binds to DNA by intercalating between the bases with little or no sequence preference. In aqueous solution, the dye has excitation/emission maxima of 493/636 nm.
Since propidium iodide is not permeant to live cells, it is also commonly used to detect dead cells in a population.
The stock solution of the two fluorochromes was prepared with 0.7 µl of SYTO 9 and 1 µl of propidium iodide in 330 µl of sterile deionized water.
Aliquots of the bacterial suspensions were exposed to PP and LPP to control the membrane permeabilization. Untreated bacterial cells were used as a negative control. The samples were incubating with the double staining mixture at room temperature for 15 min.
After the viable staining, the fluorescent cells were observed using a Nikon Eclipse E400 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with an UV lamp and a 100x magnification objective.

3.2.8.7.2 Study of the integrity of pathogenic cell membrane after exposure to myrtle fractions

The loss of integrity of the cytoplasmic membrane for food pathogenic strains exposed to various concentration (corresponding to the MIC and MLC values determined by broth dilution assay) of hexan and ethanol myrtle fractions respectively renamed as Less Polar Phase (LPP) and Polar Phase (PP), was measured as an increase in cell fluorescence due to the uptake of propidium iodide, a dye that is normally excluded from cells with intact membranes. Based on the selective activity of the two different fractions separated by liquid-liquid extraction, some strains of *C. jejuni* and *C. coli*, chosen for their different sensitivity, were exposed to the PP, contextually *L. monocytogenes*, *L. innocua* and *S. aureus* cells were exposed to the LPP.
PI is commonly used as an indicator of cytoplasmic membrane damage. PI is able to enter cells only if the membrane is permeabilized. Once inside the cytoplasm, it binds to single- and double-stranded nucleic acids, yielding fluorescence in the red wavelength region. Untreated and treated cells of each microorganism were collected by centrifugation (5 min at 13000 rpm)after different exposure times (30minutes, 2, 4, 6, 8 and 24 hours), the supernatants were removed and the cell pellets were rinsed twice with PBS. Cells stained with PI (PI; Fluka Analytical) which was added to a final concentration of 2 µg/ml. After incubation for 10 min at room temperature, samples were centrifuged and washed twice in PBS to remove the excess dye. Relative fluorescence (Fluorescence/Optical density) was measured with the BioTek Synergy HT Multi-Mode microplate reader at an excitation wavelength of 495 nm and an emission wavelength of 615 nm. Fluorescence data for each sample were normalized with the optical density at 600 nm. PI uptake experiments were carried out at least 3 times on separate days with independent coltures. Results were expressed as ΔRF (increase in RF over than control).

3.2.8.8 Preparation of the antimicrobial polyethylene film

Three antimicrobial solutions at different concentration of PP (172.8 mg DM/ml, 86 mg DM/ml and 43 mg DM/ml, respectively) have been used for the treatment of coating of the plastic film. In fact, with the help of a threaded bar of metal each solution was spread on a polythene (PE) film , permeability to the oxygen of 2300 cm3/m2·24h·bar, in a thin layer and then dried with a jet of hot air. In particular, 1ml of the each solutions have been used to perform a treatment of coating of about 1694 cm^2 of low-density PE film, obtaining 3 activated films with the following final concentrations of PP (0.025 - 0.051 and 0.102 mg of DM/cm^2). These concentrations were chosen based on results obtained from the previous test in vitro for the antimicrobial activity, in particular showed against all strains of *P. fragi spp.*(see paragraph 3.2.8.6). These correspond to the following values of arbitrary units: 1600 – 3200 and 6400 AU ml^{-1}. In addition, after the above treatments of activation with the PP solution, each film activated was assayed for the antimicrobial activity against the spoilage bacteria of raw meat, which is major represented by*Pseudomonas spp*. In order to check the adhesion of the antimicrobial solution to the film surface, part of it has been rubbed with a sterile cotton swab before testing the antimicrobial activity. Samples (2 · 2 cm) of the treated PE films were located onto
the surface of plates containing TSA (Oxoid) with the 0.75% of agar inoculated with the bulk of the cellular populations of microbial indicator previously mentioned. From each films were obtained small discs with a diameter of 21mm, for which the treated face was in contact with the agar, untreated films were also assayed as negative controls on the agar plates seeded with 1% of an overnight culture of the indicator to obtain a final concentration of 1.0 x 10^5 CFU. After incubation for 16 h to the growth temperatures of 25°C, the antagonistic activity was evaluated by observing a clear zone of growth inhibition in correspondence of the active PE film.

3.2.9 Challenge studies

3.2.9.1. In situ effect of freeze-dried myrtle extract (FDM)

Naturally contaminated ground beef was used to evaluate the *in situ* antibacterial activity of freeze-dried myrtle extract (FDM). Ground beef was used to prepare hamburgers of 200 g with different amounts of FMD. The ground beef samples were assigned to one of four different treatments: CC: Control, meat without freeze-dried myrtle; A: meat with 1% of FDM; B: meat with 3% of FDM and C: meat with 5% of FDM. Immediately after adding the myrtle freeze-dried the samples were stored at 4°C for 5 days. Microbiological analysis of two trial of each samples were performed after 0, 1, 2, 3 and 5 days of storage at 4°C. Samples (10 g) were aseptically weighed, homogenized in 90 ml of quarter strength Ringer's solution (Oxoid) for 2 min in a stomacher (LAB Blender 400, PBI, Italy; stomacher bags: Sto-circul-bag, PBI, Italy) at room temperature. Decimal dilutions in quarter strength Ringer's solution (Oxoid) were prepared and aliquots of 0.1 ml of the appropriate dilutions were spread in triplicate on the following media: Plate Count Agar (PCA, Oxoid) incubated at 30°C for 72 h; Pseudomonas Agar with cetrimide-fucidin-cephaloridine (CFC) selective supplement (PSA, Oxoid) for Pseudomonads; Violet Red Bile Glucose Agar (VRBGA, Oxoid) for the *Enterobacteriaceae*, incubated at 30°C for 24-48 h; MRS agar (Oxoid) for LAB, incubated at 30°C for 48 h under anaerobic conditions by using an Anaerogen kit (Oxoid); STAA medium (Oxoid) for *B. thermosphacta*, incubated at 25°C for 48 h. Results were calculated as the means of Log counts for three determinations.

3.2.9.2 Antilisteria activity of WEME70 in gorgonzola cheese

Gorgonzola cheese were purchased from a local grocery store, were transported to the laboratory within 15 min of purchase and stored at 4 °C until use. The cheese was cut in slices of 200 g with sterile scalpels and tweezers in a laminar air flow cabinet under aseptic conditions. All samples were stored aseptically until inoculated and treated with the myrtle hydroalcoholic extract. Prior to experiments, some samples were treated at 121°C for 15min to reduce background microflora , while other samples were not thermally treated keeping their indigenous microflora. All samples were then inoculated aseptically with overnight and diluted cultures of *L. monocytogenes OH* to obtain approximately 5 LogCFU/g. After inoculation, the samples were kept at room temperature for 20 min to allow for cell attachment. Inoculated cheese were added of WEME70 in a concentration of 10% corresponding to 12.6mg of DM/g of gorgonzola. Inoculated, control samples were added of 10% solution of EtOH 11,5% (v/v) and without any WEME. All samples were transferred individually into a sterile Petri plate and were held at 4 °C for 2 weeks. On days 0, 6, 9 and 15 samples were analyzed for remaining microbial populations. At each sampling interval, 10g of stored samples were opened and transferred aseptically to a sterile stomacher bag (Interscience Laboratories, Rockland, Ma., U.S.A.) with 90 mL of ringer solution (Oxoid), homogenized for 1 min (Seward 400 Stomacher, West Sussex, England) and then collected. Ten-fold serial dilutions were made in ringer solution and 100 µL spread plated in duplicate onto Listeria Selective Agar (Oxford formulation, Oxoid) added of Listeria Selective Supplement (Oxford formulation, Oxoid) for *L.monocytogenes* to determine the number of remaining cells. Resulting colonies were counted after 2 days of incubation at 37 °C, populations converted to log , and the remaining populations expressed as $\log_{10}$ CFU/g.

3.2.9.3. Antipseudomonas activity of WEME's polar phase coated films during the storage of meat products (beef slices)

Based on the results of the plate assays, PE films covered with1ml of the PP solution at 6400 AU ml-1were evaluated for their long-term antimicrobial effectiveness against *P.fragi* spp. naturally associated with raw beef steaks, refrigerated storage (4 °C) for 5 days.

Raw beef (top round, no nitrate-added) were transported to the laboratory within 15 min of purchase and stored at 4 °C until use. The meat was cut into 0.7 mm slices, and slices cut into 10 cm × 20 cm sections with sterile scalpels and tweezers in a laminar air flow cabinet under aseptic conditions. All samples were stored aseptically until inoculated and treated with the antimicrobial films. Prior to experiments, raw beef were surface treated with ultraviolet light (UV) for 15 min to reduce background microflora **(Cutter and Siragusa 1994)**, aseptically cut into sections (10 cm × 20 cm) and inoculated aseptically with overnight and diluted cultures of *P.fragi* spp. to obtain approximately 3 $\log_{10}$ CFU/cm^2 on the surface. After inoculation, the samples were kept at room temperature for 20 min to allow for cell attachment. Inoculated meat surfaces were covered with PE films (20 cm × 40 cm) containing PP. Inoculated, control samples were covered with PE film without any substance. All samples were held at 4 °C for 5 days. On days 0, 1, 2, 3 and 5, samples were analyzed for remaining microbial populations. At each sampling interval, 10 g of samples were transferred aseptically to a filtered stomacher bag (Interscience Laboratories, Rockland, Ma., U.S.A.) with 90 mL of ringer solution (Oxoid), homogenized for 2 min (Seward 400 Stomacher, West Sussex, England) and the filtrate collected. Ten-fold serial dilutions were made in ringer solution and 100 µL spread plated in duplicate onto Pseudomonas Agar Base (Oxoid) with Pseudomonas C-F-C Selective Agar Supplement (Oxoid) to determine the number of remaining cells. Resulting colonies were counted after 2 days of incubation at 25°C, population converted to log and the remaining populations expressed as $\log_{10}$ CFU/cm^2.

3.2.10 Data analysis

All experiments were performed in triplicates and results were expressed as mean ± Standard Deviation (SD). Differences for various parameters were assessed by analysis of variance (ANOVA; SPSS 11.5, SPSS, Inc., Chicago, IL). Statistical significance was identified at the 95% confidence level ($P \leq 0.05$). Two way ANOVA was used to evaluate the significance of the effect of strain, log (dose) and their interaction in the experiments of resting cell.

3.3 References

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3.4 Results and discussion

3.4.1 Influence of solvent and extraction time on antimicrobial activity of myrtle leaves extracts

The quality of an extract is influenced by several factors such as the plant parts used as starting material, the solvent used for extraction, the extraction procedure, and the solvent ratio. The solvents most commonly used for gaining extracts are water, methanol, ethanol and ethylacetate (Amensour et al., 2010 and Tuberoso et al., 2010) and the methods used in extraction procedures from plants are several, including soxhlet extraction, maceration, infusion, accelerated solvent extraction, pressurized liquid extraction, percolation, membrane process, decoction(Handa et al. 2008). In this study the conventional method of maceration has been used, with different time of extraction, characterized by easy application and the low cost. Distilled water and ethanol were chosen as solvents of extraction. For using in food industry primarily water is more adequate as solvent but in this choice need to be considered also the real capacity of the solvent to extract the molecules of interest. The aim of this study was to select the more suitable system of extraction to obtaina myrtle leaves extract able to control undesirable bacteria and adequate for a food system. In this study we found that myrtle leaves extract in organic solvent (40% and 70% ethanol) provided more consistent antimicrobial activity compared to the aqueous extract which seems to be weaker and with a limited spectrum. All the extracts performed have shown different antimicrobial activity against the same strains. The inhibition zones have showed a great variability among values measured, in a range between 2 to 27 mm depending from the solvent type and the extraction times as well, according to microbial strains and extracts **(Table 2a, 2b and 2c).**Overall, AME resulted significantly less effective when compared to WEME 40 and WEME 70.

The others two hydroalcoholic extracts studied contain ethanol at final concentration of 11.45% w/w, which result not harmful. Results showed that the AME inhibits the growth of only 6 indicator strains at concentration 10 % (w/v), while both WEMEs inhibit all tested microorganism, excepted *S.enteritidis*, *H.alvei 53M* and *E.coli* O157:H7 came out to be resistant to all myrtle extracts produced. The antimicrobial activity of WEMEs gently increased with the longer time of extraction but with no significant differences. The zone of inhibition produced by WEME70 , in the range of 12 to 27 mm, were higher than that produced by WEME40. The best effect of WEME70 was on growth of Listeria and Staphylococcus sp. for which were observed inhibition zone diameter values between 24 to 27 mm, followed by *Br. thermosphacta* and *P. fragi* species with inhibition values respectively in a range from 17-24 mm and from 15-16 mm. No inhibition effect has been shown from the solution at 11.5% of ethanol used as control and no changes of antimicrobial activity were observed in neutralized extracts.

Dry matter variation during the three different maceration times has been measured for each extract and it gives clues about the extraction process yield from the leaves which results to be of the 15%(v/v) for hydroalcoholic extracts and 75% (v/v) for the aqueous extract, based on the initial volume. All samples reached the maximum level of extraction of dry matter between the 24 and the 48 hours of extraction. Strong differences depending on the solvent of extraction used, were noticed: for instance, after 24 hours, the hydrolcoholic extract WEME70 had the highest value (126.3 g/l), about twice higher than the aqueous extract AME (65.3 g/l).WEME70 showed the highest value for the dry matter weight, this was attributable to the stronger capacity of extraction of ethanol present in high concentration in the solvent used (70% v/v).

Also WEME40 showed a high amount of dry matter, almost double those of the AME, with a weight (120.7% w/w) similar to WEME70 but in any case significantly lower.

pH plays a crucial rule in the antibacterial activity of the extracts, over to contribute to the organoleptic aspects such as sour taste and colour. **Table 1** shows pH values during maceration where AME showed the highest value with 5.39 after 48 hours of extraction, while WEME40 and WEME70 showed the lowest, almost 5.25 after 48 and 24 hours, respectively.

As shown, pH values underwent small changes within each extract, during the three different maceration times.

Based on data collected , as result of this preliminary screening the WEME70 at 24h of extraction with pH of 5.2 was selected for studying and therefore was exposed to further analysis and experiments.

3.4.2 Total phenolic and flavonoids content

It is well known that many plants like myrtle are an excellent source of natural phenolic compounds. The amount of these compounds is usually dependent to the geographic origin of the plant and the repartition of total phenols, tannins, flavonoids (including flavones, flavanols and condensed tannins) and proanthocyanidins also varied between different myrtle parts. Several epidemiological studies suggest an important role of these vegetable components against human disease, mainly associated with their antioxidant activity(free radical scavenging activity and the protection against oxidative stress). Moreover, in vitro studies highlighted, in addiction to many other biological activities, their role as antibacterial agents.

In fact, has been reported that antimicrobial activity of herbs and spices is due to phenolic compounds **(Shan et al. 2005)** and that oxygenated terpens such as 1,8-cineole, linalool and α-terpineol, exhibit potent antimicrobial activity **(Randrianarivelo et al. 2009)**. Also the antimicrobial activity of dried myrtle leaves extract is suspected to be associated with its content of polyphenols and oxygenated monoterpens **(Messaoud et al. 2011).** Dried myrtle leaves contain tannins, cineol, linalool, terpineol, linalyl acetate and flavonoids compounds (Taheri et al. 2013) and the presence of phenolic compounds in the leaves of myrtle is due to the presence of flavonols and galloylderivatives (Hayder et al. 2008). Polyphenolic compounds may involve multiple modes of action. They can degrade cell wall and cytoplasmic membrane, cause leakage of cellular components, change fatty acid and phospholipid constituents, influence the synthesis of DNA and RNA and destroy protein translation **(Shan et al. 2007).**

As their potential role on the antimicrobial mechanism against pathogenic and spoilage bacteria, both the TP and TFC of WEME70 were estimated through two colorimetric methods, using as standard the gallic acid and the rutin, respectively. As result of these analysis the followed values have been obtained: TP concentration corresponded to 49.46 mg GAE/g of extract, while the TFC was of 145.64 mg RE/g of extract.

These concentrations were found substantially higher than values reported by other researchers for ethanol and methanol myrtle leaves extracts. **Amensour et al. (2010)** determined a total phenolic content of 29mg GAE/g of extract, while **Wannes et al. (2010)** indicated 33.67 mg GAE/g. An exception was represented by **Gardeli et al. (2008)**who had studied the total phenol content of the methanolic extract from Greece myrtle and founda value of 373 mg GAE/g, 8 fold higher than that found in this work.

In spite of this, **Tawaha et al. (2007)** have estimated in their studies that a phenolic content higher than 20 mg GAE/g of dry weight could be considered as very high. Based on these data, we can affirm that WEME70 is very rich in polyphenols and for this reason it could be considered as great natural source of these compounds.

3.4.3 Chemical characterization of WEME70

Myrtle is used traditionally as an herb and culinary spice, and in folk medicine it is employed as an antiseptic, anti-inflammatory agent and in the treatment of diabetes mellitus (**Romani et al., 2004; Yoshimura, Amakura, Tokuhara, & Yoshida, 2008**). The extracts obtained from Myrtus communis L. leaves are very rich in polyphenols (**Romani et al., 2004**). The characteristic constituents of this plant include monoterpenoids, flavonoids, triterpenoids, and phloroglucinol-type compounds. In particular, from a phytochemical point they contain galloyl-glucosides, gallic and ellagic tannins, galloyl-quinic acids and flavonol glycosides; In addition to these rather ubiquitous ingredients that are present in many plants, myrtle contains unique oligomeric, nonprenylated acylphloroglucinols such as myrtucommulone (MC) and semimyrtucommulone (S-MC) (**Appendino et al., 2002**).
For a series of oligomeric non-prenylated phloroglucinols related to myrtucommulone A found in myrtle leaves, was observed antibacterial (**Appendino et al., 2002**)and anti-inflammatory properties, while powerful antioxidant activity (**Rosa et al., 2003**) was demonstrated for its lower homologue, semi-myrtucommulone. Apart from these constituents, the leaves of myrtle are a rich source of flavonoids, and especially of myricetin glycosides. Flavonoids represent hydroxylated phenolic substances which occur as a C6-C3 unit linked to an aromatic ring. They are synthesized by plants in response to microbial infection, so it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. In particular, for the more lipophilic flavonoids such as luteolin, quercetin and myricetin, it has been demonstrated that may also disrupt microbial membranes.
Based on the interesting antimicrobial activity showed from the WEME70 and the selective activity observed for the several fractions separated by the PP and the LPP, on the whole the identity of polyphenols was confirmed by comparison of their massa spectra.

3.4.4 *Chemical characterization of LPP and PP phase*

The identity of polyphenols present in WEME70 extract was ascertained by comparison of their massa spectra. Among polyphenolic compounds found in *Myrtle communis L*. extracts, **Romani et al, 1999** refer to some galloyl derivates and myricetin derivates. From the analysis of mass spectra of WEME70 have been identified several molecules among which two derivates of myricetin, then identified as constituents of the LPP, known as myricetin 3-O-galactoside and myricetin galloyl glucoside, with peaks at 479 and 317 m/z, corresponding to the molecular ion and to the fragment after the loss of galactose. Moreover, was also detected the presence of quercetin 3-O-rhamnoside also described by **Romani et al., 1999**, and the fragmentation pattern reported in **Figure 2** shows two signals at 447 and 301 m/z, corresponding to the molecular ion and to the fragment after the loss of rhamnose. Quercetin derivates with different sugar substituents have been previously identified in extracts from other plants such as *Olea europea* (**Baldi et al., 1995**) and *Phillyrea* (**Romani et al., 1996**).

Investigating in details the composition of the two phases (LPP and PP) separated by partition chromatography of WEME70 emerged that the MS spectrum of the LPP showed a more heterogeneus composition, with mass peaks up to 1900 Da.

Preliminary assessment of the putative bioactive compounds were carried out by means of mass spectrometry on the highest antibacterial fraction of LPP, renamed as fraction 20, disclosed the presence of several acylphloroglucinols compounds such as Myrtucommulone A and D with a m/z 667.4 and 651.79 respectively, for which is reported in literature a known antimicrobial activity against Gram positive bacteria like *S. aureus*, *S. albus* and *B. cereus* (**Rotstein et al., 1974**) .In addition to these compounds, were found five more isomeric variants of MC A **(Figure 3)** characterized from the presence of different functional groups (n- pentyl, ethyl, isobutyl) as far as we know not previously reported in literature, which considering their chemical structure could be also determinants of the antimicrobial activity previously described.

The fraction 20 was purified by HPLC and the major eluted peak at 66 min around the hydrophobic zone was collected showing at least 3 relevant peaks. High-resolution mass spectra was carried out with electrospray ionization (ESI) using a Q- TOF mass spectrometer (**Figures 4 and 5**).

The ESI-QTOF mass spectrum obtained direct by analysis of myrtucommulone (MC) extracted from M. communis, which was used as standard reference, showed three main components at m/z 667 and 651, corresponding to the value expected for respectively myrtucommulone A, myrtucommulone C and D. These structures were demonstrated through the fragmentation spectra obtained after ESI-Q-TOF-MS/MS.

Signals at m/z 669 and 651, corresponding to the pseudomolecolar ions of the above compounds, are detailed in the inset of **Figure 5.**

Therefore, is possible to assert that myrtucommulone A , C and D were contained in the LPP of myrtle leaves extract. The products MC A and MC D were quite co-eluted with a retantion time of 42 and 41,48 min respectively, while the MC C was eluted at 34 min with a m/z of 651 as for MC D. To this regard, it is important to note that myrtucommulones A and D were also found to co-elute in HPLC procedure used by (**Nicoletti et al., 2014**) during the purification from a mixture produced by an endophitic strain of Neofusicoccum australe and from extracts of C. scabrida seeds by **Carroll et al. 2008** as well. MC C and D were also isolated from the methanolic extract of *Myrtus communis L*. and for both was assigned the formula $C_{38}H_{50}O_9$ on the basis of the ion peak at m/z 651 by chemical ionization mass spectrometry (**Shaheen et al., 2006**).

Although some MC isomeric variants were found in this work that leave us suppose their possible implication in the mechanism of action towards Gram positive, none study since now to our knowledge, mentions these molecules and has evaluated the antimicrobial properties of acylfloroglucinol (MC) of different ring substitutions, or saturated side chain.

Others fractions separated from LPP, showed the presence of the acylphoroglucinol rhodomyrtone which differently was found to be potent antibiotics against Gram-positive bacteria including *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), and several *Streptococcus* strains **(Gervais et a., 2015 and Morkunas et al., 2013)**. Therefore, this compound should be with myrtucommulone D, responsible of the antimicrobial effect observed from these fractions.

In line with the results observed in the *in vitro* test for the antimicrobial activity of the LPP and PP fractions, which have showed a different spectrum of action, closely related to the Gram positive and Gram negative nature of indicators strains, does not surprise that also the chemical composition between the two phases separated from WEME70 appears completely different in terms of molecules. In any case both phases result characterized by a constant presence of polyphenolic compounds..

In fact, from the full scan negative ionization mass spectra of fraction 9 and 12 (**showed in figures 6 and 7**) was not detected none of molecules previously identified for the fractions of LPP.

At the same time also the two fractions separated from the same PP of WEME70, showed a different chemical composition. In particular, for the fraction 9 proantocyanidins as catechin and/or epicatechin result as one of major compounds among the polar constituents, showing the deprotonated molecules ($[M-H]^-$) at *m/z* 289 reported also from (**Poon 1998**), followed by gallotannin monitored with m/z 1566.48 previously described by **Romani et al., 2012**. Were also found galloyl forms of these two molecules represented by gallocatechin and epigallocatechin with m/z of 307 and 329, and was identified the ellagic acid hexoside with m/z of 465.2($[M-H]^+$). Differently, the fraction 12 is mainly composed by

clorogenic acids among which have been identificated coumaroylquinic acid, caffeoylquinic acid and feruloylquinic acid isomers with the following m/z values : 361.9, 354.6 and 368,9 respectively. Their potential involvement in the antimicrobial activity of the PP of WEME70 extract can be related to their ability to permeabilize the cellular membrane of both Gram negative and Gram positive bacteria.

In this study the fraction 12 was found able to inhibit the growth of some antibiotic resistant and dangerous Gram negative bacteria represented in particular by *Campylobacter spp.* and also spoilage strains of *P. fragi*.

The mechanism of action of these molecules can be explained by their ability to destabilize the cell integrity increasing the outer membrane permeabilization. As well known that the outer membrane (OM) of Gram-negative bacteria consists of lipopolysaccharides (LPSs) and proteins, and they are maintained together by electrostatic interactions with divalent cations required to stabilize the OM. Anionic substances could remove divalent cations from their binding sites in LPS and thus disrupting the integrity of the outer barrier (**Chen and Cooper 2002**).

Based on data reported by **Lou et al., 2011** , chlorogenic acid result able to kill pathogenic bacteria strains like *Shigella dysenteria* and *S. pneumoniae* by provoking irreversible permeability changes in cell membrane, causing cells to lose the ability to maintain membrane potential and cytoplasm macromolecules including nucleotide. This suggest that the action mode of chlorogenic acid may be different from some reported peptides and other antibiotics, which can kill the microorganisms by pore formation in the

cell membrane. The hypothesis about the strong sensitivity observed for *Campylobacter spp*. is that chlorogenic acid bound to the outer membrane, disrupted the membrane, exhausted the intracellular potential, and released cytoplasm macromolecules, which led to cell death.

These results support our findings and they confirm that one of the main mode of action related to the WEME70 is the cellular membrane damage as demostrated by viability cells and spectrofluorimetric assay previously mentioned in this thesis.

3.4.5 Cytotoxic effect of WEME70

3.4.5.2. Sensitivity of the cell lines to WEME70

The experimental system used for the experiment is represented by a cell line of adenocarcinoma of human colon-rectum, Caco-2. Although these cells comes from a colon cancer (large intestine), growing in specific conditions they become differentiated and polarized so that their phenotype, both in morphology and functional, appears like the enterocytes which cover the small intestine. The Caco-2 cells express tight junctions, microvilli, and a number of enzymes and transporters that are characteristic of such enterocytes: peptidase, esterase, P-glycoprotein, etc. The Caco-2 cells are more commonly used not as single cells, but as a confluent monolayer on plates for cell cultures (eg petri dishes). When they grow in this way, the cells become differentiated to form a monolayer of polarized epithelial cells that provides a physical and chemical barrier to the transfer of ions and small molecules. The Caco-2 monolayer is widely used throughout the pharmaceutical industry as an in vitro model of human intestinal mucosa to predict the absorption of drugs administered orally. We employed the cell viability test (MTT) in order to evaluate the potential cytotoxic effects of extract of myrtle on the cell line Caco-2. We have plated the Caco-2 cells (10,000 cells / well in a 96 multiwell), and treated the day after with scalar myrtle extract concentrations (1 - 2.5 - 5 - 10 - 20 - 40 - 80 - 100 and 200 µg).

After 24 hours of treatment, the cells were subjected to MTT assay. The results obtained from the MTT assay showed an inhibition of cell growth by 50% (IC50) induced from the extract of myrtle to the concentration of about 50 µg. Within the range of concentrations between 1-10 mM the extract does not present have cytotoxic activity, as can be clear from the graph (**Figure 8)**

3.4.6 Antimicrobial activity in vitro

3.4.6.1 Antimicrobial activity of water-ethanol myrtle extract (WEME70) in agar and in broth medium

The strength of the antibacterial activity can be determined by dilution of antimicrobial substances in agar or broth. A preliminary check for antibacterial activity is generally done in agar plate prior to more detailed studies. The sensitivity of a wide range of microorganisms to the antimicrobial extract WEME70 was tested using the agar well diffusion assay and the WEME70 titer was determined by the two-fold dilution method.

The filtered concentrate (100mg DM/ml) of WEME70 exhibited strong and broad-spectrum antimicrobial activity towards the Gram-positive bacteria *L.monocytogenes*, *L.innocua*, *S.aureus* and *B. thermosphacta* (**Table 2b**). The antimicrobial activity was observed also against the Gram-negative bacteria like *P. fragi*, and pathogenic strains like *E.coli* ATCC 25992, *C. jejuni* and *C. coli*. The WEME70 was tested against 105 strains and found to be effective against 102 strains examined in this investigation. Among all strains tested only *S. enteritidis, Hafnia alvei 53M* and *E.coli* O157:H7 have shown microbial resistance to the extract. Results were represented as the mean values with the relevant standard deviation error. The Gram-positive bacteria that exhibited in agar plate an higher sensitivity to the WEME70 tested were *L. monocytogenes*, *L. innocua* and *S. aureus* strains with values of AU $ml^{-1}$from 6400 to 25600 (**tables 3 and 4**) while *B. thermosphacta* recorded values of 800 to 1600 AU ml^{-1} (**table 5**).The same values were also showed by Gram-negatives bacteria, where *P. fragi* and *Campylobacter* species seem to be equally sensitive, with the only one exception of *C. coli FC7* strain for which was observed a value of 3200 AU ml^{-1} resulting therefore more sensitive within its genus (**tables 6 and 7**). Overall, is possible to affirm that *L. monocytogenes* appear to be the most sensitive among the pathogens tested in this screening.The demonstration of antibacterial activity against both gram positive and gram negative bacteria may be indicative of the presence of broad spectrum antibiotic compounds. This will be of immense advantage in fighting the menace of antibiotic refractive pathogens that are so prevalent in recent times.

These previous results are in line with the small number of published papers on the effects on bacterial growth of *M. communis* where the major activity was against Gram-positive strains than that Gram-negative **(Ghalem and Mohamed 2008)**.Anyway, it is difficult to compare the data obtained with the literature because several variables influence the results, such as the different chemical composition due to the environmental factors (such as geography, temperature, day length, nutrients, etc) of the plant.

That gram-negative organisms are less susceptible to the action of antibacterials is perhaps to be expected, since they possess an outer membrane surrounding the cell wall **(Ratledge and Wilkinson, 1988)**, which restricts diffusion of hydro-phobic compounds through its lipopolysaccharide covering **(Vaara, 1992)**.

The diffusion assays result to be unsuited to nonpolar substances testing (e.g. essential oil) because the components are partitioned through the agar according to their affinity with water, therefore the antimicrobial effectiveness of a WEME70 was defined in broth, in terms of its minimum inhibitory concentration (MIC), and of its minimum lethal concentration (MLC), which are cited by most researchers as a measure of the anti-bacterial performance.

To define the MIC and MLC values in this study a chemically and microbially inert stabilizer which DMSO (able to solubilize both polar and nonpolar compounds)was chosen to dissolve the WEME70, in order to improve the diffusion into the liquid medium.

Bacteriostatic and bactericidal concentrations of WEME70 was determined in broth only against the microorganisms that exhibited sensitivity in the previous assay. All results are reported in **table 8, 9 and 10**.

Among the Gram-positive bacteria, the results in terms of MIC values in a range between 12.5 and 25mg DM/ml showed no differences among the *Listeria* and *S. aureus* species, while for 33% of *B. thermosphacta* strains were also observed a MIC value of 50 mg DM/ml which corresponded also to the highest MLC value found for this specie. In fact, WEME70 at concentration of 50 mg DM/ml showed bacteriostatic and bactericidal effect against about 36% of the *B. thermosphacta* strains tested.

Otherwise, *Listeria* and *S.aureus* species, excepted of *L.monocytogenes OH* more sensitive, showed no differences in the MLC values defined in a range from 50 to 100 mg DM/ml, resulting higher resistant than *B. Thermosphacta* strains. These data are not surprising since the pathogenic species such as *Listeria* and *S. aureus* are increasingly resistant to a rising number of microbial agents and also inevitably the same antibiotics with bactericidal effect used against these organisms are less effective. As rapidly as new antibiotics are introduced, pathogens have developed efficient mechanisms to neutralize them (**Diekema et al., 2001)**.

Among the Gram-negative bacteria *P. fragi* and *Campylobacter* species showed similar behaviour recording MIC values of 25 mg DM/ml for 70% of strains. For the remaining 30% of *Campylobacter* strains were observed MIC values of 50mg DM/ml, on the contrary 30% of *P. fragi*strains showed higher values of 100 mg DM/ml.

Comparatively MLC values were from 25 to 100 mg DM/ml for both species. To sum up, WEME70 at concentration of 25 mg DM/ml showed bacteriostatic and bactericidal effect against 70% and 30% of *P. fragi* and *Campylobacter* strains, respectively.

Moreover, concentrations of 50mg DM/ml of WEME70 were able to kill 40% of *P. fragi* and 63% of *Campylobacter* strains tested. The high MLC value of 100 mg DM/ml were found for a smaller group of microorganism between the two different species. In particular, 8 strains of *P. fragi*(A1, B81, C81, D1, A81, M52, N1 and ATCC3456) and 2 strains of *Campylobacter* (*C.jejuni* 118and *C. coli* FC11) were killed only after exposure to 100 mg DM/ml of WEME70.

3.4.6.2 Effect of WEME on resting cells

The effect of water-ethanol myrtle extract, at concentrations from 100 to 0.0 mg DM/ml, on the viability of resting cells of 3 strains of *B. thermosphacta* and 3 strains of *P. fragi* suspended in phosphate buffer is reported in **Figures 9 and 10**, respectively. Results suggest variations in vulnerability of the different strains belonging to the same species. *B. thermosphacta* Gc202 and Ec71were killed after 10 minutes of exposure to 25 and 50 mg DM/ml while the strain B02 of *B. thermosphacta* was killed only after 120 minutes of exposure to 50 mg DM/ml of extract. The population of this strain was reduced of about 3 Log CFU/ml in 120 min with 25 mg DM/ml of extract (**Figure 11a**). The 3 strains of *P. fragi* assayed proved to be less sensitive to the activity of the WEME70. The strain of *P. fragi* H83 was inactivated after 60 minutes of exposure to 100 mg DM/ml and after 120 minutes of exposure to 50 mg DM/ml, whereas at 25 mg DM/ml of extract the population was reduced of about 2 Log CFU/ml (**Figure 11c**). Strains A1 and T81 of *P. fragi* were killed after 20 minutes of exposure to 100 mg DM/ml and after 120 minutes by 25 and 50 mg DM/ml (**Figure 11b and 11d**). Viable counts of cell suspensions without water-ethanol myrtle extract were unchanged (data not shown). Two way ANOVA carried out for results at 5 and 10 minutes indicate that for each species tested the effect of strain, log (dose) and interaction was always significant ($p<0.001$). The significant interaction was due to the different dose response pattern of the strains. In fact, H83 was the *P. fragi* strain which was most affected by increase in dose, while *B. thermosphacta* Ec71 showed little change in lethality as a function of the dose at both 5 and 10 min in the range of concentrations tested. Moreover the lethality of some doses was so high that within the first few minutes the viable counts fell below the detection limit of the method (10^5-10^4 CFU/ml, see **figures 11a and 11b**).

3.4.6.3. Antimicrobial activity of hexane and ethanol fractions of WEME70

The previous data underlined a different effect of WEME70 among Gram negative and Gram positive bacteria tested.

These differences, not species-specific associated, suggested a strong variability in the composition of WEME70 and the presence of different components with particular chemical properties.

The assumption above were confirmed by the antimicrobial assays performed on the collected fractions by partition chromatography which differed for their chemical profiles.

Basically, the WEME70 molecules showed a different solvent affinity when separated by liquid-liquid extraction with n-hexane, generating two myrtle phases: one suspended in ethanol at 11.5% and the other one in hexane, respectively renamed as Polar phase (PP) and Less polar phase (LPP).

The antimicrobial activity of these two phases obtained from a starting concentration of 100mg DM/ml of WEME70, was also investigated by *agar well diffusion* and *spot on the lawn* method against all the bacteria previously tested in order to define the selectivity effect of the bioactive compounds of WEME70. The partition chromatography gave a final concentration of both fractions (LPP and PP) corresponding to 15 mg DM/ml and 80 mg/ml respectively. In order to eliminate the possibility of interference by solvents used with the liquid-liquid extraction causing sensitivity differences, these were eliminated by evaporating of nitrogen gas and the experiment was performed using DMSO 5% to dissolve the LPP while ethanol solution at 11.5% was used for PP, due to a weak sensitivity of Campylobacters. The results of this experiment against Gram positive and Gram negative bacteria are showed respectively in **table 11** and **tables 12 and 13**. The values of the inhibition halo reported in the tables for each strain correspond to the average of three replicates ± standard deviation. Data indicated that DMSO and ethanol did not cause any inhibition effect when employed as controls, resulting not active on the cell inhibition growth. It has been noted that the two myrtle extract phases gave antibacterial effect against specific targets, comparatively the LPP was effective exclusively against Gram positive bacteria (*B. thermosphacta, Listeria* and *S. aureus* strains) on the contrary the PP showed its selective activity against Gram negative microorganisms (*P. fragi* and *Campylobacter spp*.). These data, highlighted and confirmed the presence of several molecules in the two PP and PP fractions, with a selective affinity according to the polarity of the solvent.

The tables showed a slight increase of the arbitrary units values compared to the previous values recorded for the whole extract WEME70, these data were confirmed as well by the MIC and MLC values of both fraction for most of pathogenic strains tested (**table 14 and 15**). This should be related to the partial purification of myrtle compounds, which during the diffusion in agar medium probably, were affected by the interference of others molecules characterized by a different polarity.

Moreover, was also determined the antimicrobial activity of collected fractions obtained by column chromatography of n-hexane myrtle phase (LPP) and by RP-HPLC of ethanol phase (PP) by agar well diffusion assay against main indicators strains in order to narrow the field of investigation and to characterize the main active compounds.

Tables 16 showed the antibacterial effect of 7 fractions separated by the LPP, recording the highest value for the fraction 20 against all Gram-positive bacteria tested (*L. monocytogenes ATCC 7644*, *S. aureus ATCC 20231* and *Br. thermosphacta ATCC 11509*). Instead, **table 17** displayed the specific effect of two fractions (indicated as 9 and 12) against the main representative Gram-negative microorganism tested which are *P. fragi* ATCC 3456,*C. jejuni* FC7and*C. jejuni* LP1 .

The resistance of Gram-negative bacteria towards lipophilic molecules present in the active LPP, is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier to the

penetration of numerous bioactive lipophilic molecules and is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside (**Nikaido, 1994** and **Gao et al., 1999**). The Gram-positive bacteria do not have such an outer membrane and the cell wall structure is different. Therefore, the antibacterial molecules can easily destroy the cell wall and the cytoplasmic membrane, causing leakage of the cytoplasm (**Kalemba and Kunicka, 2003**). The indicators strains tested in this study follow the trend described above. Has to be underlined that the diameter of inhibition zone measured in vitro using a concentration of 2mg DM/ml of fractions 9 and 12 of myrtle PP was significantly lower(in a range from 2 to 10mm) than values previously showed from the whole mixture of PP (80mg DM/ml). This value has not been taken as a discrepancy of what tested and measured before because some assays relying on diffusion of test flavonoids may not give a reliable quantitative measure of antibacterial activity because a potent antibacterial flavonoid may have a low rate of diffusion (**Zheng et al., 1996**).Rather it suggest that there is a synergistic effect of molecules contained into the PP, which tested separately gave inhibition activity against the same bacteria but in lower level.On the contrary, in spite of the lower concentration tested (2mg DM/ml) for the fraction 20 coming from the myrtle LPP (15mg DM/ml) surprisingly has been observed an enhanced value in diameter of inhibition halos measured in a range between 24 and 30mm, suggesting a different behavior in terms of activity respect to the PP compounds. In fact, it seems that these latest compounds used as singles increased their effect, probably due to the interaction with other compounds or to the affinity of these for the same cellular receptor sites.
In fact, these fractions were found very rich in polyphenols compounds (as reported in paragraph 4.3.3) especially of flavonoids molecules (flavanols, flavonol glycosides and flavanones) which have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms (**Cushnie and Lamb 2005**).It is well known that antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Moreover, has been demonstrated that lipophilic flavonoids like luteolin, myricetin and quercetin may also disrupt microbial membranes which was one of the mechanism of action detected in this study (see paragraph 4.3.5.4).

3.4.7 Effect of hexane and ethanol fractions on membrane integrity of pathogenic bacteria

3.4.7.1. Fluorescent cell viability assay
It has been proposed that bioactive compounds from plant have different modes of action that result in the loss of the microbial viability or death. These antimicrobial effects usually lead to the destabilization of the double phospholipid layer, the disruption of plasma membrane function and composition, the loss of vital intracellular components, and the inactivation of enzymatic mechanisms, thereby inhibiting electron transport for energy production and disrupting the proton motive force, protein translocation and the synthesis of cellular components. These major physiological changes can ultimately result in cell lysis and death(**Ben Arfa et al., 2006 and Turina et al., 2006**).It is well known that green-fluorescing SYTO 9 is able to enter all cells, whereas red-fluorescing PI enters only cells with damaged membranes. Once, the cell membrane integrity is ruptured, PI enters cells and competes with SYTO 9 for the same intracellular targets, causing the displacement of SYTO 9 and quenching SYTO 9 fluorescence by fluorescence energy transfer **(Barney et al., 2007).** Based on there, after an inoculum in broth containing 50µg/ml of PP and LPP of WEME70, respectively for the Gram negative and Gram positive bacteria, the viable staining was performed after 24 h of incubation at the optimal growth temperature of each strain by using LIVE/DEAD® Bacterial Viability Kit (BacLightTM).
Figures 12, 13 and 14 revealed marked changes membrane permeability of pathogenic strains like L. monocytogenes, S. aureus and C. coli after exposure to PP and LPP of WEME70 applied alone at sublethal concentrations.
The considerable changes observed to the cellular membrane by fluorescent microscopy confirm the sensitivity of the cell envelope of these bacteria to the PP and LPP, respectively for Gram negative and Gram positive. These compounds seem to penetrate the cytoplasmic membrane, increasing the permeability and causing probably the loss of cytosolic material.
The exact mechanism underlying the antimicrobial effect of the single application of the test compounds is still unknown. Nevertheless, the increased antimicrobial activity observed when cells were treated only with PP or with the LPP at sublethal concentrations may partially be explained by the different chemical structures of these compounds.
Because exposure to PP and LPP alone induced significant changes in viability and permeability of treated cells after 24 hours of contact, the membrane damage was further investigated by multi detection microplate reader to determine if the same effects could be produced with a shorter incubation time monitoring the microbial growth during these times.

3.4.7.2 Study of the integrity of pathogenic cell membrane after exposure to myrtle fractions

MIC and MLC values of LPP and PP, defined in previous experiment, were used in order to evaluate during the time their effect on the membrane integrity of pathogenic bacteria. Exposing cell suspensions of *Listeria spp.*, *S.aureus* and *Campylobacter spp*. To 0.05 – 2.0mg/ml of LPP or of PP, for different times (30minutes, 2, 4, 6, 8 and 24 hours) increased

cell permeability to the fluorescent nucleic acid stain, propidium iodide (**Fig.15 a-b-c, 16 a-b-c, 17 a-b-c-d**), relative to control suspensions that did not contain myrtle extract. The inability of propidium iodide to penetrate cells with intact cytoplasmic or plasma membranes(**Lebaron *et al.* , 1998**) was confirmed by the low level of uptake observed in cells not exposed to myrtle fractions.

The data, which are representative of triplicate experiments that gave similar results, shows that membrane damage of *Listeria* , *S.aureus* and *Campylobacter* cells commenced immediately upon addition of myrtle phases. Increased uptake of the nucleic acid stain propidium iodide, to which the cell membrane is normally impermeable, was observed. Also, the microbial load monitored by OD and plate count agar determinations showed at t_0 an inhibition of growth for all bacteria tested.

In particular, the LPP at 0.05 (w/v) induced inhibition of growth of *Listeria* and *Staphylococcus* strains after only 30 minutes of exposure, keeping the bacterial load (3.5Log CFU/ml) unchanged till to 24 hours of incubation, while controls reached values of 8.0 Log CFU/ml for *Listeria* strains, 8.4 Log CFU/ml and 8.7 Log CFU/ml for *S.aureus* ATCC 25993 and *S.aureus* 239, respectively.

The concentration of 2 mg/ml of LPP gave the same bacteriostatic effect but do not lead to the cell death. These data are in line with the results obtained by broth dilution assay, where the lower concentration of LPP after 1 day of contact was able to have a batteriostatic effect on the cells growth, while the maximum concentration tested was unable to show a bactericidal activity.

The damage of the membrane integrity was expressed as ΔRF (increase in RF over than control). **Figure 15a** show for *L. monocytogenes* treated with 0.05 mg/ml of LPP a ΔRF of 23.49 at $t_0$respect to the controls and a twice value of ΔRF equally to 40.76 at t_{24}. The highest value of ΔRFcorresponding to 89.74 was measured at t_{24}in cells treated with 2 mg/ml of LPP. *L innocua* treated with the lower amount of LPP gave significant differences from the previous strain tested at t_0 and t_2, showing only after 4 hours of exposure a membrane damage with value of ΔRF of 23.63. On the contrary, similar results were found at t_{24} for both concentrations LPP which determined a ΔRFof 34.9 and 72.19 showing greater cell damage.

Significant lower values of ΔRF were observed for *S.aureus* strains for all times analysed (**Figure 16a, 16b**), this may be due to the rapid and higher growth of the control and a lower membrane damage in cells treated. In fact, for *S.aureus* 239 the higher values measured of ΔRF were 2.78 at t_8 for 0.05 mg/ml of LPP and 8.16 at t_{24} for 2 mg/ml of LPP. Similar behaviour was showed by *S.aureus* ATCC 25993for which the highest values of ΔRF at t_{24} corresponding to 14.54 and 18.37 respectively for 0.05 and 2 mg/ml of LPP.

Effect of the myrtle PP on the membrane integrity was evaluated for four Campylobacters which are *C. coli* FC2, *C. coli*FC3, *C.jejuni* FC7 and *C. coli* FC11 as they exhibited a different sensitivity to the substance (**Figure 17a, 17b, 17c and 17d**). In particular, *C.jejuni* FC7 with MIC and MLC values of 0.05 mg/ml of PP resulted the more sensitive strains, followed by *C. coli* FC2 which was also inhibited by 0.05mg/ml but killed by 1mg/ml of PP resulting therefore a medium resistant strains like *C. coli* FC3which showed the same MLC value of the latest mentioned but an higher value of MIC corresponding to 0.1mg/ml of PP.

A great difference was found for *C. coli* FC11 which was the only one strain resistant to the concentration of 2mg/ml and for this considered as the less sensitive strain.

The previous assumptions were partially confirmed, as *C.jejuni* FC7 compared with the control showed a ΔRF 42.1 after only two hours of contact with PP at concentration of 2mg/ml, while for cells treated with a lower concentration of 0.5mg/ml PP was determined a ΔRF 28.95 even at t_0.

For *C.coli* strains FC2 and FC3 was observed similar sensitivity to the PP, showing the maximum effect on the membrane damage after longer time of exposure. In fact, after six hours cells of *C. coli* FC2 treated have displayed a ΔRF of 31.9 and 7.8 relatively to the concentration of 2 and 0.5mg / ml, while for *C. coli* FC3 at the same time and at the same concentrations were determined a ΔRF of 54.1 and 10.55 confirming their medium resistance as none of tested concentrations caused the cell death. Despite this, is interesting to note that the maximum value of cell damage at 6 hours of treatment corresponds to a reduction in the growth of *C. coli* FC3 within 2 hours post.

Finally, a ΔRF 80.9 was shown by *C. coli* FC11 after 4 hours of contact with the PP at a concentration of 2mg /ml.

Cells treated with a lower concentration corresponding to 0.5mg/ml PP exhibit a ΔRF equal to 27.18 at t_0.

Therefore, this strain showed the highest values of damage of the cellular membrane but at the same time also shows a microbial load greater than the other strains to which corresponds in fact a relative reduction of theΔRF, resulting in any case the more resistant strain to both concentration accordingly to the previous results.

This study has demonstrated that campylobacters are highly sensitive to myrtle, although some variables, such as bacterial strain, can influence the magnitude of the response. Although, we have not studied the mechanisms responsible for this behaviour, it is likely that they are related to physiological changes in the membrane composition, considering that this is the target site for myrtle.

Overall, is possible to affirm that all tested strains were affected by damage of the cell membrane caused by the polar and non-polar fractions from myrtle extract, although not always in the same order of magnitude and at the same times. This suggest that there are differences not only species-specific but also strain-specific in terms of resistance to the tested substance.

These data strongly indicate that the treatment with PP and LPP alone at sublethal concentrations leads to the loss of cell wall and cytoplasmic membrane integrity, causing cell death even after short incubation times.

The results presented in this study showed that PP and LPP alone at sublethal concentrations caused a decrease in cell viability and marked changes in the membrane permeability of pathogenic bacteria based in broth. Our findings reinforce the conclusion that a combination of antimicrobial compounds with different chemical structures, such as PP and LPP, at sufficiently low concentrations could be applied to control pathogenic growth on foods, particularly meat and dairy products.

3.4.8 Antimicrobial activity of WEME in situ

3.4.8.1 Antimicrobial activity of freeze-dried myrtle extract (FDM)in ground beef

In situ experiments were carried out to evaluate the efficacy of antibacterial activity of freeze-dried myrtle extract (FDM) in ground beef during refrigerated storage conditions.

The ground beef samples were assigned to one of four different treatments: CC: Control, meat without freeze-dried myrtle; A: meat with 1% of FDM; B: meat with 3% of FDM and C: meat with 5% of FDM.

The microbiological changes of the ground beef during storage at 4°C are shown in **Table 18**. In meat without freeze-dried myrtle (control) all bacterial populations increase during storage at 4°C. In ground meat with 5% of FDM, except for *Pseudomonas* spp., microbial population decreased significantly. Total viable count (TVC) increased during the storage at 4°C in all the samples of ground beef 52nalysed. In the control meat samples the TVC was initially approximately 5.65 log CFU/g, which increased steadily with storage time and reached close to 8.82 log CFU/g at 5 days of storage. At the end of the second and third day of storage, in the samples treated with different amount of myrtle, bacterial populations were significantly ($P<0.05$)lower than in the control although after 5 days of storage at 4°C the TVC reached the same concentration in all the samples 52nalysed. The results showed that the antimicrobial activity of freeze-dried myrtle extract was most effective against *B. thermosphacta*. Initially the population of *B. thermosphacta* in the meat control was approximately 6.05 log CFU/g, which increased steadily with storage time and reached close to 7.91 log CFU/g after 5 days. After 3 days of storage *B. thermosphacta* counts in all the meat treated samples (A, B and C) was significantly ($P<0.05$)lower than in the control (**table 18**). The antimicrobial activity of FDM was recorded against *Enterobacteriaceae* after 1 day of storage at 4°C in the samples treated with 3% and 5% of the substance. After 2 and 3 days the number of *Enterobacteriaceae* in the samples treated with 5% of myrtle was significantly ($P<0.05$) lower than in the control of 1.31 and 0.58 log CFU/g meat, respectively. A similar result was obtained also for Lactic Acid bacteria population, which decrease significantly after 2 and 3 days in the samples, treated with 3% and 5% of myrtle.

Finally, none of the FDM concentrations showed significant effects on the growth of *Pseudomonas* spp. (**Table 18**).

3.4.8.2. Antilisteria activity of WEME's PE film coated in Gorgonzola cheese

The antimicrobial activity of WEME70 was also assayed in situ for activity against growth of *L. monocytogenes OH* inoculated in Gorgonzola cheese. As shown in **Fig. 18**, the growth of indicator strain gradually decreased during the storage period of 15 days at 4°C, compared with the two controls, respectively gorgonzola cheese and gorgonzola cheese added of ethanol at 11.5%(v/v), where on the contrary *L. monocytogenes* grew unchallenged.

Accordingly to the previous result recorded in vitro for the MIC value of 12.5mg DM/ml, the WEME70at the same concentration clearly inhibit the development of the pathogen during the storage period, showing immediately a reduction of 1.3 Log in terms of microbial load at time 0 (corresponding to 30 minutes of contact with WEME70) after which was observed a slight increase in microbial growth up to a value of 1.9 Log during the first 9 days. These values appear to be to be significantly lower than displayed by the controls which reached values of 3.2 Log at the end of the experiment. Subsequently to the bacteriostatic effect, it was also observed the bactericidal effect of the extract on the indicator strain after 15 days of storage, corresponding to a completely absence of *L. monocytogenes* OH in the treated Gorgonzola sample.

3.4.8.3. Antipseudomonas activity of WEME's polar phase coated films during the storage of meat products (beef steaks)

The PE films used for the active packaging manufacture were chosen for their technological characteristics such as solder ability, resistance to tensile stress, transparency, etc. and before and after the activation treatment they were always shown to keep their quality after being coated with myrtle PP.

The activation of PE films with the PP of WEME70was performed by coating method yielded positive results; in fact, after coating of PE film with the PP at different concentrations, the films showed to be always active against *P.fragi* in agar inhibition assays. In all the cases untreated films did not show any antimicrobial activity. An example of the detection of antimicrobial activity of the PP coated PE films is shown in **Fig. 19**. There was no difference in inhibition intensity when films were treated with a solution of PP at 1600 and 3200 AU ml^{-1}but an higher inhibition was observed in agar plate for the PP solution at 6400 AU ml^{-1}. The growth inhibition area was closely confined to the film area on the plate confirming that the distribution of the substance was homogeneous on the surface of film. In order to assess the PP activity in situ, the developed antimicrobial films with the highest activity were also used in challenge tests of storage of meat products artificially contaminated by *P. fragi* as shown in **Fig. 20.**

The results of the viable counts of *P.fragi* on raw beef slices at different times (0, 3 and 5 days) of storage at 4°C are reported in **Fig. 21.**

The trend of the *P.fragi* population appeared to be the same at the end of the storage period of the beef slices in PE films both with and without PP treatment. However, significant differences were observed in the viable count of *P.fragi* at the beginning of the storage period in the samples packed with the PE film treated. In particular, the spoilage strain was reduced of almost 1 log after 30 minutes of contact with the treated film and then showed a lower load of 0.7 Log after 1 day at 4°C compared with the control.

Unfortunately, the batteriostatic effect was lost during the storage at 2, 3 and 5 days and the microbial count increased reaching same values of meat packaged with untreated film.

For active antimicrobial packaging to be effective, an adequate procedure of activation is necessary in order to assure that the antimicrobial is linked to the film and to keep the antimicrobial activity during the film shelf life. Moreover, the activated film has to exert its preservative antimicrobial potential during the storage (**Mauriello et al., 2004**).

Although, the PP coated PE film showed good antimicrobial effect in agar plate against the indicator strain and a batteriostatic effect was also observed inhibiting the grow of *P. fragi* at the first times of the experiment, the efficacy of the activated film was lost during the storage, proving to be not useful in controlling of spoilage growth.

3.5 Discussion

Rapid and reliable inhibition of microorganisms in food samples is essential for prevention of disease which are the major cause of morbidity and mortality worldwide, but it is also important to save on the cost of waste of infected products, (**Rathnayaka, 2011**). The number of multidrug resistant microbial strains and the appearance of strains which reduced susceptibility to antibiotics are continuously increasing. Such increase has been attributed to indiscriminate use of broad spectrum antibiotics, therefore the development of new antimicrobial substances food grade and economically competitive to reduce or eliminate the presence of undesirable microorganisms in food, are always needed.

In this context, natural antimicrobial compounds may play an important role provided the impetus to the search for new antimicrobial substances from various source like medicinal plants.

In this study an hydroalcoholic leaves extract from myrtle plant was tested for its antimicrobial activity against foodborne pathogens and spoilage bacterial strains, both Gram-positives and Gram-negatives.

The choice of the solvent to use for the extraction was done considering the final purpose to use in food systems. Different solvents have been reported to have the capacity to extract different molecules depending on their solubility or polarity in the solvent (**Marjorie MC, 1999**).

Though specifically it was observed that, the type of solvent followed by the concentration of solvent of extraction were important factors to improve the antimicrobial efficacy, while the time of extraction has not affected variation of the antimicrobial activity. Out of the three solvents used for extraction, ethanol at 70% showed better characteristics as a solvent of extraction for antimicrobial compounds, followed by ethanol at 40% and then distilled water.
The WEME70 obtained after 24 hours of maceration showed the highest activity against the tested organisms, none significant differences was shown increasing the timing of extraction. The final concentration of ethanol residual from the extraction corresponded to 11.5% and was not found damaging on cells.
Although extensive information on the antibacterial activity of Myrtus communis is available , sometimes reports are contradictory. Some authors have reported that myrtle extracts are more effective against gram-positive bacteria, while others have considered that it presents a higher bactericidal capacity against gram-negative.
In the present study, myrtle leaves hydroalcoholic extract have shown a high efficacy at inhibiting the growth of both gram-negative and gram-positive bacteria.
105 indicators, pathogenic and food spoilage strains, belonging to the Gram positive and Gram negative species were tested for their sensitivity to the WEME70 which showed a broad spectrum of activity. Among the pathogenic Gram positive bacteria *Listeria monocytogenes OH* was found more sensitive to the myrtle effect with MIC and MLC values respectively of 12.5 and 25 mg DM/ml, recording a diameter of inhibition zone of 26 ± 0.05 mm and a titer of 25600 AU ml^{-1}. Results obtained through in vitro assays have been also confirmed by a challenge study carried out, inoculating *L. monocytogenes OH* in gorgonzola cheese added of WEME70 in concentration of 12.5 mg/g and stored at refrigerating temperature for 2 weeks, where the initial concentration of the pathogenic strain gradually decreased during 9 days until to disappear after 15 days.
Among Gram negative bacteria, *P. fragi* and *Campylobacter* spp. seems to appear equally sensitive to the myrtle leaves hydroalcoholic extract. Surprisingly, there are no previous reports in the literature about the effect of myrtle on *Campylobacter*, in spite of its importance as a foodborne pathogen. Therefore, positive data obtained against this specie result very remarkable and interesting. This work has shown that although the sensitivity of campylobacters to myrtle extract is high.
The viable staining procedure by using the LIVE/DEAD BacLightTM Bacterial Viability Kit was chosen to rapidly assess the bacterial viability in the myrtle-treated samples. This method is used to microscopically to evaluate the cellular membrane damage in live and dead bacteria in environmental samples or in laboratory conditions, and several applications can be found in the recent literature (**Decker et al., 2001**). In the LIVE/DEAD BacLightTM bacterial viability kit the SYTO 9 labels all the bacteria in a population staining them green, while propidium iodide penetrates only bacteria whose cell membrane has been damaged, staining them red. Therefore, undamaged cells will be stained green and scored as live whereas cells whose membrane is damaged will appear red and scored as dead (**Ercolini et al, 2006**) . In each experiment performed in this work, the contact of the PP and LPP of WEME70 with cells of pathogenic strains which are *L. monocytogenes*, *S. aureus* and *C. coli* turned all or part of the cells into red.
Study of the membrane integrity of pathogenic after exposure to the polar and nonpolar phase of WEME70 was also performed by a highly sensitive spectrofluorimetric method developed and validated for the determination of the loss of membrane integrity of all microorganism tested compared with untreated cells, monitoring the cell viable count as well by OD using a microplate multi-mode reader and plate count agar.
Results have demonstrated that all pathogenic strains involved in this study are highly sensitive to myrtle compounds, although some variables, such as bacterial strain, can influence the magnitude of the response.
In fact, Gram positive bacteria appear to be more inactivated by LPP of WEME70 showing no growth at the lower and higher concentrations tested respectively of 0.05 and 2 mg/ml while Campylobacter strains seems to be less sensitive to the same concentrations of PP which are able to damage the membrane integrity and to inhibit the growth but unable to kill bacteria.
Although we have not studied the mechanisms responsible for this behavior, it is likely that they are related to physiological changes in the membrane composition, considering that mains molecules identified by mass spectrometry for WEME70 are represented by acylphloroglucinols and clorogenic acids for which is well described in literature the strong antimicrobial activity due to their ability to damage and permeabilize the outer membrane.

3.6. Conclusion

The overall results demonstrate that the hydroalcoholic myrtle extract WEME70 has an antimicrobial effect on populations of food spoilage and pathogenic bacteria. The direct contact of the populations with the WEME70 resulted effective for an irreversible inactivation of the L. monocytogenes population and for a bacteriostatic effect on all the others strains. This activity is most likely associated with the phenolic compounds in the extract that can effect cellular membranes altering their permeability and release of intracellular constituents but also interfere with membrane functions (electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity). The essential oils from many plants have recently been of increased interest in research as well as the food industry that are looking for natural additives to replace synthetic antioxidants in food products. The antimicrobial activity of the hydroalcoholic leaves extract of Myrtus communis suggest that this plant justifies further study. According to these results, the use of myrtle film is encouraged especially for food products where the superficial contaminants come immediately in contact with the antimicrobial film but also for food preparation where myrtle could be commonly used for flavouring. The effect would be a fast inactivation and control of the population, which coupled with appropriate condition of storage, might improve the quality and safety and prolong the shelf-life of the food products packed in antimicrobial films or characterized by myrtle extract as natural ingredient .

Therefore, for M. communis grown as foliage for cutting and used for flower compositions, is possible to redirect the inevitable waste (leaves damaged by phyto-pathogens or insects parasitic and abiotic agents) to a micro-chain, possibly local, for the extraction of useful secondary metabolites identified, eliminating or almost the commercial value of production waste.

Of course, this extract require further in vivo and in situ investigations such as the antagonistic activity against foodborne pathogens in food matrices using different vehicles such as the microencapsulation technology. Moreover, after specific studies on the sensory evaluation in food systems added with myrtle, the suitable extract or directly its biological active molecules may be eventually used as natural preservatives.

3.7 References

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3.8 Bacteriocin production by *Lb. curvatus* 54M16 and its technological properties as starter culture

The ability of *Lb. curvatus* 54M16 strain to produce bacteriocin was investigated through *in vitro* tests as the spot on lawn method and critical dilution assay against several indicators strains, food pathogens and spoilage bacteria. Finally, the main technological properties of the strain were determined in order to evaluate its suitability as starter culture in food preparations.

3.9 Materials and methods

3.9.1 Bacterial strains and culture conditions

L. curvatus 54M16 object of this study, isolated from fermented sausage from Valle di Diano was supplied from the collection of the Department of Agriculture, Division of Microbiology - University of Naples. Twelve bacterial strains belonging to different species among which food borne pathogens and spoilage microorganisms, have been used as indicators: 3 strains of *L. monocytogenes*, *L. innocua* C6, 3 strains of *S. aureus*, *Clostridium perfringens* 13124, *Clostridium sporogenes* SPOL, *Clostridium butyricum* BUT, *Bacillus cereus* BC, *E. coli* O157:H7. All strains were grown in an appropriate nutrient medium to the optimal conditions of time and temperature which are indicated in **table 19**. *Lb. curvatus 54M16* cultures were maintained in appropriate broths with 25% (v/v) sterile glycerol and stored at -20°C. Plating and overlay medium were prepared by adding 1.5 and 0.75% agar, respectively, to the TSB medium listed in **Table 19**.

3.9.2 Bacteriocin production by Lb. curvatus 54M16 and measurement of bacteriocin activity

Lb. curvatus 54M16 strain was grown in MRS broth (Oxoid, UK) for 16 h at 30 °C. The cells were removed by centrifugation (10,000 g for 10 min at 4 °C) and the cell-free supernatant fluid (CFSF) was adjusted to pH 7.0 with 2 mol l^{-1} NaOH and filter-sterilized through 0.22 µm pore size filters (Sterile Acrodisc, Gelman Sciences, USA). Aliquots (10 µl) of the CFSF were assayed for bacteriocin activity using the spot-on-lawn method (**Villani et al., 1993**) against indicator strains listed in **Table 20**. Inhibition due to proteinaceous substances was assessed by spotting near the spot containing the CFSF 5 µl of proteolytic enzymes (10 mg ml^{-1}; trypsin, pronase E, α-chymotrypsin and pepsin, all from Sigma, Chemical Co., St. Louis, MO, USA, were tested; the enzymes were dissolved in 50 mmol l^{-1} Tris–HCl, pH 7.0) which resulted in the disappearance of the inhibition zone near the enzyme spot. Bacteriocin activity was measured by the spot-on-lawn method and critical dilution assay as previously described (**Villani et al., 1995**). Briefly, 10 µl of serial two-fold dilutions of CFSF were spotted on Tryptone Soya Agar (TSA, Oxoid, UK) plates previously inoculated with the selected indicator strain (about 10^6 CFU ml^1). After diffusion (30 min at room temperature) the plates were incubated at 30 °C for 24 h and examined for zone of inhibition.
The antimicrobial titer was defined as reciprocal highest dilution showing an inhibition of sensitive bacterial cells of the indicator strain. It was defined as the amount of Arbitrary Units (AU) ml^{-1} and finds with the following calculation: AU/ml = 1000/A x D (where 1000 = µl; A= µl spotted on the agar plate; D= reciprocal of dilution factor).

3.9.3 Amplification of lactobacilli bacteriocin genes

DNA extraction was carried out from a single colony of *Lb. curvatus* 54M16 by using an InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA), following the conditions described by the supplier. DNA was quantified by using the Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific, Milan, Italy).
Bacteriocin specific primers used for amplification of different gene fragments as well as oligonucleotide probes for hybridization studies were obtained from MWG (Ebersberg, Germany).
Oligonucleotides targeting several bacteriocin genes used in this study are reported in Table 20.
PCR amplifications were performed in 50 µl total volume including 2 µl (ca. 50 ng) of the target DNA (isolated and quantified as above described), 5.0 µl of *Taq* DNA polymerase 10 x buffer (Invitrogen, SG Milanese, Italy), 2.5 µl of 50 mM MgCl, 0.5 µl of dNTP mix (25 mM each), 0.1 µl of each primer (0.1 mM), and 0.5 µl of *Taq* DNA polymerase (5 U/µl) (Invitrogen). PCR program consisted of an initial template denaturation step for 2 min at 96°C, followed by 35 cycles (1 min at 96°C, 30 sec at 47°C, 3 min at 72°C) and one additional final cycle at 72°C for 5 min. The PCR amplification fragments were separated by agarose (1.5% w/v) gel electrophoresis at 100 V for 1.5 hours **(Hequet et al., 2007).**
Lactobacillus curvatus LTH1174 and *Lactobacillus sakei* LTH673, were used as positive and negative controls, respectively.

3.9.4. Evaluation of bacteriocin production in different growth conditions

L. curvatus 54M16 was grown in MRS broth (Oxoid) for 0, 24, 48 and 72 hours and incubated at different temperatures (10, 15, 20, 25 and 30°C). The cells were removed by centrifugation (10 000 g for 10 min) and the supernatant fluids were adjusted to pH 7.0 with 2 mol l^{-1} NaOH and treated at 80°C for 10 min. Aliquots (50 µl) of the heat-treated supernatant fluids were assayed for bacteriocin activity using the agar-well diffusion method and critical dilution assay described by **Villani et al. 1993**, by inoculating *L. monocytogenes* ATCC 7644 (1% v/v) and incubating at 30°C.
The changes in cell density (optical density at 600nm), the bacteriocin activity (AU ml^{-1}) and pH at different times of incubation were measured at hourly intervals.

3.9.5 Partial purification of bacteriocin

The bacteriocin-producing strain inoculated at 2% in MRS broth (1000 ml) was grown to the stationary phase by incubating at 30 °C for 10 hours. Crude extract was prepared as follow. The bacteria cells were removed by centrifugation (10000g, 15 min at 4°C) from the culture and the supernatant was passed through a 0.22 pm Millex-GV filter (Millipore) . A concentrated extract, used for SDS-PAGE, was obtained by adding ammonium sulphate to the cell free supernatant (CFS) to a concentration of 50% (w/v). After incubation for 2 h at 4 °C the precipitated proteins were collected by centrifugation (9,000 *g* for 45 min at 4°C) dissolved in 5 ml of 50 mmol l^{-1} potassium phosphate buffer pH 7.0 and exhaustively dialyzed using a natural cellulose membrane with a cut-off of 3500 Da (3,500 MWCO, Spectra por 3; Spectrum Medical Industries) against the same buffer (50mM potassium phosphate buffer pH 7.0) for 24 h at 4°C. The dialyzed fractions were used as the crude sample.

3.9.6 Bacteriocin detection by SDS-PAGE analysis

The apparent molecular mass of the crude sample of bacteriocin(s) was estimated by Tricine-SDS-PAGE done on 0.75 mm gels as described by **Schagger & Von Jagow (1987)**, using standard peptides as molecular weight markers. Concentrating and separating gels were separated by a spacer gel. The polyacrylamide concentration of the 3 gels was 4, 16.5 and 10% (w/v), respectively. The separating gel contained 10% (v/v) glycerol. Migration was performed in a vertical slab gel apparatus (Mighty small, Hoeffer Instruments), at a constant voltage (200V) for 45 min.
The gel was cut in two parts. The first half was stained with Coomassie brillant blue R (Sigma) while the other was assayed for antimicrobial activity by the direct method as previously described by **(Bhunia et al., 1987**, **Villani et al., 2001)** with modifications**.** Briefly, the gel containing the sample was fixed immediately for 2 h in a solution of 20% isopropanol and 10% acetic acid and washed in deionized water for 6 h. This gel was placed into a sterile petri dish and overlaid with 10 ml of TSA soft agar (TSA broth plus 0.75% agar) containing about 10^6 cells of the indicator bacteria. The plate was incubated at 30°C for 18 h and the gel was examined for the presence of a inhibitory zone.

3.9.7 HPLC purification of peptides and mass spectrometry analysis

The crude sample of bacteriocin(s) was fractionated by HPLC using a 1100MSD Modular System (Agilent, Palo Alto, CA, USA) using a reversed-phase Vydac C4 column (250 mm x 2.1 mm, 5µm, 300Å; Vydac, Hesperia, CA, USA) at 0.2 ml/min flow rate**.** Sample elution was achieved using a gradient from 5 to 70% of solvent B in 90 min. Solvent A was a 0.1% trifluoroacetic acid (TFA) in water (v/v); solvent B was 0.1% TFA in acetonitrile (v/v). A volume of 100 µl sample was injected for each chromatographic run. The UV detector was set at 220 nm.
HPLC purified bacteriocin(s) was analyzed by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS, Voyager DE-PRO; PerSeptive Biosystems, Framingham, MA, USA) equipped with a N2 laser (337 nm, 3 ns pulse width). α-cyano-4-hydroxycinnamic acid was used as matrix. The sample (1 µl) was loaded on a stainless steel plate and dried. Then was added 1 µl of a matrix solution comprised of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 0.1% TFA in H_2O/acetonitrile (1/1, v/v). For each sample, mass spectrum acquisition was performed in the positive linear or reflector mode accumulating 200 laser pulses. The accelerating voltage was 20 kV. External mass calibration was performed with low-mass peptide standards (PerSeptive Biosystems, Framingham, MA). The mass spectrometer was calibrated in the mass range 500–2500 *m/z* using 1 µl of standard Low Mass Range Peptide Mix (brand name mix1, Applied Biosystems, Monza, Italy); a resolution of 100.000 was calculated.
LC/ESI-Q-ToF-MS/MS spectra were performed by a quadrupole-time-of-flight (Q-ToF) Ultima hybrid mass spectrometer (Waters, Manchester, UK), equipped with an electrospray ion source (ESI) operating in the positive ion mode, and a nanoflow high-pressure pump system model CapLC (Waters, Manchester, UK). Samples (1 µl) were loaded on a 5 mm × 100 µm i.d. Zorbax™ 300 SB C18 trap column (Agilent Technologies, USA), and MCs were separated on a 15 cm × 100 µm i.d. Atlantis C18 capillary column at 1 µl/min flow rate, using aqueous 0.1% TFA (mobile phase A) and 0.1% TFA in 84% aqueous acetonitrile (mobile phase B). The chromatography was carried by linear gradient at room temperature, according to the following programme: from 0% B at time 0–60% B in 40 min, then to 90% B in 5 min, at last

to 0% B in 5 min; the equilibrium time between analyses was 5 min. LC-MS was performed operating in both (continuum) MS mode and in MS/MS mode for data dependent acquisition (DDA) of peptide fragmentation spectra. The spectra were acquired at the speed of 1 scan/sec. The source conditions were the following: capillary voltage: 3000 V; cone Voltage: 100 V; Extractor: 0 V; RF Lens: 60. Raw data were processed by MassLynx™ version 3.5 software (Waters, Manchester, UK). Mass spectrometer calibration was carried out on the basis of the multiple charged ions from fibrinopeptide-Glu (mw 1570.57 Da) introduced separately.

3.9.8 Preparation of cell suspension and extract for screening of technological properties

Lb. curvatus 54M16 was cultivated in MRS broth at 30°C for 24 h in order to determine enzymatic activities. The supernatant was separated by centrifugation (10,000 g, 10 min, 4°C), constituting the extracellular extract (EE). Then, cell pellets were washed twice in 0.85% (w/v) NaCl, containing 20mM $CaCl_2$ and suspended in a 1.5% initial volume of 50 mM Tris-HCl, pH 7.0, constituting the whole-cell suspension (WC). To prepare cell-free extract (CFE), cells were collected as stated above, washed twice in 50mM Tris-HCl pH 7.0 and suspended in a 1.5% initial volume of 50 mM Tris-HCl, pH 7.0. Then, an equivalent volume of glass beads (0.5 mm in diameter; Sigma, St. Louis, MO) was added to the cell-suspension and cell disruption was carried out in a mini-Bead-Beater (Biospec Products, Washington, N.C.) by six shakings for 30 s each with 1 min intervals on ice. The unbroken cells were removed by centrifugation (20,000 g, 10 min, 4°C) and the supernatant obtained constituted the CFE.

3.9.9 Acidifying activity of Lb. curvatus 54M16 and NaCl effect on microbial growth

Lb. curvatus 54M16 was cultivated in ME broth (1% meat extract, 0.5% peptone, 1% NaCl, pH 6.5) with and without 0.3% of glucose, supplemented with different % of NaCl. The test was performed in triplicate, in order to determine the acidifying activity. Each broth culture was monitored at the following times 0, 4, 8, 12, 24, 34 and 48 hours to record the pH values . Moreover, the same broth supplemented with 4% and 6% NaCl, was also used to evaluate the effect of NaCl on the microbial growth.

3.9.10 Nitrate reductase activity

The nitrate reductase activity was evaluated on agar substrate using the method described by Miralles et al. (1996) and by spectrophotometric assay as well.

Agar method. In an agar medium YT agar plates (1.0% tryptone, 0.5% of yeast extract, 0.025% of potassium nitrate and 1.5% of agar at pH 7.0) supplemented with 1 g l^{-1} KNO_3 , were practiced the wells of 6 mm and loaded with 30µl of cell suspension of L. curvatus 54M16 grown overnight, centrifugated (13000 g for 5 min, 4°C) and then resuspended in 50 mM pH 7.0 phosphate buffer. After incubation at 30°C for 7 hours and at 20°C and 15 °C for 24 and 72 hours, respectively, the plates were flooded with 1 ml of a 1:1 solution of NIT1 (0.8 g sulphanilic acid in 100 ml of acetic acid 5 N) and NIT2 (0.6 g N-N-dimethyl-1-Naphthylamine in 100 ml of acetic acid 5 N) for the detection of nitrite. The appearance of red haloes surrounding the wells indicated the presence of nitrate reductase activity.

Spectrophotometric assay. Ten millilitres of YT-broth (YTA without agar) supplemented with 250 ppm of KNO_3 were inoculated with 100 µl of an overnight culture of *Lb. curvatus* 54M16. After incubation at 30 and 20 °C for 24 h and at 15°C for 72 h a fraction of overnight cultures was used for the determination of dry weight while another fraction was used to detect nitrate reductase activity by spectrophotometric assay according to **Gerhardt et al.,1994**. Aliquots of one hundred microlitres of the overnight culture were added to 250 µl of Griess I (0.5 g of sulphanilic acid in 150 ml of 5 N acetic acid), 250 µl of Griess II (0.5 g of Naphthylamine in 50 ml of distilled water and 100 ml of 5 N acetic acid) and 2 ml of distilled water and incubated at room temperature for 15 min after shaking in a vortex for 1 min. By reading optical density (OD) at 540 nm was determined the Nitrite production from nitrate. Relative activity was calculated as the rate: $OD_{540\ nm}$/mg dry weight.

3.9.11 Catalase assay

Catalase activity was measured on resting cells according to Aebi's method (Aebi, 1974) in parallel, after growth in YT broth and in YT broth supplemented with 250 ppm of KNO_3 for 24 h at 30 °C. Five millilitres of each culture (resting cells with a $OD_{600\ nm}$ = 1.0) were centrifuged at 13,000g for 5 min and the resulting pellet mixed with 1.5 ml of 60 mM H_2O_2 in

20 mM phosphate buffer pH 7.0. The activity was measured spectrophotometrically at 240 nm after 3 min of incubation at room temperature. Results were expressed in arbitrary units: micromoles of degraded H_2O_2/min/ml/ of cells with OD600 = 1.0

3.9.12 Superoxide dismutase activity

The cell pellet of 54M16 strain was harvested by centrifugation of 2 ml of culture at 13000g for 5 min and washed once in 50 mM K_2HPO_4, pH 7.8. For SOD extraction the cell pellet (approximately 100 mg wet weight) was suspended in 1 ml of 50 mM K_2HPO_4 pH 7.8 and disrupted with glass beads (0.10–0.11 mm) on a vortex mixer for 5 min. After lysis, the suspension was centrifuged and the supernatant (cell extract) was used for determination of SOD activity as described below. 10µl of cell extract were added to 1.0 ml of 150 µM nitroblue tetrazolium (NBT), 10 mM methionine, 1.2 µM riboflavin, 50 mM K_2HPO_4 pH 7.8 and incubated at room temperature in a light chamber with a 60 W bulb lamp for 8 min. Under this condition, ribo- flavin is excited by a photon and is able to oxidise the methionine. This donation of an electron results in the production of a superoxide molecule (O_2) that is able to reduce NBT resulting in a colour change which was measured spectrophotometrically at 560 nm against the appropriate solution. The presence of SOD leads to a reduction in the colour change. Results were expressed as percentage of optical density (OD) at 560 nm according to the following equation: SOD activity = (1- *s/c*)100, where *s* is the OD of the sample and *c* the OD of the control, consisting of a solution without adding cell extract and incubated under the same conditions **(Mauriello *et al.* 2004)**

3.9.13 Proteolytic activity on sarcoplasmic and myofibrillar proteins

Extraction of sarcoplasmic and myofibrillar proteins. Sarcoplasmic proteins were extracted according to **Fadda et al. (1999)**. Lean pork minced meat and freeze-dried samples of 'Naples-type' salami were diluted 1 : 10 (w/v) with 20 mmol l^{-1} phosphate buffer pH 6·5, homogenized in a Stomacher 400 blender for 3 min and centrifuged at 13 000 g for 20 min at 4°C. The supernatant (sarcoplasmic fraction) was sterilized by filtration (0·22 µm, Millipore). The resulting pellet was used for myofibrillar preparation as described by **Sanz et al. (1999)**. It was resuspended in 200 ml of 0.03 mol l^{-1} phosphate buffer pH 7·4 containing 0·1% (v/v) Triton X-100 and homogenized for 2 min in a Stomacher 400 blender. After centrifugation at 13 000 g. for 20 min at 4°C, the pellet was washed three times by resuspending in the same buffer to remove muscle proteases. The resulting pellet was weighed and resuspended in nine volumes of 0·1 mol l^{-1} phosphate buffer pH 6·5 containing 0·7 mol l^{-1} KI. After Stomacher homogenization for 8 min, the suspension was centrifuged at 13 000 g for 20 min at 4°C and supernatant water-diluted 10 times to avoid possible inhibition of bacterial proteases by the presence of KI. The extract was filter sterilized (0·22 µm) and stored at 4°C (**Mauriello et al. 2002**).

Proteolytic activity in agar medium. The proteolytic activity was evaluated on an agar medium containing 0.5% of tryptone, 0.25% of yeast extract, 0.1% glucose and 1.5% of agar, pH 6.9 to which was added, after sterilization the 20% v/v of the sarcoplasmic extract and before sterilization 20% v/v of the extract myofibrillar.

In each substrate, they poured into Petri dishes, were drilled the wells loaded with 30µl of resuspended cell suspension in 1 ml 50 mM phosphate buffer pH 7.0. Simultaneously 30µl enzyme pronase 0.015% and 30µl of 50 mM phosphate buffer pH 7.0 was used respectively as positive and negative controls.

After 48 hours of incubation at 30 ° C, the agar layer was removed and immersed for 5 minutes in a dye solution consisting of: Coomassie brilliant blue 0.05% (w /v), methanol 50% (v/v) and acetic acid 9,2% (v/v) and subsequently decolored for 24 h in a solution containing methanol 25% (v/v) and acetic acid 5% (v/v). The proteolytic activity was indicated by the appearance of a halo of clarification around the inoculated wells.

3.9.14 Antimicrobial activity of 54M16 against L. monocytogenes inoculated in fermented sausages

Dry fermented sausage were hand made in laboratory in a small scale, using meat from the same supplier and the same breeds (Large White and Duroc) . The minced meat together with the mixture of ingredients, reported in Table 21 , was placed in the mixer and mixed for a maximum time of 5 minutes. The mixture obtained was divided into aliquots of which one was used as a control while the others were inoculated with *L. monocytogenes* ATCC 7644, with and without *Lb. curvatus* 54M16 strain added at the same concentration.

One more sample was inoculated only with *Lb. curvatus* and used as control for this strain.

The strains were added to the meat in the same amount to achieve the final concentration of 10^4 CFU/g. They were dissolved in 100 ml of distilled water, added to the mixture, distributed homogeneously and left for 30 minutes at room temperature.

Subsequently, the sausage mixture was stuffed into a natural casing and placed in a fermentation

chamber. The sausages were kept at 23°C and 95% relative humidity (RH) for 4h and 50% RH for 6h. After a pause at 15-23°C and 85-90% RH for 4h, the sausages were dried at 23°C and 90-95%
RH for 3h, at 50% RH for another 3 h and subjected to a further pause step. They were then dried at 20-18°C and 65-60% RH for 3 days and finally ripened at 14°C and 76% RH for 28 days up to
moisture content of 27-34%. Three sausages in each trial were taken during the ripening to the following days (0, 3, 6, 9, 10, 15, 20 e 44) for the microbial counts performed by serial dilution in ringer solution and plating on Listeria Selective Agar (Oxford formulation) by spread plate method for the enumeration of *L. monocytogenes* 7644, incubating at 37°C for 48 h. MRS agar was used for *Lb. curvatus* 54M16 counting and in order to determine the number and the presence of the strain of *L. curvatus* 54M16 at each sampling point, the plates of MRS agar of the last dilutions (with approximately 20-30 colonies) were overlaid with 5 ml of TSA soft-agar (0.75% agar) inoculated with ca. 10^6 CFU ml^{-1} of *L. monocytogenes* ATCC 7644 and incubated for an additional 24 h at 32°C. Colonies surrounded by clear zones of inhibition were presumptively counted as *L. curvatus* 54M16.

3.9.15 Statistical analysis

Statistical analyses were performed using the SPSS 13.0 software for Windows (SPSS Chicago, IL, USA). All the values are the mean values (± Standard Deviation, SD) obtained from three independent assays for each trait.

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3.14 Results

3.14.1 Determination of antimicrobial properties and identification of Lb. curvatus 54M16

Lb. curvatus strain previously isolated from fermented sausage of Vallo di Diano, was screened for its antimicrobial activity and was found able to inhibit growth of all *L. monocytogenes* and *L. innocua* strains, resulting bacteriocin producer. Therefore was studied in detail the production conditions, physical and chemical properties and the activity of strain in food matrix.

The inhibitory spectrum of bacteriocin(s) was tested by critical dilution assay of the cell free supernatant against Gram-negative and Gram-positive bacteria, including several pathogenic strains. **Table 22** shows that the bacteriocin(s) produced has a great activity versus *Listeria* genus. In fact, all four strains of *Listeria* were inhibited, showing highest values of AU ml^{-1} in a range between 800-12800. On the contrary, Gram-negative bacteria and *S. aureus* strains as well did not show any sensitivity to the antimicrobial substance produced by *Lb. curvatus* strain.

The effect of incubation temperature on the production of the antibacterial compound by *L. curvatus* 54M16 in MRS broth is shown in **Table 23**. Inhibitory activity was detected when the producer strain was incubated at 15, 20, 25 and 30°C. Maximum yield of the inhibitory activity of 6400 AU ml-1 was detected after 24 h of incubation at 20, 25 and 30°C and after 48h of incubation at 15°C. Moreover, *L. curvatus* 54M16 grew and produced acid at 15, 20, 25 and 30°C but not at 10°C.

3.14.2 Detection of bacteriocin genes

In order to detect the presence of the *curA* gene encoding for the curvacin A, the gene *sakP* encoding for the sakacin P and of the genes and s*akX*, *sakTa*, *sakT*, which encodes respectively for sakacin X, sakacin T_{α} and sakacin $T_{ß}$, PCR experiments described by **Remiger et. al. (1996)** and **Macwana & Muriana (2012)** were performed. Gel Electrophoretic analysis of PCR products showed that sakacin X, T and P encoding genes were present in *L. curvatus* 54M16 DNA (data not shown) as they were demonstrated in *L. curvatus* 2711 **(Hequet et al 2007)** and in *L. sakei* 5 **(Vaughan et al. 2003)**.

3.14.3 Detection of bacteriocin activity by SDS-PAGE

Tricine SDS-PAGE was used to estimate the molecular mass of crude bacteriocins produced by *L. curvatus* 54M16. The gel stained with Coomassie blue was overlayed with the a culture of *L. monocytogenes* 7644 in order to detect the inhibitory activity.
The presence of a clear zones of inhibition in correspondence of the were highlighted in the area above and below 2.5 KDa (**Figure 22**).

3.14.4 MALDI-ToF/MS analysis of bacteriocins
The crude samples of peptides mixture were separated by reverse phase HPLC. Subsequently, single fractions obtained were analyzed by MALDI-TOF-MS.
Five main signals were detected whose m/z values were in the range compatible with those of bacteriocins (**Figure 23**). MALDI-TOF-MS and ESI-Q/TOF-MS/MS allowed to determine the amino acid sequence. Further confirm was obtained by peptide mapping after reduction, carboxymethylation and tryptic digestion of the HPLC fractions. The peptides for the components at 24.19 min, 31.02 min and 35.89 min were shown to correspond to sakacin X, sakacin T□ □and sakacin T□ respectively (**Table 24**), expressed at the composite bacteriocin locus identified in malt isolated *L. sakei* 5 (Vaughan et al., 2003) and in *L.□ □curvatus* 2711 (Hequet et al., 2007). For the component at 45.43 min only a partial fragmentation spectrum was obtained, but the sequence was sufficient to identify it as a sakacin P variant.

3.14.5 Technological properties of Lb. curvatus
Based on results obtained and reported in table 22 is possible to affirm that the strain was able to growth in presence of NaCl in a concentration between 1 and 6%. Moreover, the higher acidifying activity was showed when inoculated in ME broth with the 1% of NaCl and the 0.3% of glucose, with a decreasing of the pH value of 1.2 unit.
All the technological properties of *L. curvatus* 54M16 are reported in **Table 25**. The results of the
proteolytic activity assayed by agar plate methods against sarcoplasmic and myofibrillar proteins, showed that the strain was able to hydrolyse sarcoplasmic proteins but not myofibrillar proteins; in
addition the strain is not able to reduce nitrates by the agar plate method, while it shows only a slight nitrite production from nitrate as determined by spectrophotometric assay, showing instead
high value of SOD and low value of catalase enzymes.

3.14.6 Antilisterial activity of Lb. curvatus 54M16 in fermented sausage
The antimicrobial activity of *Lb. curvatus* 54M16 was assayed *in situ* against *L. monocytogenes* ATCC 7644 intentionally inoculated in fermented sausages. Results are illustrated in **Figure 24** . The highest inhibitory activity was recorded after 10 days of repening, where the growth of the pathogen strain was reduced of 1.65 log in presence of *Lb. curvatus* 54M16.
However during the whole experiment the pathogenic strain keeps growing in all samples, showing at the end of the experiment only a reduction of 0.5 log compared to the sample without 54M16.

3.15 Discussion
Food represent given their physical and chemical properties a supportive environment for the growth and the proliferation of spoilage and pathogenic microbial species which are able to enter the body through the gastrointestinal tract where the often occur first symptoms of infection. The severity of the food diseases in humans varies, moving from mild symptoms to life-threatening conditions and the hazard intensity depends by several key factors moving from the health state, the immunity system conditions of the host and the antimicrobial resistance of bacteria, corresponding to the ability of microbes to resist the effects of drugs. The risks of contamination are ever- present, due to a large number of combinations of food-borne disease agents and food vehicles, that's why require prevention and monitoring throughout the food chain.
The process of globalization, the intensification of international trade and migration have accelerated the spread of food dangerous pathogens and contaminants, increasing our universal vulnerability **(Tauxe et al. 2010).** A recent estimate suggests that approximately 30% of all newly globally emerging infections over the past 60 years included pathogens commonly transmitted through food **(Jones et al., 2008).**
Fermented sausages represent a traditional product with a high gastronomic value appreciated and they are appreciated worldwide. Despite the incoming of new technologies and starter cultures addition, the traditional sausages especially in Italy are produced without added starter culture and additives such as nitrate and nitrite and often they naturally present different and interesting microorganisms. Therefore when it is required is preferable to use as starter culture microorganism from meat source.
Currently the lactic acid bacteria are used extensively for the formulation of starter cultures to be used also in the production of fermented meat sausages, due to their positive effect on the hygienic property, safety of use and on the sensory and organoleptic properties of the finished product. Indeed, the lactic acid bacteria show their inhibitory activity metabolizing carbohydrates in the food matrix and releasing numerous natural preservatives such as: alcohols, lactic acid,

acetic acid as well as CO_2 and other chemical compounds derived from some types of fermentations. Some strains have many physiological and biochemical characteristics by which directly influence the technological and sensory properties of fermented sausages and are also able to inhibit the growth of spoilage or pathogens microorganisms through the production of bacteriocins. Objective of this work was to study the technological characteristics and antagonistic properties of *Lb. curvatus* 54M16 isolated from fermented sausage of Vallo di Diano, both *in vitro* and *in situ* in order to investigate its suitability to be used as starter and protective culture for sausage fermentation.

A great variety of bacteriocins produced by species of *Lactobacillus* have been described and bacteriocin-producing lactobacilli strains have been isolated from such different

sources. Meat strains of *Lb. curvatus* produce several sakacins and curvacins **(Cui et al., 2012)**. *L. curvatus* 54M16 carried the genes for production of sakacin X, T and P as demonstrated by the performed PCR studies. It is not unusual the production of more bacteriocins from strains of *Lactobacillus* (**Vaughan et al., 2003; Hequet et al., 2007**). The production of sakacins X, T and P has been already described for the strain of *L. sakei* 5 (**Vaughan et al., 2003**). The bacteriocin produced by *L. curvatus* 54M16 exhibits a great activity *in vitro* against all Lysteria strains tested The ability to inhibit certain organisms outside of their genus is also typical of Gram-positive bacteriocins (**Jack *et al.* 1995**). On the contrary, no inhibition of tested Gram-negative bacteria and of *S. aureus* was observed. Anyway, the antilisterial activity results interesting and promising especially for food industry.

The high activity of this strain in vitro was recorded against the *L. monocytogenes* ATCC 7644 strain for which was measured a value of 6400 AU ml^{-1} after 24 hours of incubation at 20, 25 and 30°C and also after 48 hours at 15°C. Moreover *Lb. curvatus* 54M16 was found able to growth in the presence of 6%

NaCl. Therefore we assayed the some in vitro technological properties of the strain, in order to investigate its suitability to be used as starter culture for sausage fermentation.

The use of commercial starter cultures for fermented sausages production is now a well established procedure. Therefore the use of autochthonous strains is essential in the artisanal manufacture of traditional fermented products, since such strains are well adapted to the particular environment and specific manufacturing technology (**Villani et al., 2007**). The strain 54M16 of *L. curvatus* showed good acidifier capability, lowering after 48 hours of incubation of about 1 unit the pH value in meat extract broth, added of 4.0% and 6.0% NaCl,. Moreover it is able to hydrolyse sarcoplasmic but not myofibrillar proteins, as demonstrated by agar plate methods. In a study of Fadda et al. (1999) strains of *L. curvatus* and *L. sakei* were shown to be capable of hydrolyzing 97, 45, 37, and 26 kDa sarcoplasmic fractions during incubation at 30°C in a sarcoplasmic extract, whereas, as reported in a previous work (**Villani et al., 2007**) *L. curvatus* BVL7 and CVL12 showed a reduction in the 22.7 and 11.5 kDa bands and an increase in the 13

kDa band.

Generally, the nitrate reductase activity is not common in lactobacilli strains even if some authors report different data showing that some strains possess nitrate reductases and heme-dependent catalase activity (**Ammor & Mayo, 2007**). In the present study, the strain 54M16 showed only a slight nitrite production from nitrate and low value of catalase enzymes, showing instead antioxidant activity with high value of SOD.

Although the promising and satisfactory antilisterial activity of *L. curvatus* observed in vitro tests, the strain showed slightly inhibitory activity in situ reducing only of 0.5 log at the end of the ripening process the microbial growth of *L. monocytogenes* 7644 intentionally inoculated in dry fermented sausage. Further studies are necessary to investigate the possibility to improve the antilisterial activity in fermented sausage through different strategies. Moreover, more investigations are needed to verify the antagonistic activity of the bacteriocin produced in situ, perhaps using other antagonistic substances in order to evaluate the potential synergy and increase safety in the contaminated products. In conclusion, the use of the strain in the sausage meat production could be extremely important not only to improve the organoleptic characteristics of the finished product, through its technological activities mainly because of its proteolytic activity, but may also help to ensure the safety of use by the production of its bacteriocin active against L. monocytogenes thus avoiding the possible food and dangerous infections of which is responsible for this microorganism.

APPENDIX 1 – PUBBLICATIONS

Journal article # 1. Technological properties and bacteriocins production by Lactobacillus curvatus 54M16 and its use as starter culture for fermented sausage manufacture

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Abstract
Lactobacillus curvatus 54M16 was isolated from traditional fermented sausages of Campania region (Italy) and identified by 16S rRNA and hsp60 gene sequencing and by SDS-PAGE of whole cell proteins. The strain produced more than one bacteriocin, carrying the genes for sakacin X, T and P as demonstrated by PCR studies. The ability of L. curvatus 54M16 to produce multiple bacteriocins is confirmed by molecular mass spectrometry analysis and N-terminal amino acid sequencing. Among the bacteria tested, the pathogens Listeria monocytogenes and Bacillus cereus and the meat spoilage Brochotrix thermosphacta were sensitive to the bacteriocins. No inhibition of tested Gram-negative bacteria, some strains of Lactobacillus and of strains of Staphylococcus aureus was observed.

All the in vitro conditions tested for the production of the bacteriocins (temperature, pH and NaCl), indicate that the strain of L. curvatus 54M16 is able to grow and to produce the antagonistic substances at pH 4.5 and in the presence of 4% NaCl. The strain has good acidifier capability and it is able to hydrolyse sarcoplasmic but not myofibrillar proteins, lipids and to reduce nitrates. Moreover it shown high values of SOD, while the aminopeptidase activity is restricted to whole cells that hydrolyse at high rates the amino acids l-arginine, l-valine, l-phenylalanine and l-lysine. The suitability of L. curvatus 54M16 as starter culture was assayed during sausage fermentation. Its use can improve the quality and safety of the traditional fermented sausages prepared without antimicrobial additives.

Keywords
Bacteriocins; Sakacins; Lactic Acid Bacteria; Lactobacillus curvatus; Listeria monocytogenes; Fermented sausages; Starter

cultures

Journal article # 2. Antimicrobial activity of Myrtus communis L. water-ethanol extract against meat spoilage strains of Brochothrix thermosphacta and Pseudomonas fragi in vitro and in meat

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Abstract
The antimicrobial activity of hydro-alcoholic extract of Myrtus communis L. was investigate in vitro and in situ against different meat spoilage biotypes of Pseudomonas fragi and Brochothrix thermosphacta. Water-ethanol myrtle extract (WEME) was prepared from myrtle leaves and used to study the antimicrobial activity, Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC). Naturally contaminated ground beef was used to evaluate in situ antibacterial activity of freeze-dried myrtle extract at different concentrations. In vitro antimicrobial activity of WEME was significantly more effective against B. thermosphacta than P. fragi strains. MIC and MLC values were in the range of 12.5–50 and 25–100 mg DM/ml, respectively, for B. thermosphacta and P. fragi strains. In situ results showed that the microbial population, except for Pseudomonas spp., decreased significantly in ground meat with added 5 % of freeze-dried myrtle extract. In conclusion, the findings demonstrate the effectiveness of myrtle extract to control or prevent the proliferation of meat spoilage bacteria.

Keywords
Mirtus communis L. Antimicrobial activity Spoilage bacteria Water-ethanol extract MIC MLC

Poster # 1.

Technological properties and bacteriocin production by Lactobacillus curvatus 54M16 isolated from fermented sausage

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INTRODUCTION

Food quality and safety have become the master target for food industries. Therefore, natural antimicrobial substances, like peptides from lactic acid bacteria (LAB), may be considered as promising biopreservatives and represent an alternative to reduce the addition of chemical additives in foods. The bacteriocins produced by LAB offer several desirable properties: (i) are generally recognised as safe substances, (ii) are not active and nontoxicon eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) are usually pH and heat-tolerant, (v) they have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation.

In this study the strain 54M16 of *Lactobacillus curvatus*, isolated from naturally fermented Italian sausages, was investigated for its technological and antagonistic properties.

MATERIALS AND METHODS

1. ANTIMICROBIAL ACTIVITY BY AGAR WELL DIFFUSION METHOD AND CRITICAL DILUTION ASSAY

2. INFLUENCE OF CULTURE MEDIA AND GROWTH CONDITION ON BACTERIOCIN PRODUCTION

3. EXTRACTION OF BACTERIOCIN BY AMMONIUM SULPHATE PRECIPITATION

4. DETECTION OF BACTERIOCIN BY SDS – PAGE

5. IDENTIFICATION OF *Lb. curvatus* 54M16 BY RESTRICTION ENDONUCLEASES ANALYSIS - PULSED FIELD GEL ELECTROPHORESIS (REA – PFGE)

6. PURIFICATION OF PEPTIDES AND CHEMICAL CHARACTERIZATION BY HPLC AND MALDI TOF - MS

7. TECHNOLOGICAL PROPERTIES OF *Lb. curvatus* 54M16

- NITRATE REDUCTION BY AGAR PLATE AND SPECTROPHOTOMETRIC ASSAY
- CATALASE AND SUPEROXIDE DISMUTASE (SOD) ACTIVITY BY AEBI'S METHOD
- LIPOLYTIC ACTIVITY BY TITRATION METHOD
- PROTEOLYTIC ACTIVITIES BY AGAR PLATE ASSAY

RESULTS

The inhibitory activity of *Lb. curvatus* 54M16 was tested toward several food pathogenic and spoilage bacteria.

Antimicrobial activity of *Lb. curvatus* 54M16

INDICATOR STRAINS	AGAR WELL DIFFUSION DIAMETER OF INHIBITION ZONE (mm)	CRITICAL DILUTION ARBITRARY UNITS (AU/ml)
L. monocytogenes OH	14	12800
L. monocytogenes SA	14	12800
L. monocytogenes CAL	12	800
L. monocytogenes 7644	13	3200
Br. thermosphacta 7R1	6	400
Br. thermosphacta 11509	7	1600
E. faecalis 226	6	400
P. fragi 3456	0	0
S. aureus 20231	0	0
S. aureus 19095	0	0
H. alvei 53M	0	0
E. coli 32	0	0
S. proteamaculans 20p	0	0

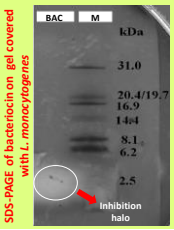
EFFECT OF GROWTH CONDITIONS ON BACTERIOCIN PRODUCTION

Substrates	pH				O.D.600 nm Times (h)				AU ml^{-1}			
	0	7	24	48	0	7	24	48	0	7	24	48
MRS	6.5	5.53	4.26	4.07	0.03	0.48	1.29	1.44	0	3200	6400	12800
BHI	7.0	6.84	6.05	6.07	0.01	0.25	0.63	0.54	0	100	400	400
APT	6.5	6.06	4.38	4.25	0.18	0.28	1.16	1.49	0	200	800	800
TS	6.5	6.45	5.35	5.40	0.04	0.28	0.62	0.60	0	200	800	800
WYE1	6.2	4.75	4.68	4.61	N.D.	N.D.	N.D.	N.D.	0	1600	1600	400
WYE2	6.59	5.33	5.20	5.14	N.D.	N.D.	N.D.	N.D.	0	3200	3200	800
WYE3	6.58	4.60	3.89	3.80	N.D.	N.D.	N.D.	N.D.	0	3200	3200	1600
WYE4	6.23	4.72	4.64	4.62	N.D.	N.D.	N.D.	N.D.	0	3200	3200	800
WYE5	6.59	5.33	5.18	5.14	N.D.	N.D.	N.D.	N.D.	0	3200	3200	1600
WYE6	6.53	4.60	3.89	3.80	N.D.	N.D.	N.D.	N.D.	0	3200	3200	1600
YE	6.13	5.90	5.17	N.D.	N.D.	N.D.	N.D.	N.D.	0	400	1600	N.D.
YE + Glucose (1%)	6.11	5.92	4.13	N.D.	N.D.	N.D.	N.D.	N.D.	0	400	1600	N.D.

Changes in pH, cell density (O.D. at 600nm) and bacteriocin production (AU ml-1) during the growth of L. curvatus 54M16 in different medium at 30°C for 48h

EXTRACTION AND DETECTION OF BACTERIOCIN

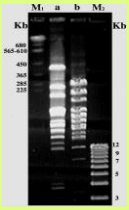
Broth-culture of *Lb. curvatus* 54M16 incubated at 30°C for 16h
↓
Centrifugation at 7000 rpm for 10 min
↓
Precipitation of bacteriocin with ammonium sulphate at 50% of saturation
↓
Centrifugation at 7000 rpm for 40 min at 4°C
↓
Supernatant → Measuring of bacteriocin activity
Pellet → Dialysis in 50mM sodium-phosphate buffer pH 7.0



SDS-PAGE of bacteriocin on gel covered with *L. monocytogenes*

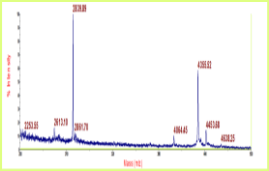
REA – PFGE

REA-PFGE analysis was performed in order to characterize and differentiate the strain of *Lb. curvatus* 54M16 from other closely related strains. REA-PFGE fingerprints of strains Lactobacillus curvatus LTH1174 and 54M16 were compared. A clear differentiation of these two strains was obtained by Not I restriction enzyme.



Line a: Not I REA-PFGE patterns of *Lb. curvatus* LTH1174
Line b: Not I REA-PFGE patterns of *Lb. curvatus* 54M16
M_1: *Saccharamyces cerevisiae* PFGE size standard
M_2: 1Kb DNA Plus Ladder

HPLC AND MALDI – TOF - MS



MALDI TOF-MS analysis of single fraction obtained through HPLC system

HPLC retention time	Measured MW (Da, monoisotopic)	Expected MW (Da, monoisotopic)	Peptide name
24.19 min	4360.1	4362.12	Sakacin X
31.02 min	4068.9	4070.22	Sakacin T_B
32.01 min	2841.1	2842.32	IP-TX
35.89 min	4640.6	4644.32	Sakacin T_A
45.04 min	4453.6	4434.02	Sakacin P variant

For accurate structural characterization was carried out a RP-HPLC separation of culture medium and the single components detected were analyzed by MALDI – TOF mass spectrum .

NITRATE REDUCTION

Measurement (mm) of the red haloes surrounding the wells by agar plate method

Temperature	Red haloes
15°C	0
20°C	0
30°C	0

Determination of Nitrate by spectrophotometric method

Temperature	mM nitrite/mg dry weight
15°C	0.0120 ± 0.001
20°C	0.0191 ± 0.004
30°C	0.0155 ± 0.001

CATALASE ASSAY

Measured on resting cells after growth in YT- broth according to Aebi's method by reading adsorption at 240 nm. The result was 9.4 ± 1.2 µmoles of degraded H_2O_2/min/ml/ of cells with OD600 = 1.0

SOD

Superoxide dismutase (SOD) activity determined as described by Casaburi et al. (2005) by spectrophotometric method showed a value of 91 ± 12.4 expressed as percentage of optical density (OD) at 560 nm according to the following equation:

SOD ACTIVITY = (1 – s/c)100

where s is the OD of sample and c the OD of the control, consisting of a solution without adding cell extract.

LIPOLYTIC AND PROTEOLYTIC ACTIVITY

LIPOLYTIC Tritation method (% PALMITIC ACID)	0
PROTEOLYTIC Sarcoplasmic/Myofibrillar proteins (mm of clear zone surrounding the wells)	12/0

REFERENCES:
- Mauriello, G., A. Casaburi, and F. Villani. 2002. Proteolytic activity of Staphylococcus xylosus strains on pork myofibrillar and sarcoplasmic proteins and use of selected strains in the production of Naples type salami. J. Appl. Microbiol, 92:482 -490.
- Aebi, H. 1974. Catalase. In H. U. Bergmeyer (Ed.), Methods of analytic analysis (pp.673-684). New York and London: Academic Press.
- Moschetti, G., G. Blaiotta, M. Aponte, G. Mauriello, F. Villani, and S. Coppola. 1997. Genotyping of Lactobacillus delbrueckii subsp. bulgaricus and determination of number and forms of rrn operons in Lactobacillus delbrueckii and its subspecies. Res. Microbio. 148:501–510.

Poster #2.

Antimicrobial activity of Myrtus communis L. hydro-alcoholic extract against meat spoilage strains of *Brochothrix thermosphacta* and *Pseudomonas fragi*

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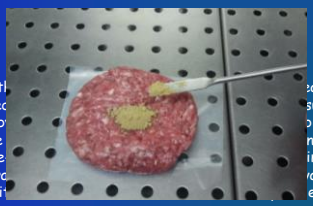
INTRODUCTION

Myrtle (*Myrtus communis* L.) is a widely used spice that has many applications in the perfumery, food and pharmaceutical industries (Sumbul et al., 2011). Essential oils and organic solvent extracts of leaves and steams were assayed for their antimicrobial activities against food-borne and clinical pathogens and food spoilage bacteria, including *Escherichia coli, Klebsiella aerogenes, Proteus vulgaris, Salmonella* typhi, *Pseudomonas aeruginosa, P. vulgaris, P. mirabilis, Campylobacter jejuni, Mycobacterium tuberculosis, Staphylococcus aureus, Micrococcus luteus, Streptococcus pneumoniae, S. pyogenes, S. agalactiae, Listeria monocytogenes, Bacillus subtilis* and some molds and yeasts (Sumbul et al., 2011). The effective antimicrobial properties of myrtle extracts lead to consider these antimicrobials as an alternative way to preserve food. However, most of the antimicrobial assays were performed against only a few strains of each one of the many different species, sometimes leading to conflicting results on the efficacy against a particular species. In addition, the antimicrobial activity of myrtle extract against important spoilers such as *P. fragi* and *Br. thermosphacta* has not yet been demonstrated.

The aim of this work was to investigate, *in vitro* and *in situ*, the antimicrobial activity of hydroalcoholic extract of *Myrtus communis* leaves against strains of *Pseudomonas fragi* and *Brochothrix thermosphacta* isolated from fresh and spoiled meat.

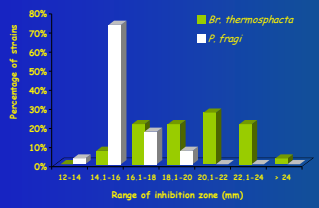


AND METHODS

1. Preparation of water-et… …aves. Powder (10 g) was mixed with 100 mL of 70% ethanol (WE) and left to shake for 24 h. Extract was filtered, centrifuge… …sure at 40° C.
2. Antimicrobial activity (b… …bitory Concentration (MIC) and Minimum Lethal Concentration (MLC) (by serial two-fold broth dilution method) of WEME were … …nd 33 of *Br. thermosphacta* isolated from fresh and spoiled meat
3. In situ effect of freeze… …inal dry of WEME was stored at 4° C until use, whereas the other was freeze-dried and stored under vacuum at 4° C.. Natura… …valuate *in situ* antibacterial activity of freeze-dried myrtle extract. Ground beef was used to prepare hamburgers of 200 g wi… …extract (0%, 1%, 3% and 5%) and microbiological analysis were performed after 0, 1, 2, 3 and 5 days of storage at 4° C.

RESULTS AND DISCUSSION

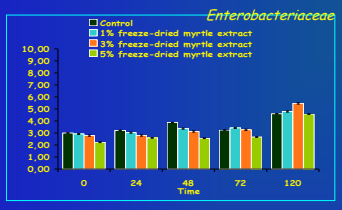
Antimicrobial activity

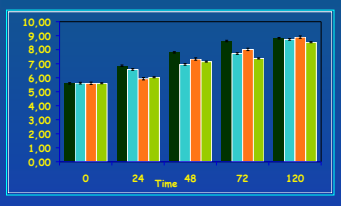


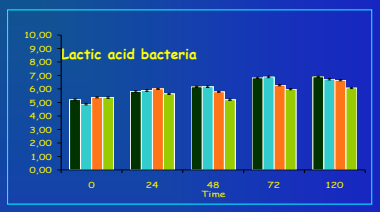
MIC and MLC of WEME

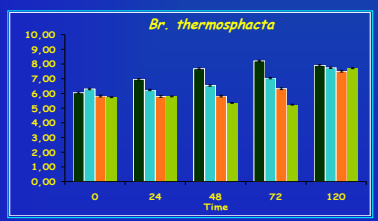
	MIC[a]			MLC[b]		
	12,5	25	50	25	50	100
Strains						
Br. thermosphacta	25%	43%	33%	64%	33%	-
P. fragi	-	73%	27%	33%	40%	27%

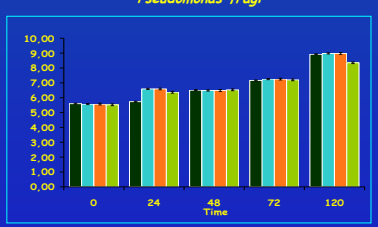
Freeze-dried myrtle extract in meat











List of tables and figures

Table 1. Evolution of dry matter (g/l) and pH of myrtle extracts during the maceration times

	Maceration (hours)		
	24	**48**	**120**
Dry matter (g/l)			
WEME40	120.7[a]	121.1[a]	120.3[a]
WEME70	126.3[b]	125.6[b]	126.1[b]
AME	65.3[c]	64.9[c]	64.6[c]
pH			
WEME40	5.28[a]	5.25[a]	5.26[a]
WEME70	5.25[a]	5.29[ab]	5.35[b]
AME	5.27[a]	5.39[b]	5.31[a]

Values within a column for each extracts having different letters are significantly different from each other at $p < 0.05$.

Table 2 (a) Effect of different extraction times and solvents on the antimicrobial activity of myrtle extracts from dried leaves (100 mg/ml) by agar well diffusion assay

	WEME 40		
Extraction time	**24**	**48**	**120**
B.thermosphacta 7R1	3 ± 0.5 (*)	4 ± 0.1	5 ± 0.4
B. thermosphacta 11509 ATCC	8 ± 0.2	7 ± 0.3	8 ± 0.5
B. thermosphacta D274	5 ± 0.6	5 ± 0.4	7 ± 0.4
B. thermosphacta FC74	3 ± 0.5	5 ± 0.5	6 ± 0.3
L.monocytogenes 7644 ATCC	3 ± 0.5	4 ± 0.1	6 ± 0.2
L.monocytogenes SA	6 ± 0.1	6 ± 0.4	6 ± 0.3
L.monocytogenes CAL	6 ± 0.5	5 ± 0.3	5 ± 0.2
L.monocytogenes OH	5 ± 0.5	5 ± 0.3	5 ± 0.4
L.innocua 1770	3 ± 0.3	3 ± 0.2	4 ± 0.2
S.aureus 19095	4 ± 0.5	4 ± 0.1	5 ± 0.3
S.aureus RIMD 31092	2 ± 0.2	2 ± 0.1	3 ± 0.1
S.aureus 14458	3 ± 0.1	4 ± 0.2	4 ± 0.2
S.aureus NCTC 9393	4 ± 0.2	3 ± 0.1	4 ± 0.1
S.aureus 20321	2 ± 0.2	4 ± 0.2	3 ± 0.2
S.aureus K6278-97	5 ± 0.1	5 ± 0.3	5 ± 0.4
P.fragi 3456 ATCC	4 ± 0.1	3 ± 0.1	5 ± 0.3
P.fragi M891	4 ± 0.5	3 ± 0.2	5 ± 0.2
P.fragi A2	3 ± 0.2	2 ± 0.1	4 ± 0.1
P.fragi I82	3 ± 0.2	3 ± 0.1	4 ± 0.2
P.fragi F2	5 ± 0.4	5 ± 0.4	5 ± 0.3
S.enteritidis	0 ± 0.0	0 ± 0.0	0 ± 0.0
H.alvei 53M	0 ± 0.0	0 ± 0.0	0 ± 0.0
E.coli ATCC 25992	3 ± 0.1	4 ± 0.2	3 ± 0.2
E.coli O157:H7	0 ± 0.0	0 ± 0.0	0 ± 0.0

WEME40: water ethanol myrtle extract by maceration with 40% of ethanol;

Table 2 (b) Effect of different extraction times and solvents on the antimicrobial activity of myrtle extracts from dried leaves (100 mg/ml) by agar well diffusion assay

	WEME 70		
Extraction time	**24**	**48**	**120**
B.thermosphacta 7R1	22.0 ± 0.1	21.0 ± 0.4	22.0 ± 0.2
B. thermosphacta 11509 ATCC	24.0 ± 0.2	24.0 ± 0.3	25.0 ± 0.3
B. thermosphacta D274	17.0 ± 0.3	18.0 ± 0.9	18.0 ± 0.2
B. thermosphacta FC74	17.0 ± 0.2	16.0 ± 0.6	17.0 ± 0.1
L.monocytogenes 7644 ATCC	27.0 ± 0.2	26.0 ± 0.5	27.0 ± 0.2
L.monocytogenes SA	26.0 ± 0.6	25.0 ± 0.5	25.0 ± 0.2
L.monocytogenes CAL	25.0 ± 0.5	25.0 ± 0.6	26.0 ± 0.3
L.monocytogenes OH	26.0 ± 0.5	25.0 ± 0.3	26.0 ± 0.1
L.innocua 1770	24.0 ± 0.4	24.0 ± 0.2	25.0 ± 0.5
S.aureus 19095	26.0 ± 0.3	25.0 ± 0.5	26.0 ± 0.4
S.aureus RIMD 31092	25.0 ± 0.2	25.0 ± 0.1	26.0 ± 0.3
S.aureus 14458	26.0 ± 0.4	25.0 ± 0.5	25.0 ± 0.6
S.aureus NCTC 9393	24.0 ± 0.3	25.0 ± 0.1	25.0 ± 0.2
S.aureus 20321	26.0 ± 0.6	24.0 ± 0.5	26.0 ± 0.3
S.aureus K6278-97	25.0 ± 0.5	24.0 ± 0.6	26.0 ± 0.2
P.fragi 3456 ATCC	15.0 ± 0.8	14.0 ± 0.3	15.0 ± 0.1
P.fragi M891	16.0 ± 0.4	16.0 ± 0.2	17.0 ± 0.5
P.fragi A2	15.0 ± 0.2	15.0 ± 0.4	18.0 ± 0.7
P.fragi I82	15.0 ± 0.1	13.0 ± 0.6	15.0 ± 0.3
P.fragi F2	15.0 ± 0.2	14.0 ± 0.6	16.0 ± 0.2
S.enteritidis	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
H.alvei 53M	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
E.coli ATCC 25992	12.0 ± 0.1	11.0 ± 0.5	10.0 ± 0.6
E.coli O157:H7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

WEME70: water ethanol myrtle extract by maceration with 70% of ethanol;

(*)The results are expressed as diameter of inhibition zone in mm and are means ± standard deviations (n = 3).

Table 2 (c) Effect of different extraction times and solvents on the antimicrobial activity of myrtle extracts from dried leaves (100 mg/ml) by agar well diffusion assay

	AME		
Extraction time	**24**	**48**	**120**
B.thermosphacta 7R1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
B. thermosphacta 11509 ATCC	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
B. thermosphacta D274	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
B. thermosphacta FC74	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
L.monocytogenes 7644 ATCC	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
L.monocytogenes SA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
L.monocytogenes CAL	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
L.monocytogenes OH	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
L.innocua 1770	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S.aureus 19095	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S.aureus RIMD 31092	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S.aureus 14458	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S.aureus NCTC 9393	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S.aureus 20321	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S.aureus K6278-97	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P.fragi 3456 ATCC	5.0 ± 0.2	4.0 ± 0.2	5.0 ± 0.3
P.fragi M891	7.0 ± 0.3	6.0 ± 0.3	7.0 ± 0.1
P.fragi A2	4.0 ± 0.1	5.0 ± 0.1	5.0 ± 0.2
P.fragi I82	7.0 ± 0.2	5.0 ± 0.4	6.0 ± 0.2
P.fragi F2	5.0 ± 0.0	5.0 ± 0.2	4.0 ± 0.1
S.enteritidis	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
H.alvei 53M	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
E.coli ATCC 25992	4.0 ± 0.2	3.0 ± 0.1	4.0 ± 0.2
E.coli O157:H7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

AME: aqueous myrtle extract by maceration with distilled water

Table 3. Antimicrobial activity of WEME70 against several strains belonging to *Lysteria* genus

Strains	Inhibition zone (mm)[a]	(AU ml-1)[b]
L. monocytogenes OH	26 ± 0.5	25600
L.innocua	19 ± 0.5	12800
L. monocytogenes ATCC 7644	27 ± 0.2	12800
L. monocytogenes ATCC 932	24 ± 0.2	25600
L. innocua ATCC 1770	24 ± 0.4	12800
L. innocua IFI1	14 ± 0.5	25600
L. monocytogenes SA	26 ± 0.6	12800
L. monocytogenes CAL	25 ± 0.5	12800

Table 4. Antimicrobial activity of WEME70 against several strains of *Staphylococcus aureus*

Strains	Inhibition zone (mm)[a]	(AU ml-1)[b]
NCTC 9393	27 ± 0.2	6400
19095	25 ± 0.5	6400
K6278-97	25 ± 0.5	6400
RIMD 31092	25 ± 0.2	12800
14458	26 ± 0.4	12800
ATCC 20231	26 ± 0.6	12800
ATCC 25993	16 ± 0.6	12800
CETC 239	21 ± 0.6	12800

[a]Values of inhibition zones were expressed as mean of three experiments ± Standard

[b]Values of antimicrobial activity of WEME (100mg DM/ml) were expressed as AU ml^{-1}

Table 5. Antimicrobial activity of WEME70 against several strains of *Brochothrix thermosphacta*

Strains	Inhibition zone (mm)[a]	(AU ml-1)[b]
Fc74	17.0 ± 0.1	800
Bc201	17.0 ± 0.1	800
F175	17.0 ± 0.2	800
D274	17.5 ± 0.1	1600
Dc71	19.0 ± 0.2	1600
M175	21.0 ± 0.2	1600
Gc74	22.0 ± 0.2	1600
Ec71	24.0 ± 0.4	1600
E1203	16.0 ± 0.2	800
Cc203	18.0 ± 0.2	800
Dc201	18.0 ± 0.2	800
Ec204	19.0 ± 0.2	1600
D171	19.0 ± 0.3	1600
Cc74	19.0 ± 0.4	1600
G01	19.5 ± 0.1	1600
Fc203	20.0 ± 0.2	1600
Gc202	21.0 ± 0.5	1600
I03	22.0 ± 0.3	1600
D01	24.0 ± 0.5	1600
ATCC 11509	24.0 ± 0.5	1600
Cc75	27.0 ± 0.5	1600
B05	21.0 ± 0.1	1600
Hc202	16.0 ± 0.1	800
Lc71	17.0 ± 0.1	800
B02	19.0 ± 0.2	1600
Ic71	20.0 ± 0.1	1600
7R1	22.0 ± 0.2	1600
Fc201	22.0 ± 0.3	800
Fc204	22.0 ± 0.3	800
Ec72	23.0 ± 0.2	800
Hc201	23.0 ± 0.4	800
C01	24.0 ± 0.1	800

Table 6. Antimicrobial activity of WEME70 against several strains of Pseudomonas fragi

Strains	Inhibition zone (mm)[a]	(AU ml-1)[b]
F2	15.0 ± 0.1	1600
L2	15.0 ± 0.1	1600
T81	15.0 ± 0.1	1600
Y1	15.0 ± 0.1	1600
M51	15.0 ± 0.1	800
I1	15.0 ± 0.2	800
M12	15.0 ± 0.4	800
X1	17.0 ± 0.3	1600
E1	18.0 ± 0.2	1600
U1	20.0 ± 0.4	1600
M854	12.0 ± 0.1	800
G1	15.0 ± 0.1	800
H81	15.0 ± 0.1	1600
A2	15.0 ± 0.2	800
H83	15.0 ± 0.2	800
I82	15.0 ± 0.2	800
X83	15.0 ± 0.2	800
M102	15.0 ± 0.3	800
M891	16.0 ± 0.5	800
B83	16.5 ± 0.5	800
F1	18.0 ± 0.4	1600
L81	19.0 ± 0.1	1600
A1	15.0 ± 0.1	800
B81	15.0 ± 0.1	800
C81	15.0 ± 0.1	800
D1	15.0 ± 0.1	800
A81	15.0 ± 0.1	800
N1	15.0 ± 0.1	800
ATCC 3456	15.0 ± 0.1	1600
M52	17.0 ± 0.3	1600

[a]Values of inhibition zones were expressed as mean of three experiments ± Standard

[b]Values of antimicrobial activity of WEME70 (100mg DM/ml) were expressed as AU ml^{-1}

Table 7. Antimicrobial activity of WEME70 against Campylobacter strains

Strains	Inhibition zone (mm)[a]	(AU ml-1)[b]
C.coli LPH1	19.0 ± 0.2	1600
C.jejuni LPH2	18.0 ± 0.1	800
C.jejuni LPH3	20.0 ± 0.4	800
C.jejuni LPH4	19.0 ± 0.5	800
C.jejuni LPH5	22.0 ± 0.6	800
C.jejuni LPH6	26.0 ± 0.8	800
C.jejuni LPH7	18.0 ± 0.6	1600
C.jejuni LPH8	18.0 ± 0.5	800
C.jejuni LPH9	20.0 ± 0.5	1600
C.jejuni LPH10	26.0 ± 0.8	800
C.jejuni LP1	26.0 ± 0.2	1600
C.jejuni 11351	23.0 ± 0.6	800
C.jejuni ATCC 11168	17.0 ± 0.5	800
C.coli LP	18.0 ± 0.1	800
C.coli 7571	17.0 ± 0.6	800
C.jejuni 118	19.0 ± 0.2	800
C. jejuni FC1	12.0 ± 0.5	800
C. coli FC2	26.0 ± 0.3	1600
C. coli FC3	23.0 ± 0.9	1600
C. jejuni FC4	22.0 ± 0.8	1600
C. jejuni FC5	24.0 ± 0.6	1600
C. coli FC6	26.0 ± 0.5	800
C. jejuni FC7	27.0 ± 0.2	3200
C. coli FC8	25.0 ± 0.5	1600
C. jejuni FC9	28.0 ± 0.6	800
C. jejuni FC 10	23.0 ± 0.4	800
C. coli FC11	21.0 ± 0.5	800

[a]Values of inhibition zones were expressed as mean of three experiments ± Standard

[b]Values of antimicrobial activity of WEME70 (100mg DM/ml) were expressed as AU ml^{-1}

Table 5. Antimicrobial activity of WEME70 against several strains of *Pseudomonas fragi*

Strains	Inhibition zone (mm)[a]	(AU ml-1)[b]
F2	15.0 ± 0.1	1600
L2	15.0 ± 0.1	1600
T81	15.0 ± 0.1	1600
Y1	15.0 ± 0.1	1600
M51	15.0 ± 0.1	800
I1	15.0 ± 0.2	800
M12	15.0 ± 0.4	800
X1	17.0 ± 0.3	1600
E1	18.0 ± 0.2	1600
U1	20.0 ± 0.4	1600
M854	12.0 ± 0.1	800
G1	15.0 ± 0.1	800
H81	15.0 ± 0.1	1600
A2	15.0 ± 0.2	800
H83	15.0 ± 0.2	800
I82	15.0 ± 0.2	800
X83	15.0 ± 0.2	800
M102	15.0 ± 0.3	800
M891	16.0 ± 0.5	800
B83	16.5 ± 0.5	800
F1	18.0 ± 0.4	1600
L81	19.0 ± 0.1	1600
A1	15.0 ± 0.1	800

B81	15.0 ± 0.1	800
C81	15.0 ± 0.1	800
D1	15.0 ± 0.1	800
A81	15.0 ± 0.1	800
N1	15.0 ± 0.1	800
ATCC 3456	15.0 ± 0.1	1600
M52	17.0 ± 0.3	1600

[a]Values of inhibition zones were expressed as mean of three experiments ± Standard

[b]Values of antimicrobial activity of WEME70 (100mg DM/ml) were expressed as AU ml^{-1}

Table 6. Antimicrobial activity of WEME70 against *Campylobacter* strains

Strains	Inhibition zone (mm)[a]	(AU ml-1)[b]
C.coli LPH1	19.0 ± 0.2	1600
C.jejuni LPH2	18.0 ± 0.1	800
C.jejuni LPH3	20.0 ± 0.4	800
C.jejuni LPH4	19.0 ± 0.5	800
C.jejuni LPH5	22.0 ± 0.6	800
C.jejuni LPH6	26.0 ± 0.8	800
C.jejuni LPH7	18.0 ± 0.6	1600
C.jejuni LPH8	18.0 ± 0.5	800
C.jejuni LPH9	20.0 ± 0.5	1600
C.jejuni LPH10	26.0 ± 0.8	800
C.jejuni LP1	26.0 ± 0.2	1600
C.jejuni 11351	23.0 ± 0.6	800
C.jejuni ATCC 11168	17.0 ± 0.5	800
C.coli LP	18.0 ± 0.1	800
C.coli 7571	17.0 ± 0.6	800
C.jejuni 118	19.0 ± 0.2	800
C. jejuni FC1	12.0 ± 0.5	800
C. coli FC2	26.0 ± 0.3	1600
C. coli FC3	23.0 ± 0.9	1600
C. jejuni FC4	22.0 ± 0.8	1600
C. jejuni FC5	24.0 ± 0.6	1600
C. coli FC6	26.0 ± 0.5	800
C. jejuni FC7	27.0 ± 0.2	3200
C. coli FC8	25.0 ± 0.5	1600

C. jejuni FC9	28.0 ± 0.6	800
C. jejuni FC 10	23.0 ± 0.4	800
C. coli FC11	21.0 ± 0.5	800

[a]Values of inhibition zones were expressed as mean of three experiments ± Standard

[b]Values of antimicrobial activity of WEME70 (100mg DM/ml) were expressed as AU ml^{-1}

Table 8. Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of WEME against strains of *B. thermosphacta* and *P. fragi*

P. fragi	MIC[a]	MLC[a]	*B. thermosphacta*	MIC[a]	MLC[a]
F2	25	25	Fc74	12.5	25
L2	25	25	Bc201	12.5	25
T81	25	25	F175	12.5	25
Y1	25	25	D274	12.5	25
M51	25	25	Dc71	12.5	25
I1	25	25	M175	12.5	25
M12	25	25	Gc74	12.5	25
X1	25	25	Ec71	12.5	25
E1	25	25	E1203	25	25
U1	25	25	Cc203	25	25
M854	25	50	Dc201	25	25
G1	25	50	Ec204	25	25
H81	25	50	D171	25	25
A2	25	50	Cc74	25	25
H83	25	50	G01	25	25
I82	25	50	Fc203	25	25
X83	25	50	Gc202	25	25
M102	25	50	I03	25	25
M891	25	50	D01	25	25
B83	25	50	ATCC 11259	25	25
F1	25	50	Cc75	25	25
L81	25	50	B05	25	50
A1	100	100	Hc202	50	50
B81	100	100	Lc71	50	50
C81	100	100	B02	50	50
D1	100	100	Ic71	50	50
A81	100	100	7R1	50	50
N1	100	100	Fc201	50	50
ATCC 3456	100	100	Fc204	50	50
M52	100	100	Ec72	50	50
			Hc201	50	50
			Dc72	50	50
			C01	50	50

[a]Values of MIC and MLC were expressed as mg of DM/ml of WEME

Table 9. Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of WEME70 against strains of *Listeria* and *S. aureus*

Listeria	MIC[a]	MLC[a]	*S.aureus*	MIC[a]	MLC[a]
L.monocytogenes OH	12.5	25	*S. aureus* 239	12.5	50
L.innocua	12.5	50	*S. aureus* ATCC 25993	25	50
L.monocytogenes 932	12.5	50	*S. aureus* NCTC 9393	25	50
L. monocytogenes ATCC 7644	12.5	100	*S. aureus* 19095	25	100
L. monocytogenes ATCC 932	25	100	*S. aureus* K6278-97	25	100
L. innocua ATCC 1770	25	100	*S. aureus* RIMD 31092	25	100
L. innocua IFI1	25	100	*S. aureus* ATCC 20231	25	100
L. monocytogenes SA	25	100			
L. monocytogenes CAL	25	100			

[a]Values of MIC and MLC were expressed as mg of DM/ml of WEME

Table 10. Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of WEME70 against clinically and foodborne Campylobacter strains

Clinical strains	MIC[a]	MLC[a]	*Food strains*	MIC[a]	MLC[a]
C.coli LPH1	25	50	*C. jejuni FC1*	25	50
C.jejuni LPH2	25	25	*C. coli FC2*	25	50
C.jejuni LPH3	25	25	*C. coli FC3*	25	50
C.jejuni LPH4	25	50	*C. jejuni FC4*	25	25
C.jejuni LPH5	50	50	*C. jejuni FC5*	25	50
C.jejuni LPH6	25	50	*C. coli FC6*	25	50
C.jejuni LPH7	25	50	*C. jejuni FC7*	25	25
C.jejuni LPH8	50	50	*C. coli FC8*	25	50
C.jejuni LPH9	25	50	*C. jejuni FC9*	25	50
C.jejuni LPH10	25	25	*C. jejuni FC 10*	50	50
C.jejuni LP1	25	25	*C. coli FC11*	50	100
C.jejuni 11351	50	50			
C.jejuni 11168	50	50			
C.coli LP	25	25			
C.coli 7571	50	50			
C.jejuni 118	50	100			

[a]Values of MIC and MLC were expressed as mg of DM/ml of myrtle polar phase (PP)

Table 11. Gram sensitivity to the myrtle phases separated by partition chromatography

Strains	Diameter of inhibition zone (mm)[a]				AU ml^{-1}
	PP[b]	LPP[b]	Control PP [b]	Control LPP [b]	
L. monocytogenes CAL	- [c]	17 ± 0,2	-	-	12800
L. monocytogenes OH	-	19 ± 0,2	-	-	12800
L. monocytogenes SA	-	18 ± 0,1	-	-	6400
L. monocytogenes ATCC 7644	-	18 ± 0,2	-	-	6400
L. innocua 1770	-	16 ± 0,3	-	-	6400
L. innocua IFI1	-	14 ± 0.5	-	-	6400
L. monocytogenes ATCC 932	-	20 ± 0.2	-	-	6400
S. aureus K6278-97	-	19 ± 0,3	-	-	12800
S. aureus RIMD 31092	-	22 ± 0,2	-	-	12800
S. aureus NCTC 9393	-	19 ± 0,1	-	-	6400
S. aureus 14458	-	21 ± 0,2	-	-	12800
B. thermosphacta ATCC 11509	-	17 ± 0,2	-	-	6400
B. thermosphacta 7R1	-	11 ± 0,2	-	-	6400
B. thermosphacta B02	-	13 ± 0,2	-	-	6400
B. thermosphacta B05	-	12 ± 0,3	-	-	6400
B. thermosphacta C01	-	12 ± 0,2	-	-	1600
B. thermosphacta Cc75	-	13 ± 0,1	-	-	6400
B. thermosphacta D274	-	13 ± 0,2	-	-	6400
B. thermosphacta D275	-	11 ± 0,3	-	-	6400
B. thermosphacta Dc201	-	10 ± 0,3	-	-	6400
B. thermosphacta Dc71	-	13 ± 0,2	-	-	6400
B. thermosphacta E1203	-	14 ± 0,2	-	-	6400
B. thermosphacta Ec204	-	13 ± 0,1	-	-	6400
B. thermosphacta Fc201	-	13 ± 0,1	-	-	1600
B. thermosphacta Fc203	-	9 ± 0,2	-	-	6400
B. thermosphacta Fc204	-	13 ± 0,2	-	-	1600
B. thermosphacta Fc74	-	14 ± 0,1	-	-	6400
B. thermosphacta Gc202	-	14 ± 0,3	-	-	6400
B. thermosphacta Gc74	-	13 ± 0,2	-	-	6400
B. thermosphacta Hc201	-	14 ± 0,2	-	-	1600
B. thermosphacta Hc202	-	10 ± 0,1	-	-	6400
B. thermosphacta I03	-	14 ± 0,2	-	-	6400

Table 12. Gram-negative sensitivity to the myrtle phases separated by partition chromatography

P. fragi	Diameter of inhibition zone (mm)[a]				AU ml^{-1}
	PP [b]	LPP [b]	Control PP [b]	Control LPP [b]	
F2	13 ± 0,2	- [c]	-	-	3200
L2	13 ± 0,2	-	-	-	3200
T81	12 ± 0,1	-	-	-	3200
Y1	13 ± 0,3	-	-	-	3200
M51	11 ± 0,4	-	-	-	3200
I1	14 ± 0,2	-	-	-	1600
M12	11 ± 0,1	-	-	-	3200
X1	15 ± 0,2	-	-	-	3200
E1	12 ± 0,3	-	-	-	3200
U1	13 ± 0,2	-	-	-	3200
M854	12 ± 0,2	-	-	-	3200
G1	12 ± 0,3	-	-	-	3200
H81	11 ± 0,2	-	-	-	3200
A2	15 ± 0,2	-	-	-	3200
H83	12 ± 0,1	-	-	-	1600
I82	15 ± 0,3	-	-	-	3200
X83	13 ± 0,2	-	-	-	1600
M102	14 ± 0,3	-	-	-	3200
M891	13 ± 0,1	-	-	-	1600
B83	10 ± 0,3	-	-	-	3200
L81	11 ± 0,2	-	-	-	3200
A1	11 ± 0,2	-	-	-	1600
B81	12 ± 0,2	-	-	-	3200
C81	8 ± 0,3	-	-	-	3200
D1	13 ± 0,1	-	-	-	3200
A81	11 ± 0,2	-	-	-	1600
N1	13 ± 0,2	-	-	-	1600
ATCC 3456	12 ± 0,2	-	-	-	3200

Table 13. Gram-negative sensitivity to the myrtle phases separated by partition chromatography

Campylobacter strains	Diameter of inhibition zone (mm)[a]				AU ml^{-1}
	PP [b]	LPP [b]	Control PP [b]	Control LPP [b]	
C.coli LPH1	19 ± 0.6	- [c]	-	-	3200
C.jejuni LPH2	28 ± 1.0	-	-	-	1600
C.jejuni LPH3	20 ± 0.6	-	-	-	1600
C.jejuni LPH4	20 ± 0.6	-	-	-	1600
C.jejuni LPH5	23 ± 0.6	-	-	-	1600
C.jejuni LPH6	27 ± 0.6	-	-	-	1600
C.jejuni LPH7	19 ± 1.2	-	-	-	3200
C.jejuni LPH8	18 ± 1.5	-	-	-	1600
C.jejuni LPH9	21 ± 0.8	-	-	-	3200
C.jejuni LPH10	27 ± 0.6	-	-	-	1600
C.jejuni LP1	26 ± 0.5	-	-	-	3200
C.jejuni 11351	24 ± 0.6	-	-	-	1600
C.jejuni 11168 ATCC	18 ± 0.6	-	-	-	1600
C.coli LP	19 ± 0.4	-	-	-	1600
C.coli 7571	18 ± 0.5	-	-	-	1600
C.jejuni 118	19 ± 0.4	-	-	-	1600
C. jejuni FC1	26 ± 0.5	-	-	-	1600
C. coli FC2	24 ± 0.5	-	-	-	3200
C. coli FC3	22 ± 0.4	-	-	-	3200
C. jejuni FC4	26 ± 0.4	-	-	-	3200
C. jejuni FC5	24 ± 0.8	-	-	-	3200
C. coli FC6	26 ± 0.5	-	-	-	1600
C. jejuni FC7	29 ± 0.8	-	-	-	6400
C. coli FC8	26 ± 0.6	-	-	-	3200
C. jejuni FC9	28 ± 0.6	-	-	-	1600
C. jejuni FC10	23 ± 0.5	-	-	-	1600
C. coli FC11	21 ± 0.8	-	-	-	1600

[a]Values of inhibition zones were expressed as mean of three experiments ± Standard

[b] PP: myrtle polar phase at 11.5% of ethanol (80mg DM/ml); LPP: mytle less polar phase resuspended in DMSO at 5% (15 mg DM/ml); Control PP : ethanol at 11.5%; Control LPP: DMSO at 5%

[c] (-) no inhibition zone

Table 14. Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of myrtle polar phase (PP) against clinically and foodborne *Campylobacter* strains

Clinical strains	MIC[a]	MLC[a]	*Food strains*	MIC[a]	MLC[a]
C.coli LPH1	0.05	2	*C. jejuni FC1*	0.05	1
C.jejuni LPH2	0.05	1	*C. coli FC2*	0.05	1
C.jejuni LPH3	0.05	1	*C. coli FC3*	0.1	1
C.jejuni LPH4	0.05	1	*C. jejuni FC4*	0.05	1
C.jejuni LPH5	0.05	1	*C. jejuni FC5*	0.05	1
C.jejuni LPH6	0.05	1	*C. coli FC6*	0.05	1
C.jejuni LPH7	0.05	2	*C. jejuni FC7*	0.05	0.05
C.jejuni LPH8	0.05	1	*C. coli FC8*	0.05	1
C.jejuni LPH9	0.05	1	*C. jejuni FC9*	0.05	1
C.jejuni LPH10	0.05	1	*C. jejuni FC 10*	0.1	2
C.jejuni LP1	0.05	0.05	*C. coli FC11*	0.05	> 2
C.jejuni 11351	0.05	1			
C.jejuni 11168	0.1	> 2			
C.coli LP	0.05	0.1			
C.coli 7571	0.05	1			
C.jejuni 118	0.1	1			

[a]Values of MIC and MLC were expressed as mg of DM/ml of myrtle polar phase (PP)

Table 15. Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of myrtle less polar phase (LPP) against *Listeria* and *Staphylococcus* strains

Food strains	MIC[a]	MLC[a]	*Food strains*	MIC[a]	MLC[a]
L.monocytogenes 932	0.05	> 2	*S.aureus ATCC 25993*	0.05	>2
L.innocua	0.05	> 2	*S. aureus 239*	0.05	>2
L. innocua IFI1	0.05	> 2	*S. aureus 19095*	0.05	>2

[a]Values of MIC and MLC were expressed as mg of DM/ml of myrtle less polar phase (LPP)

Table 16. Antimicrobial activity of LPP fractions separated by column chromatography

Fractions [a]	B. thermosphacta ATCC 11509	L. innocua 1770	L. monocytogenes ATCC 7644	S.aureus ATCC 20231	P. fragi ATCC 3456	C. jejuni FC7
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	10 ± 0.1 [b]	7 ± 0.2	11 ± 0.3	9 ± 0.5	-	-
9	9 ± 0.4	6 ± 0.3	7 ± 0.4	8 ± 0.6	-	-
10	9 ± 0.5	6 ± 0.5	8 ± 0.2	6 ± 0.6	-	-
11	11 ± 0.1	8 ± 0.1	9 ± 0.1	7 ± 0.1	-	-
12	11 ± 0.3	9 ± 0.2	10 ± 0.2	8 ± 0.3	-	-
13	-	-	-	-	-	-
14	-	-	-	-	-	-
15	-	-	-	-	-	-
16	-	-	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-
19	8 ± 0.5	8 ± 0.3	6 ± 0.4	6 ± 0.1	-	-
20	30 ± 0.2	28 ± 0.1	24 ± 0.1	24 ± 0.3	-	-
21	-	-	-	-	-	-
22	-	-	-	-	-	-
23	-	-	-	-	-	-

[a] All collected fraction were assayed at concentration of 2mg DM/ml

[b] Diameter of inhibition zone rounding wells (mm) ± standard deviation

- Antimicrobial activity not detected

Table 17. Antimicrobial activity of PP fractions collected by RP-HPLC

Fractions [a]	L. monocytogenes ATCC 7644	S.aureus ATCC 20231	B. thermosphacta ATCC 11509	P. fragi ATCC 3456	C. jejuni FC7	C. jejuni LP1
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	-	-
9	-	-	-	4 ± 0.1 [b]	3 ± 0.2	2 ± 0.2
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	-	-	7 ± 0.2	10 ± 0.2	8 ± 0.3
13	-	-	-	-	-	-
14	-	-	-	-	-	-
15	-	-	-	-	-	-
16	-	-	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-
19	-	-	-	-	-	-
20	-	-	-	-	-	-
21	-	-	-	-	-	-
22	-	-	-	-	-	-
23	-	-	-	-	-	-
24	-	-	-	-	-	-
25	-	-	-	-	-	-
26	-	-	-	-	-	-
27	-	-	-	-	-	-
28	-	-	-	-	-	-
29	-	-	-	-	-	-

[a] All collected fraction were assayed at concentration of 2mg DM/ml

[b] Diameter of inhibition zone rounding wells (mm) ± standard deviation

- Antimicrobial activity not detected

Table 18. Microbial changes in ground beef treated with different concentration of freeze-dried myrtle extract during storage at 4°C

Microbial population (Log CFU/g)	Treatment[a]	Storage time (days)[b]				
		0	1	2	3	5
Total viable counts						
	Control	5.65 ± 0.27 Aa	6.86 ± 0.10 Ba	7.81 ± 0.29 Ca	8.62 ± 0.10 Da	8.82 ± 0.15 Da
	A	5.61 ± 0.29 Aa	6.59 ± 0.26 Ba	6.95 ± 0.05 Bb	7.73 ± 0.12 Cb	8.74 ± 0.17 Da
	B	5.49 ± 0.30 Aa	5.92 ± 0.02 Ab	7.33 ± 0.12 Bc	8.02 ± 0.12 Cc	8.89 ± 0.48 Da
	C	5.53 ± 0.25 Aa	6.03 ± 0.12 Ab	7.15 ± 0.19 Bc	7.37 ± 0.02 Bb	8.53 ± 0.46 Ca
Pseudomonas spp.						
	Control	5.60 ± 0.16 Aa	5.73 ± 0.55 Aa	6.51 ± 0.16 Aa	7.16 ± 0.07 Ba	8.92 ± 0.03 Ca
	A	5.57 ± 0.13 Aa	6.59 ± 0.41 Ba	6.49 ± 0.12 Ba	7.25 ± 0.05 Ca	8.98 ± 0.17 Da
	B	5.42 ± 0.12 Aa	6.49 ± 0.03 Ba	6.67 ± 0.19 Ba	7.26 ± 0.01 Ca	9.08 ± 0.07 Da
	C	5.52 ± 0.15 Aa	6.37 ± 0.07 Bb	6.53 ± 0.05 Ca	7.20 ± 0.04 Da	8.35 ± 0.11 Eb
Br. thermosphacta						
	Control	6.05 ± 0.49 Aa	6.96 ± 0.08 Ba	7.69 ± 0.17 Ca	8.22 ± 0.27 Da	7.91 ± 0.09 Da
	A	6.30 ± 0.12 Aa	6.23 ± 0.12 Ab	6.54 ± 0.01 Bb	7.04 ± 0.04 Cb	7.74 ± 0.03 Db
	B	5.82 ± 0.15 Aa	5.80 ± 0.01 Ac	5.82 ± 0.01 Ac	6.32 ± 0.02 Bc	7.51 ± 0.02 Cc
	C	5.74 ± 0.01 Aa	5.79 ± 0.11 Ac	5.35 ± 0.31 Ad	5.24 ± 0.06 Ad	7.71 ± 0.09 Bb
Lactic acid bacteria						
	Control	5.21 ± 0.28 Aa	5.81 ± 0.03 Ba	6.16 ± 0.015Ca	6.82 ± 0.003 Da	6.90 ± 0.14 Da
	A	4.88 ± 0.42 Aa	5.89 ± 0.09 Ba	6.17 ± 0.14 Ca	6.89 ± 0.02 Da	6.73 ± 0.43 Da
	B	5.38 ± 0.32 Aa	6.01 ± 0.34 Ba	5.79 ± 0.01 Bb	6.27 ± 0.03 Cb	6.64 ± 0.20 Da
	C	5.36 ± 0.12 Aa	5.64 ± 0.12 Ba	5.21 ± 0.33 Bc	5.98 ± 0.07 Cc	6.08 ± 0.07 Cb
Enterobacteriaceae						
	Control	3.00 ± 0.36 Aa	3.22 ± 0.11 Aa	3.87 ± 0.03 Ba	3.25 ± 0.12 Ba	4.60 ± 0.27 Ba
	A	2.92 ± 0.01 Aa	3.03 ± 0.03 Ab	3.37 ± 0.49 Aa	3.42 ± 0.10 Aa	4.79 ± 0.22 Ba
	B	2.79 ± 0.03 Aa	2.80 ± 0.13 Ac	3.13 ± 0.02 Ba	3.27 ± 0.01 Ca	5.47 ± 0.12 Db
	C	2.23 ± 0.12 Aa	2.59 ± 0.01 Ac	2.56 ± 0.03 Ab	2.67 ± 0.02 Bb	4.58 ± 0.18 Ca

Table 19 Sensitivity of indicator strains to Lb. curvatus 54M16 as determined by the critical dilution assay

Indicator strains	Substrates[a]	Incubation temperature	Sources[b]	AU ml^{-1} [c]
Bacillus cereus BC	TSB	37°C	CDAS	0
Clostridium perfringens 13124	TSB	37°C	ATCC	0
Clostridium sporogenes SPOL	TSB	37°C	CDAS	0
Clostridium butyricum BUT	TSB	37°C	CDAS	0
Escherichia coli 0157:H7	TSB	37°C	CDAS	0
Staphylococcus aureus 20231	TSB	37°C	DSMZ	0
Staphylococcus aureus 19095	TSB	30°C	ATCC	0
Staphylococcus aureus 14458	TSB	30°C	ATCC	0
Listeria monocytogenes 7644	TSB	30°C	ATCC	3200
Listeria monocytogenes OH	TSB	30°C	Carminati	12800
Listeria monocytogenes CAL	TSB	30°C	Carminati	800
Listeria innocua C6	TSB	30°C	CDAS	800

[a] TSB, Tryptone Soya Broth (Oxoid, CM0129)

[b] CDAS: collection of Department of Agricultural Science, Division of Microbiology, University of Naples Federico II; ATCC: American Type Culture Collection;
Dr D. Carminati, Istituto Sperimentale Lattiero-caseario, Lodi, Italy; DSMZ: Deutsche Sammlung fu¨r Mikroorganismen und Zellkulturen, Braunschweig, Germany;

[c] Titers of *Lb. curvatus* 54M16 using a given strain as indicator.

Table 20. PCR primers used in this study amplifying lactobacillus bacteriocin genes

Primers	Sequences (5’ 3’)	PCR product	Bacteriocin	References
CurAF CurAR	GTA AAA GAA TTA AGT ATG ACA TTA CAT TCC AGC TAA ACC ACT	171-bp	Curvacin A	Remiger et al. 1996
SakXF SakXR	AGC TAT GAA AGG TAT TGT CGG G TAA GAT TTC CAG CCA GCA GC	150-bp	Sakacin X	Macwana & Muriana 2012
SakTαF SakTαR	TCG GTG GCT ATA CTG TCT AAA CA TGT CCT AAA AAT CCA CCA ATG C	160-bp	Sakacin Tα	
SakTβF SakTβR	AAG AAA TGA TAG AAA TTT TTG GAG G TGT GAA ATCC AAT CTT GTC CTG	151-bp	Sakacin Tβ	
SakPF SakPR	ATG GAA AAG TTT ATT GAA TTA TTA TTT ATT CCA GCC AGC GTT	186-bp	Sakacin P	Remiger et al. 1996

Table 21. Ingredients composition of sausage mix

Ingredients	**Quantity %**
pork shoulder	70
bacon	18
pork fat	12
NaCl	2.7
red wine	0.5
meat flavour	1

Table 22. Effect of temperature on bacteriocin production after 24, 48 and 72 h in MRS broth

	Temperature																			
	10°C				15°C				20°C				25°C				30°C			
Times (h)	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72
pH	6.04	6.04	6.03	6.01	6.04	5.78	5.16	4.75	6.04	4.74	4.43	4.27	6.04	4.42	4.09	4.08	6.04	4.15	3.98	4.12
O.D. $_{600nm}$	0.05	0.05	0.05	0.04	0.05	0.27	0.57	0.79	0.05	0.77	1.15	1.24	0.05	1.03	1.31	1.16	0.05	1.15	1.21	1.28
AU ml^{-1}	0	50	50	50	0	3200	6400	1600	0	6400	3200	3200	0	6400	3200	3200	0	6400	3200	3200

Table 23. *Acidifying activity and effect of NaCl on microbial growth*

Substrate[a]	Glu %[b]	NaCl %[c]	pH[d]	ΔpH[e]
ME	0	1	6.5	0.9
ME	0	4	6.5	0.8
ME	0	6	6.5	0.7
ME	0.3	1	6.5	1.2
ME	0.3	4	6.5	1.1
ME	0.3	6	6.5	0.9

[a] ME (Lab-Lemco beef extract, Oxoid);

[b] Glu: amount of glucose supplemented to the ME broth;

[c] Nacl : amount of sodium chloride supplemented to the ME broth

[d] pH of ME broth not inoculated

[e] Acidifying activity measured as decrease of pH value after 48 hours of incubation at 30°C

Table 24. MALDI-TOF analysis of single fractions obtained by HPLC system

HPLC retention time	**Measured MW**	**Expected MW**	**Peptide name**
24.19 min	4360.1	4362.12	Sakacin X
31.02 min	4068.9	4070.22	Sakacin T_{β}
32.01 min	2841.1	2842.32	IP-TX
35.89 min	4640.6	4644.32	Sakacin T_{α}
45.04 min	4453.6	4434.02	Sakacin P variant

Table 25. Technological properties of *Lb. curvatus* 54M16

	54M16 Activities					
	Nitrate reductase		Catalase [c]	SOD[d]	Proteolytic [e]	
Temperature	Diameters (mm)[a]	O.D./mg dry weight [b]	µmoles H2O/ml cells	% O.D.540$_{nm}$	Sarcoplasmic proteins	Myofibrillar proteins
15°C	0	0.012 ± 0.001	-	-	-	-
20°C	0	0.191 ± 0.004	-	-	-	-
30°C	0	0.155 ± 0.004	9.4 +1.2	9.1+12.4	-	-
37°C	-	-	-	-	12	0

[a] Nitrate reductase by agar test: measures of red haloes diameter (mm) surrounding the wells at 15, 20 and 30°C

[b] Nitrate reductase by spectrometric assay: measures of O.D./mg of dry weight at 15, 20 and 30°C

[c] Catalase activity determined in YT broth as µmoles of degraded H_2O/min/ml of cells with OD=1.0

[d] SOD (Superoxide dismutase activity) : expressed as % of O.D. at 540 nm

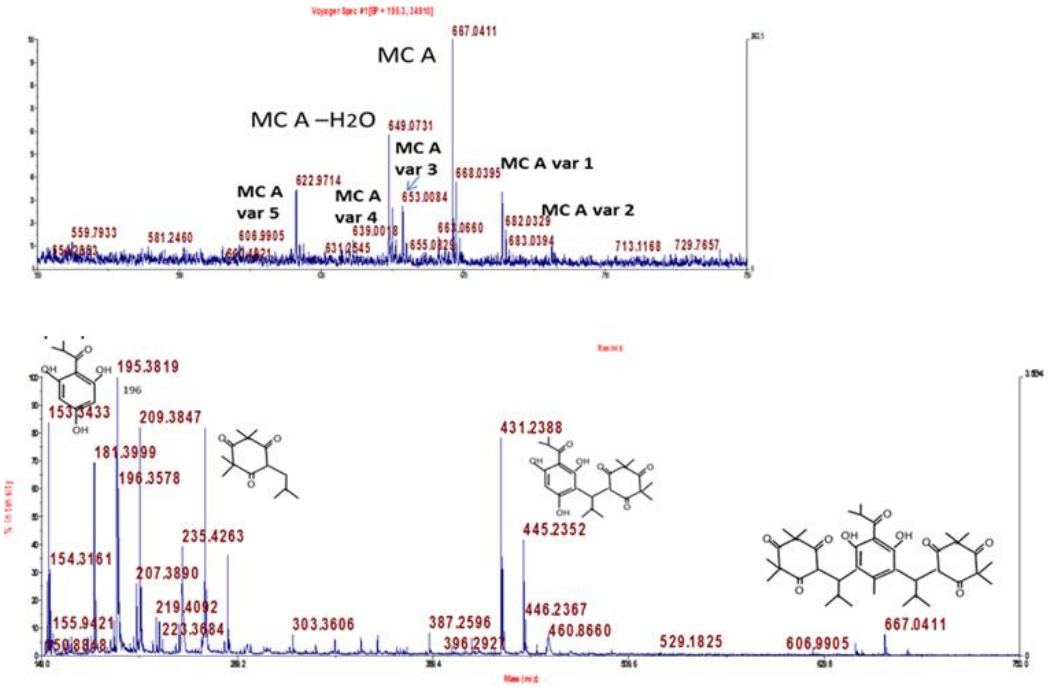
[e] Proteolytic activity was checked by the measure of clear zone surrounding the wells (mm)

- Not detected

Figure1. - **Aphrodite driving a chariot drawn by Erotes; attic red figure, c. 450-400 BC, Museo Archeologico Etrusco, Florence, Italy (www.theoi.com).**

Figure 2. Analaysis MALDI-TOF-MS of LPP from WEME70

Figure 3. MALDI-TOF-MS spectra of fraction 20 obtained by chromatography on silica column gel of LPP



MCA: myrtucommulone A; MA var 1: myrtucommulone with isobutyl; MA var 2: myrtucommulone with n-pentyl; MA var 3: myrtucommulone with n-pentyl; MA var 3: myrtucommulone with n-ethyl; MA var 4: myrtucommulone with 2 molucules of ethyl; MA var 5: myrtucommulone without isopropyl.

Figure 4. Purification of fraction 20 by HPLC

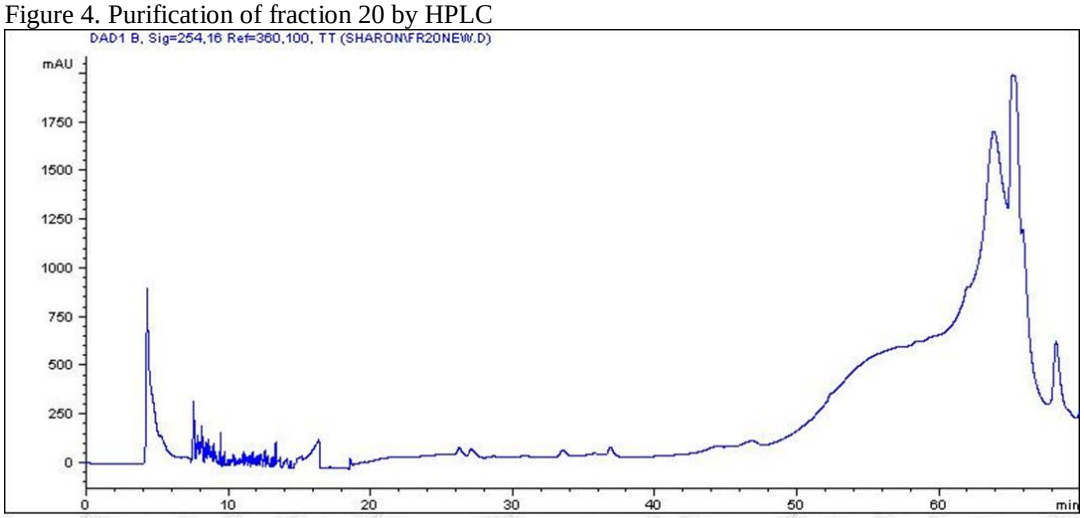


Figure 5. Electrospray ionization quadrupole time-of-flight mass spectrometry of myrtucommulone A and D

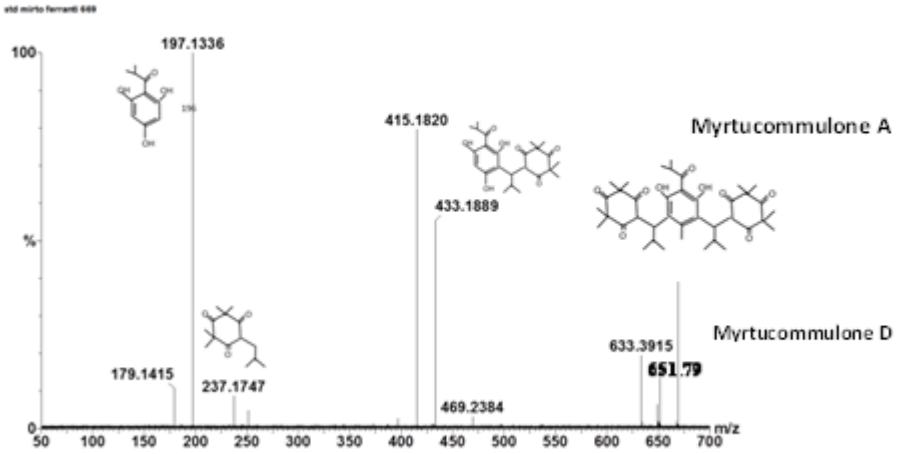


Figure 6. **MALDI-TOF-MS spectra of fraction 9 separated by RP-HPLC of PP**

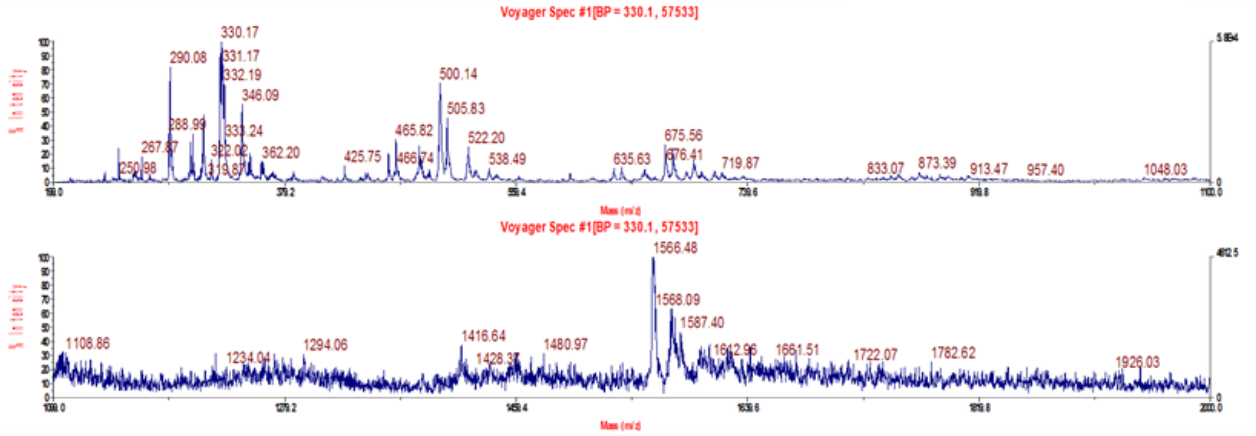


Figure 7. **MALDI-TOF-MS spectra of fraction 12 of PP**

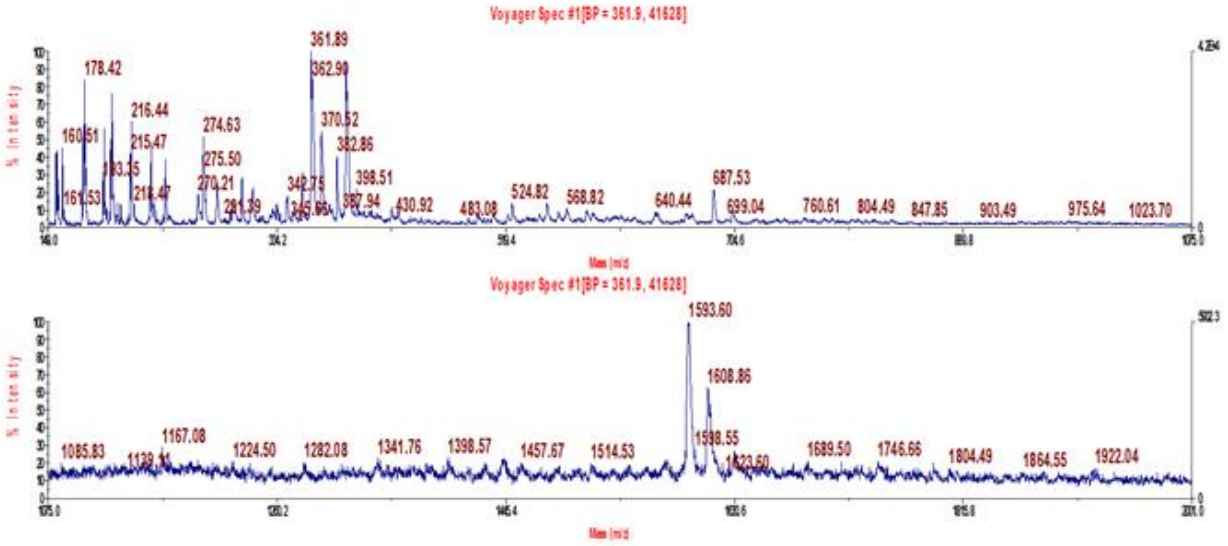


Figure 8. **Caco-2 cell viability test (MTT)**

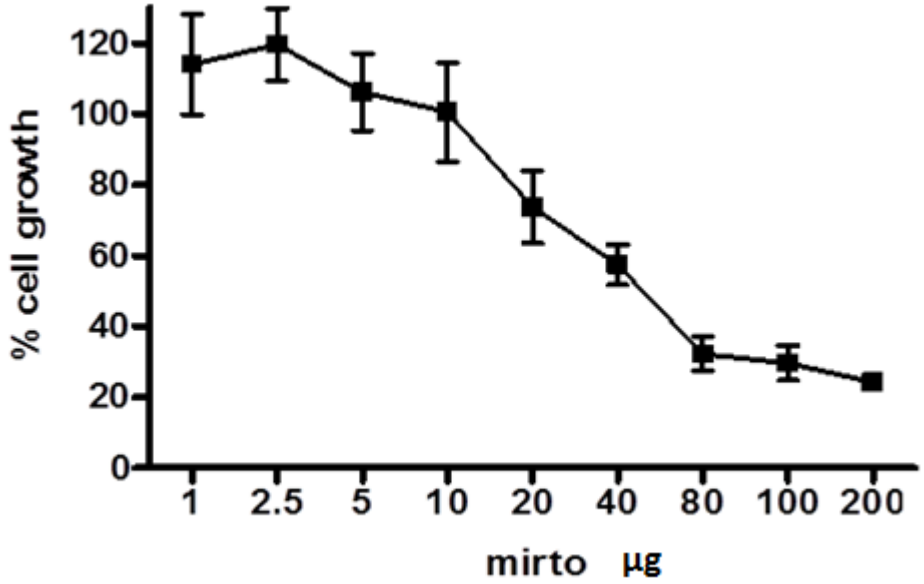
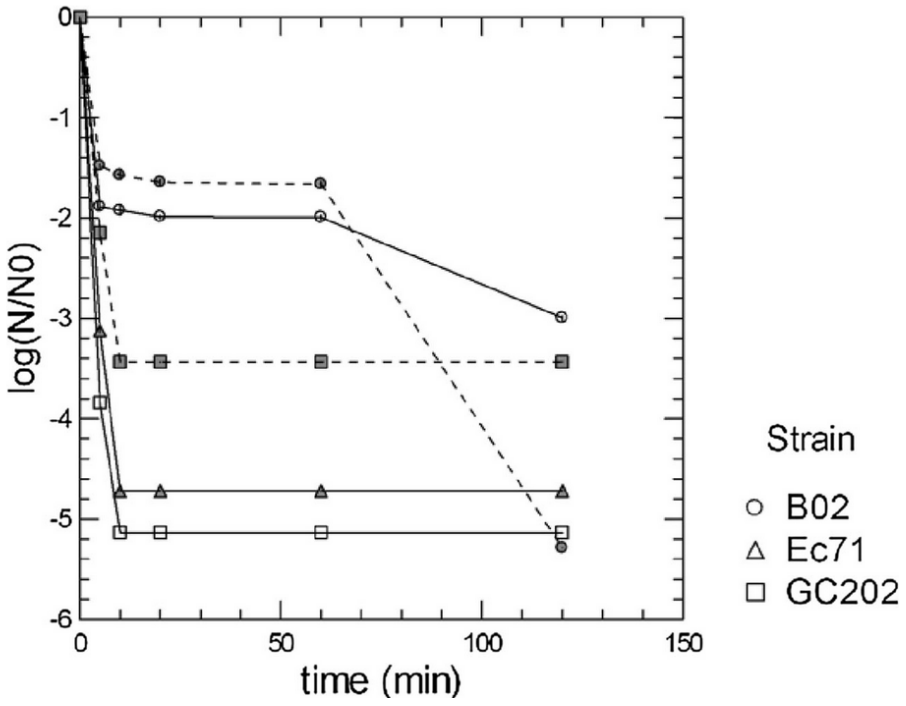


Fig 9. Effect of WEME at different concentration on non-growing cells of B. thermosphacta

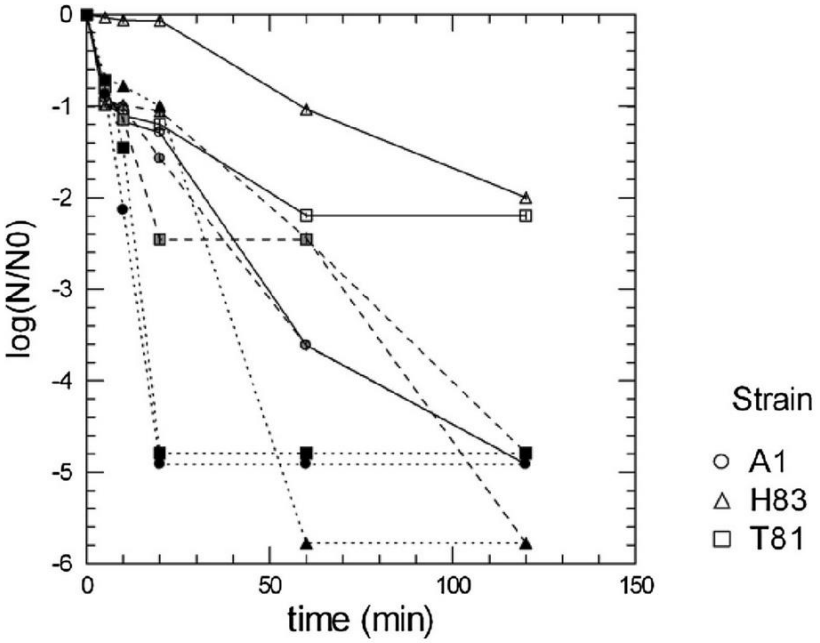


white symbols: WEME at 25 mg of DM/ml; gray symbols: WEME at 50 mg of DM/ml;

B02, Ec71 and GC202 *B. thermosphacta* strains suspended in 50 mM potassium phosphate buffer, pH 7.

Values are shown as mean ± standard error bars

Figure 10. Effect of WEME70 at different concentration on non-growing cells of *P. fragi*



white symbols: WEME at 25 mg of DM/ml; - gray symbols: WEME at 50 mg of DM/ml; - black symbols: WEME at 100 mg of DM/ml;

A1, H83 and T81 *P. fragi* strains suspended in 50 mM potassium phosphate buffer, pH 7.

Values are shown as mean ± standard error bars

Figure 11 (a) Effect on WEME70 on resting cells

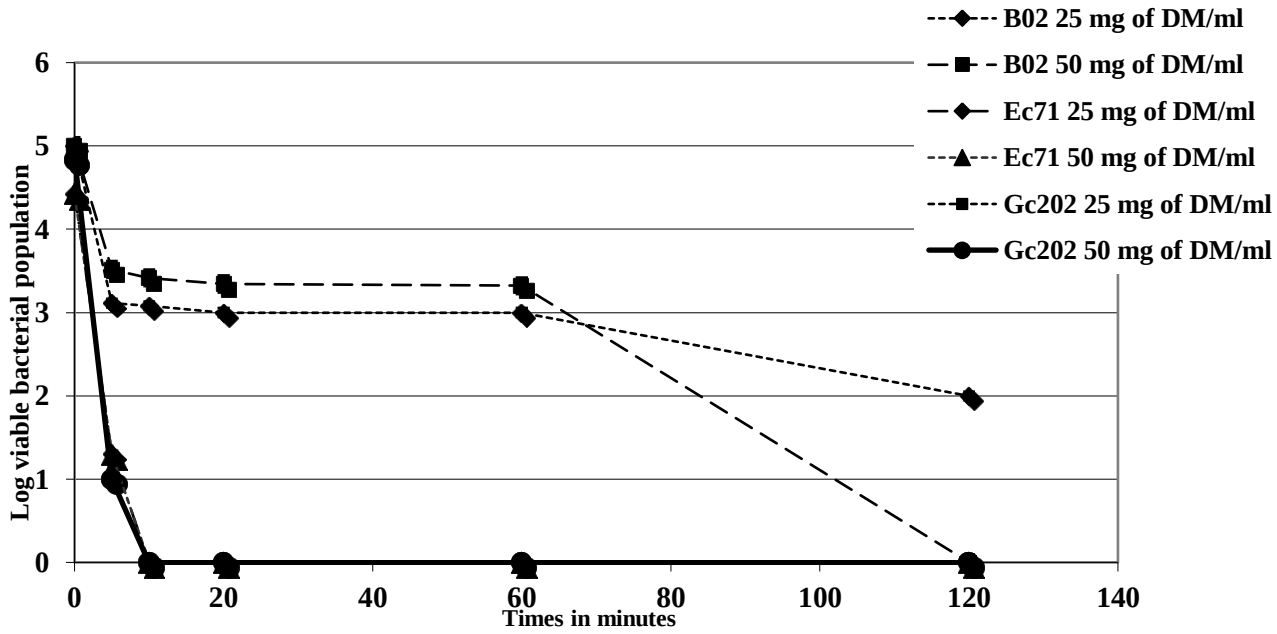


Figure 11 (b) Effect on WEME70 on resting cells

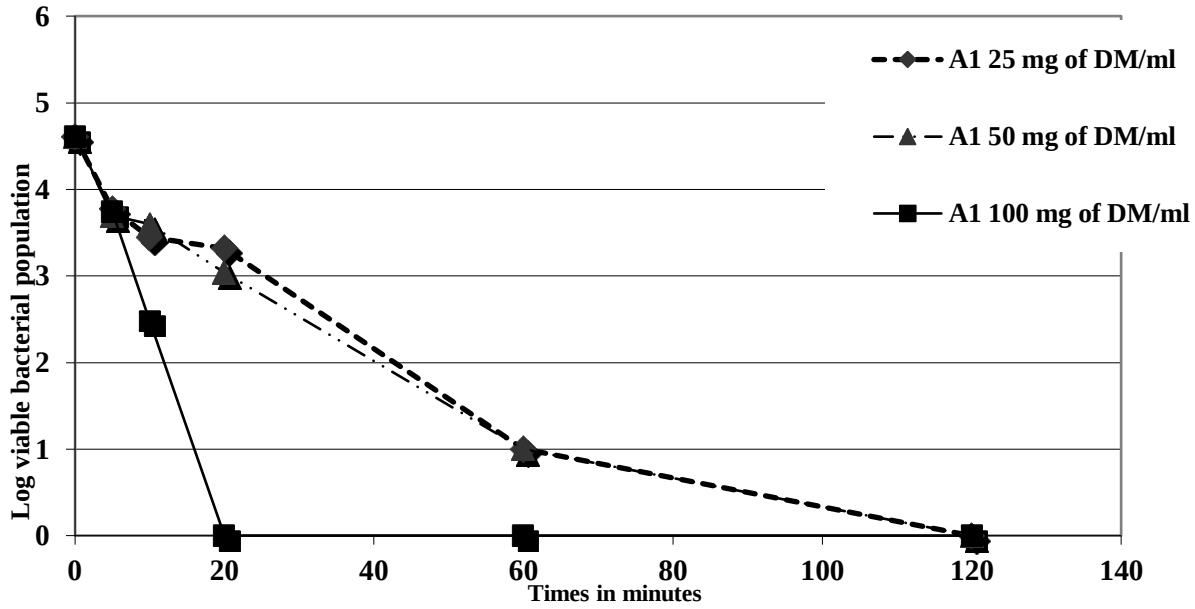


Figure 11 (c) Effect on WEME70 on resting cells

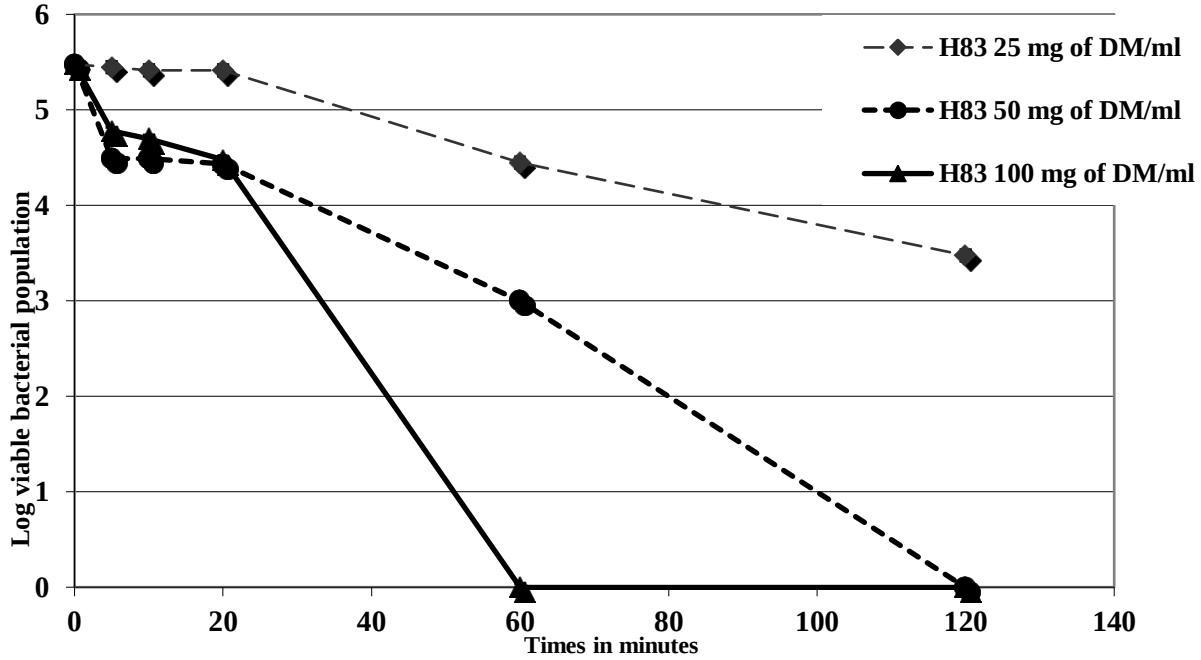


Figure 11 (d) Effect on WEME70 on resting cells

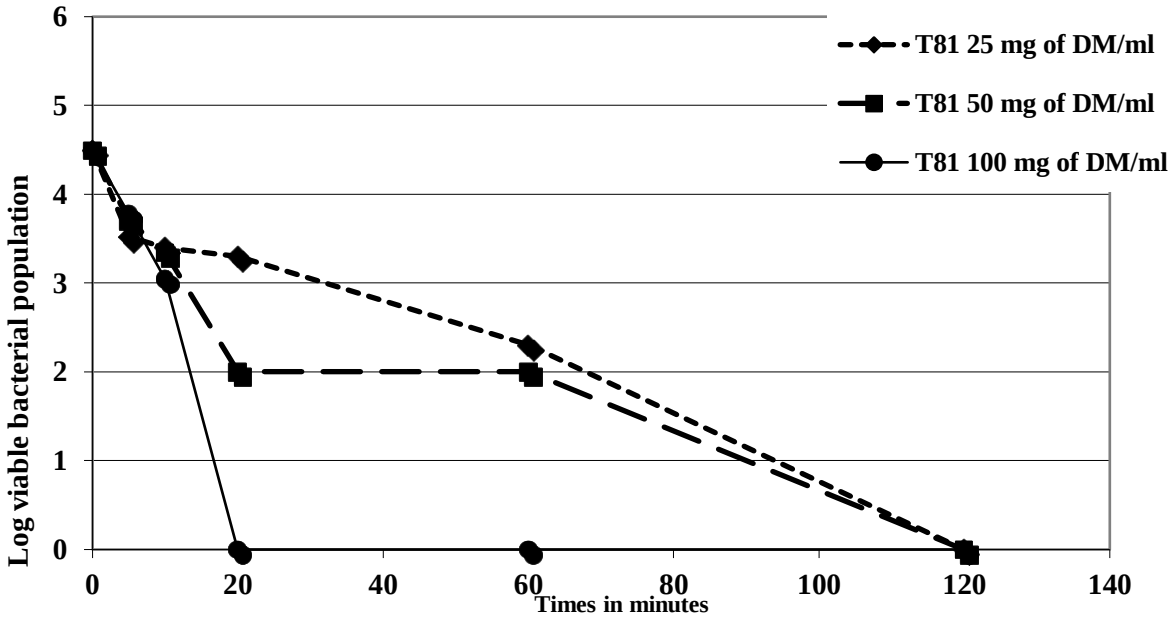


Figure 12. Viable staining performed after the inoculum of L. monocytogenes in broth containing 0.05mg/ml of LPP of WEME70 and after 24 h of incubation at the optimal growth temperature by using LIVE/DEAD® Bacterial Viability Kit (BacLightTM). The membrane integrity changes during cells treatment with myrtle fraction (A) Viable (green) rod-shaped cells stained with Syto 9 (B) viable (green) and dead (red) cells treated with the LPP.

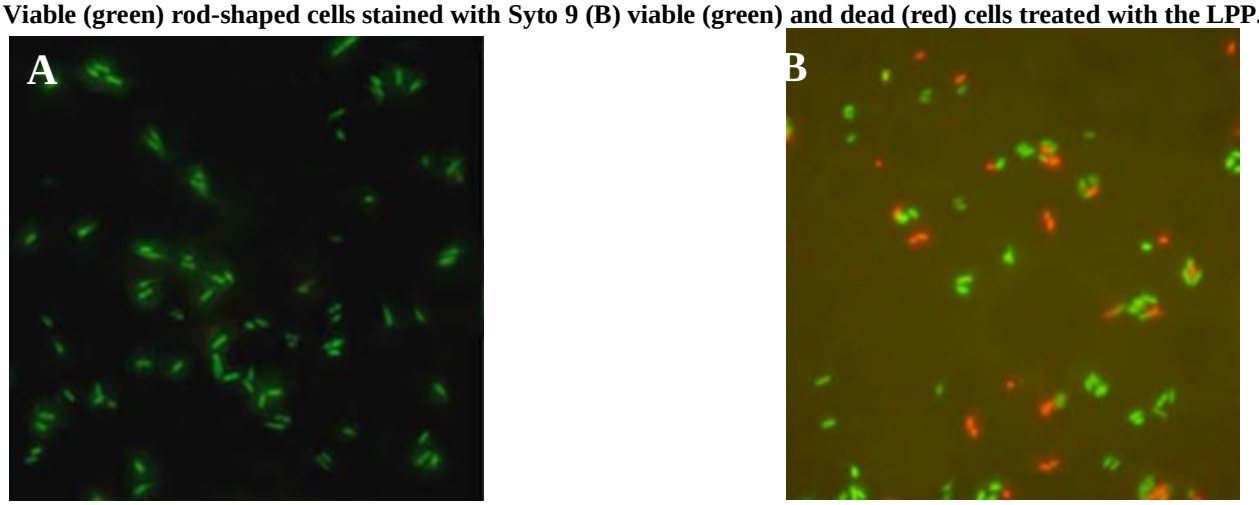


Figure 13. Viable staining performed after the inoculum of *C. coli* in broth containing 0.05 mg/ml of PP and after 24 h of incubation at the optimal growth temperature by using LIVE/DEAD® Bacterial Viability Kit (BacLightTM). The membrane integrity changes during cells treatment with myrtle extract (A) Viable (green) spiral form cells stained with Syto 9 (B) viable (green) and dead (red) cells treated with PP.

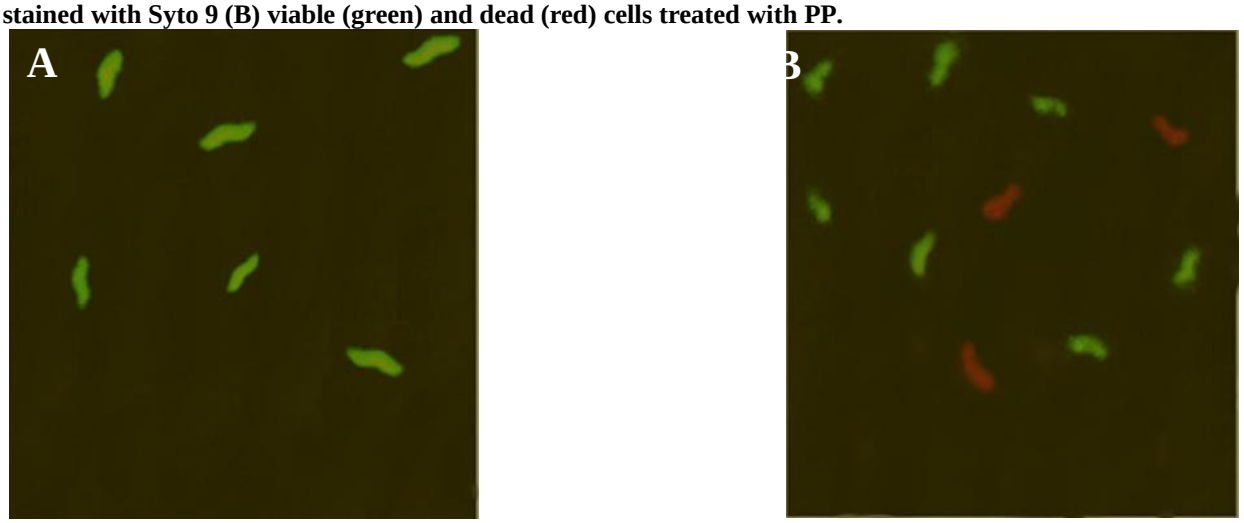


Figure 14. **Viable staining performed after the inoculum of S. aureus in broth containing 0.05 mg/ml of LPP and after 24 h of incubation at the optimal growth temperature by using LIVE/DEAD® Bacterial Viability Kit (BacLightTM). The membrane integrity changes during cells treatment with myrtle extract (A) Viable (green) coccal-shape cells stained with Syto 9 (B) viable (green) and dead (red) cells treated with LPP.**

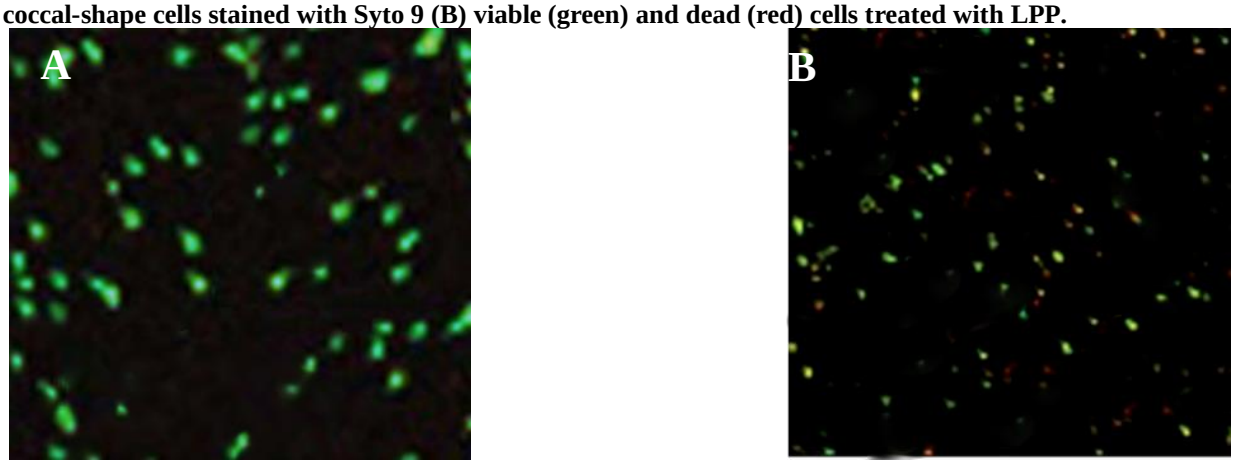


Figure 15 (a) Membrane integrity of cells of *L. innocua* exposed to myrtle hexane fraction (LPP)

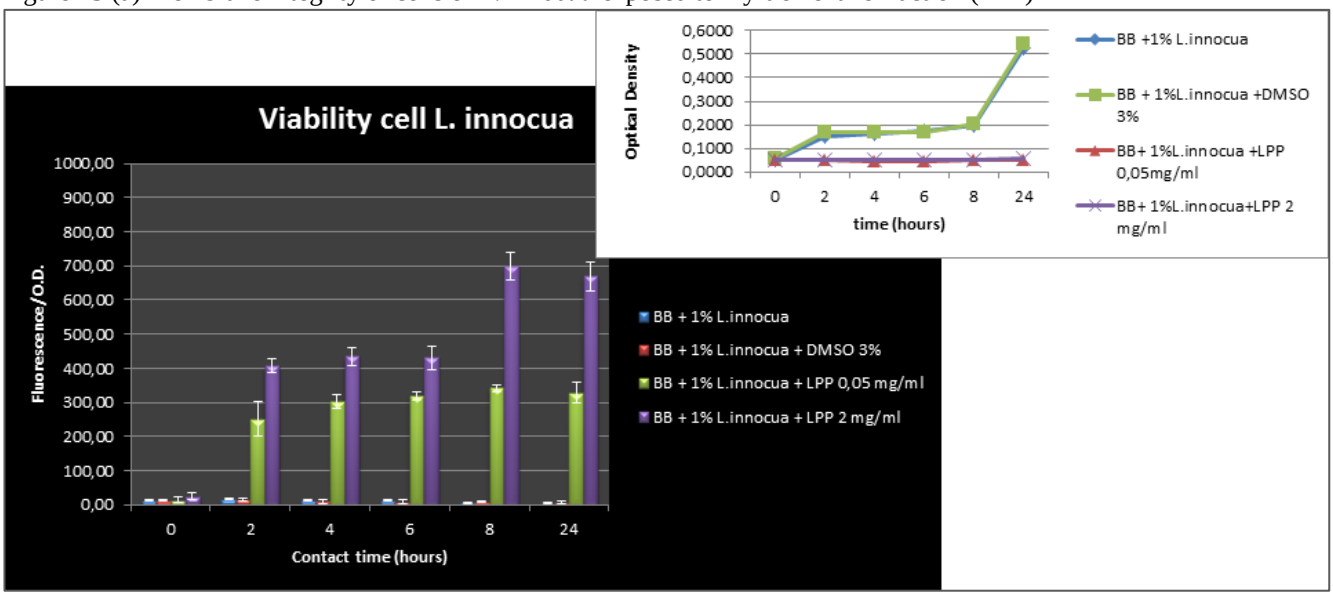


Figure 15 (b) Membrane integrity of cells of *L. monocytogenes* exposed to myrtle hexane fraction (LPP)

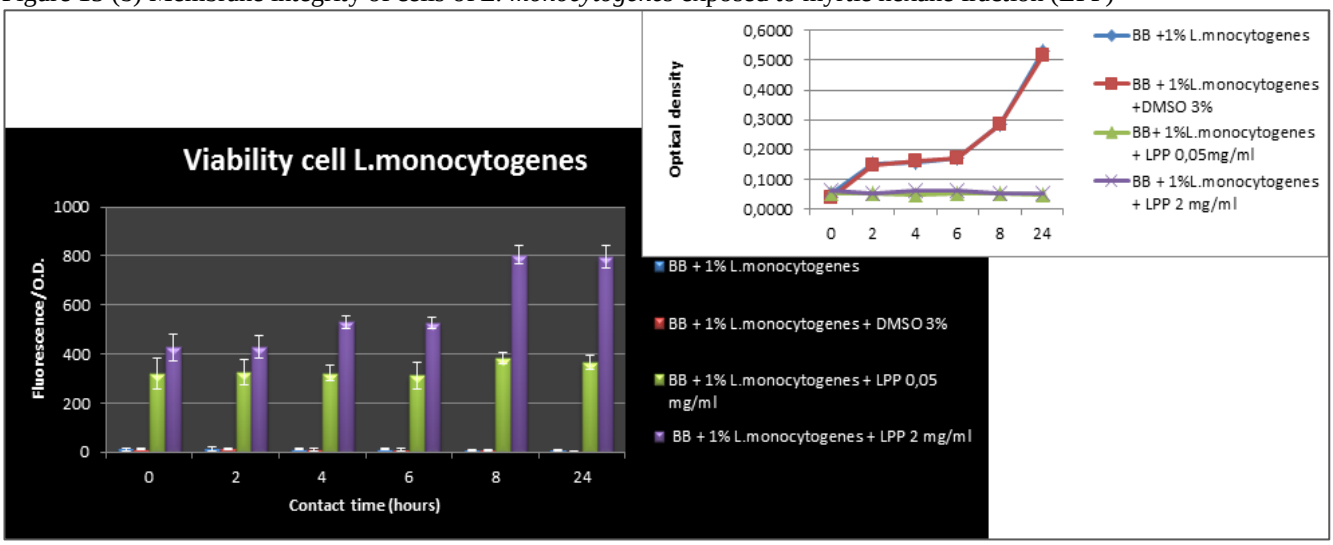


Figure 16(a). Membrane integrity of cells of *S. aureus* strains exposed to myrtle hexane fraction (LPP)

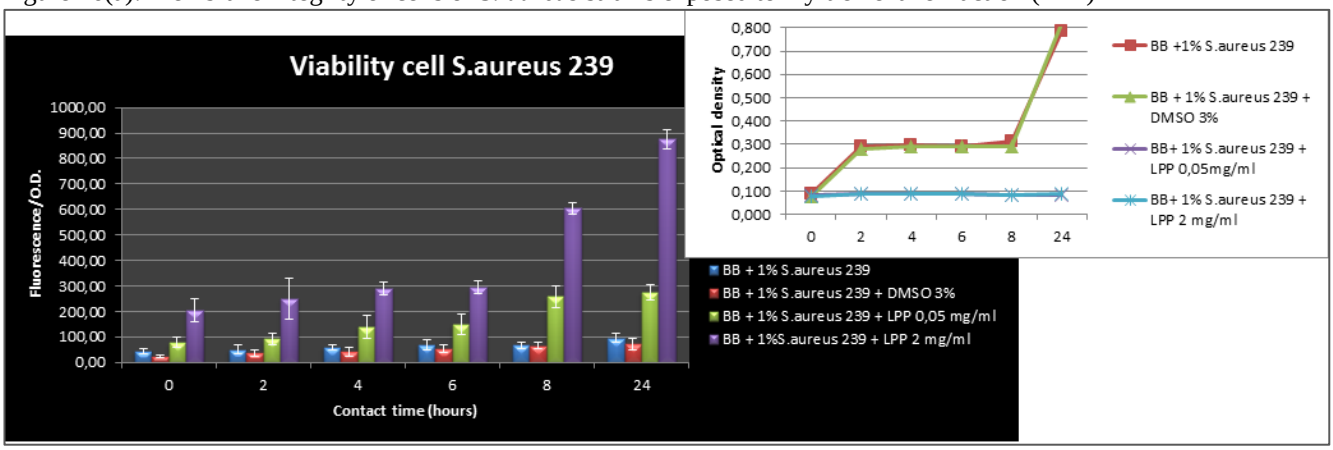


Figure 16 (b)

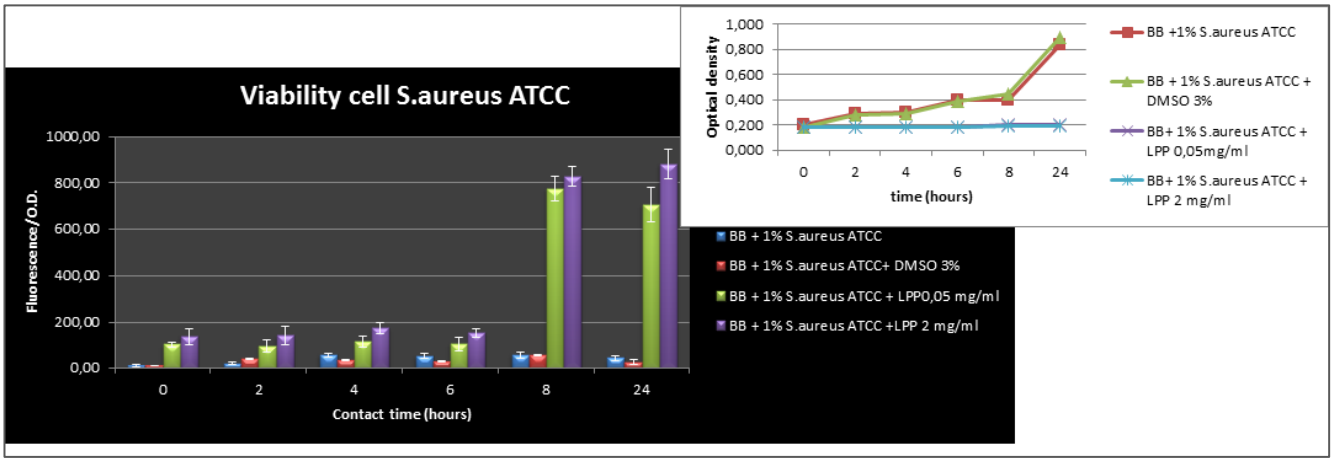


Figure 17(a) Membrane integrity of cells of *C. jejuni* exposed to myrtle ethanol fraction (PP)

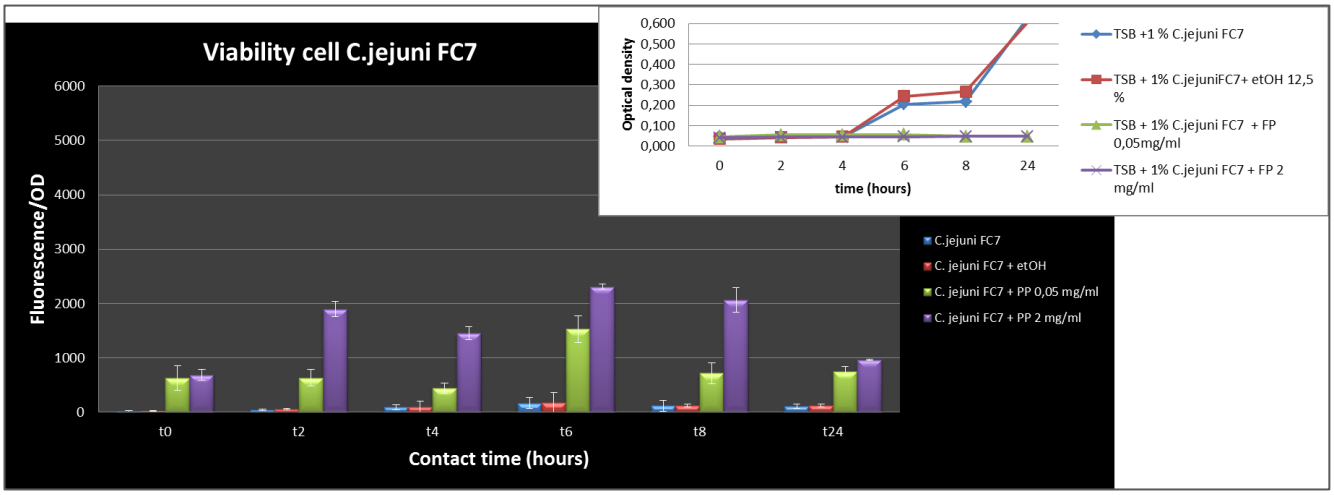


Figure 17(b) Membrane integrity of cells of *C. coli* exposed to myrtle ethanol fraction (PP)

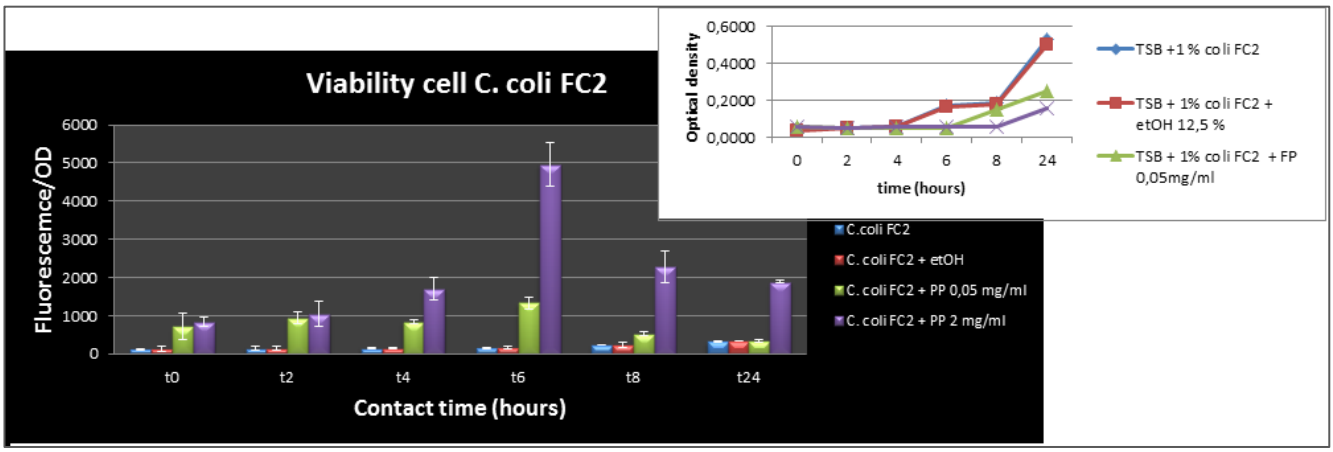


Figure 17(c)

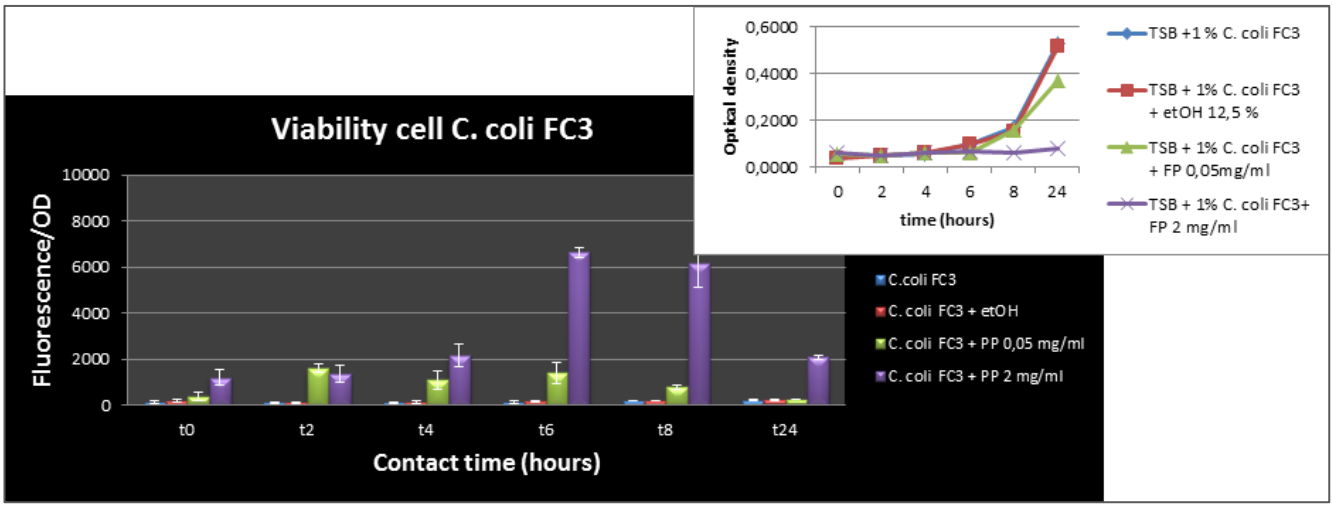


Figure 17(d) Membrane integrity of cells of *C. coli* exposed to myrtle ethanol fraction (PP)

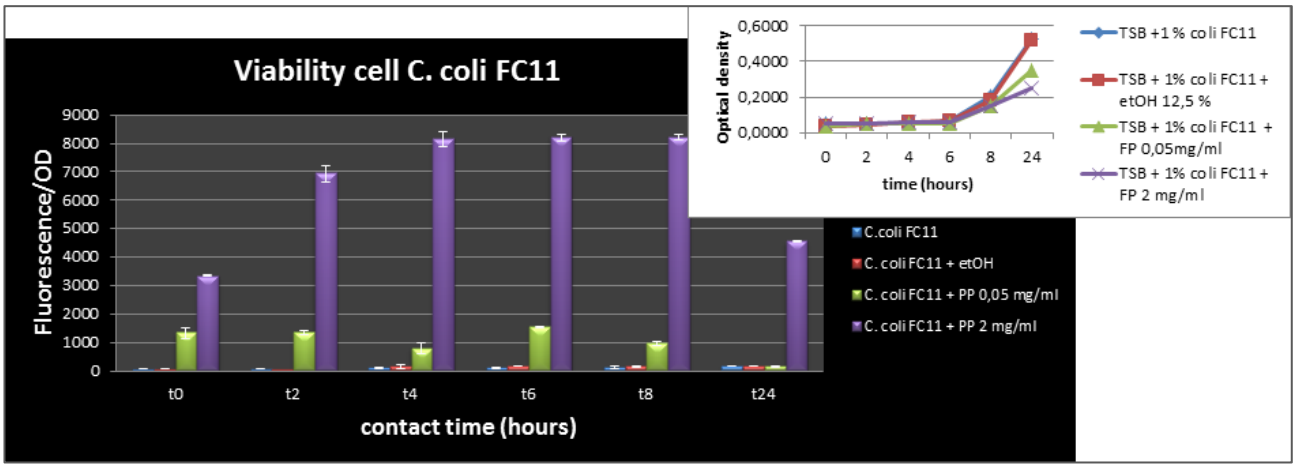


Figure 18 Antimicrobial activity of WEME70 against *L. monocytogenes OH* in Gorgonzola cheese

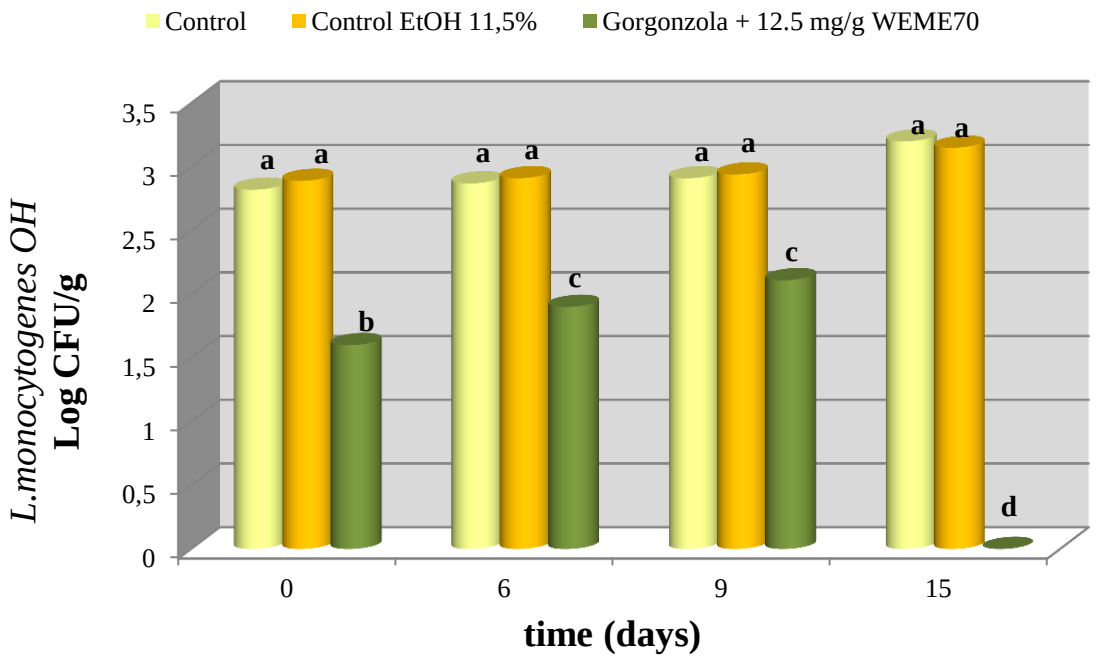
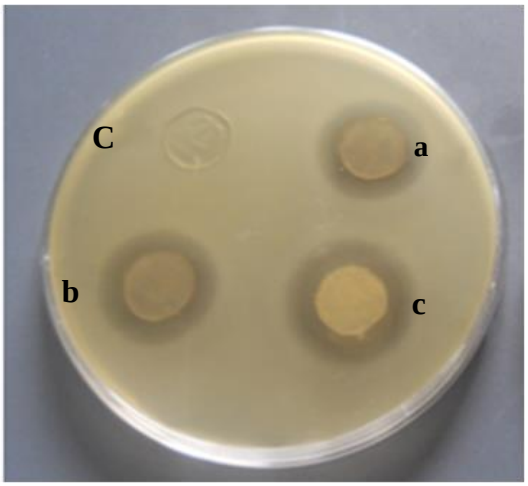
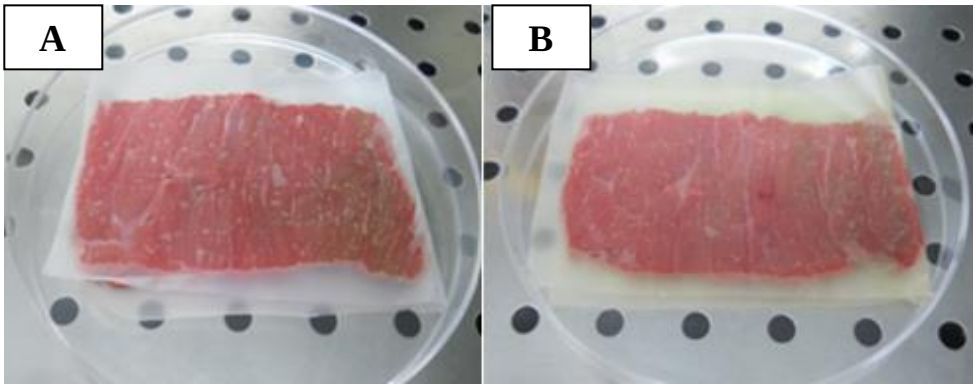


Figure 19. Antimicrobial activity of the PE film coated with the PP solutions at 1600 – 3200 and 6400 AU ml-1 against the indicator strain of *P. fragi F2*



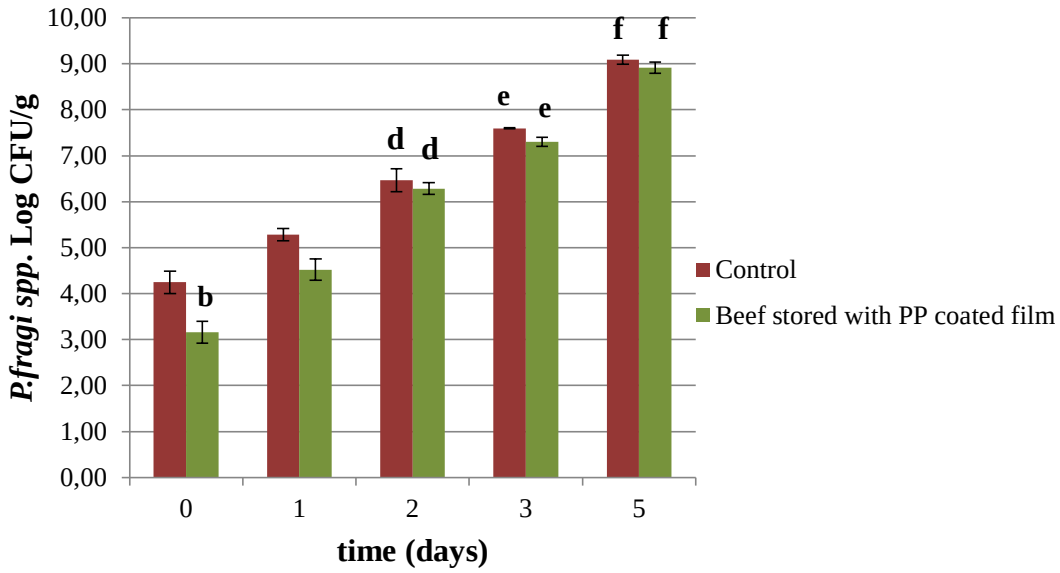
CT: untreated film covered with 11.5% EtOH solution(v/v) ; **(a)** treated film with PP at 1600 AU ml^{-1}; **(b)** treated film with PP at 3200 AU ml^{-1}; **(c)** treated film with PP at 6400 AU ml^{-1}.

Figure 20. Antimicrobial activity of PE film coated with PP of myrtle leaves extract 6400 AU ml^{-1} against the indicator strain *P.fragi* inoculated in beef



A: untreated PE film; **B**: treated PE film

Fig. 21 Antibacterial activity of PP coated PE film against *P. fragi spp*. in raw beef



Data represent the means of experiments performed in duplicate ± standard deviation

Mean values was elaborated by one way ANOVA test with $p<0.05$

Figure 22. **SDS- PAGE of bacteriocin produced by Lb. curvatus 54M16 covered with L. monocytogenes ATCC 7644**

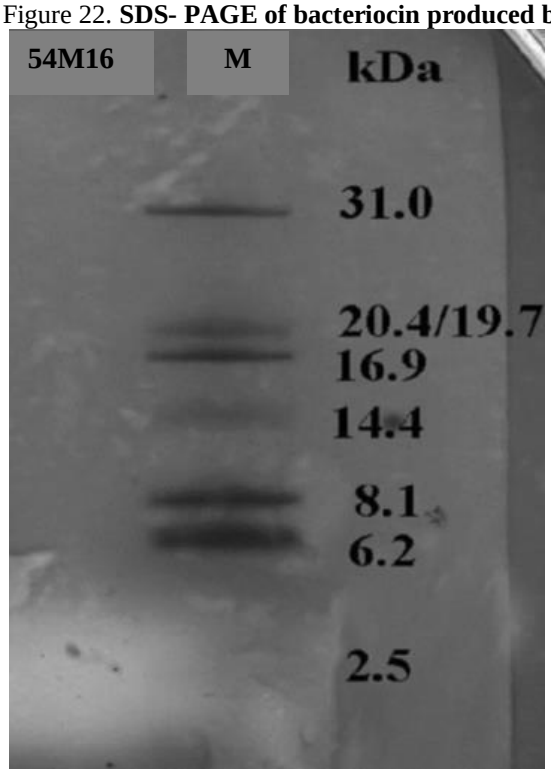


Figure 23. **MALDI-TOF-MS of peptide fraction separated by RP-HPLC**

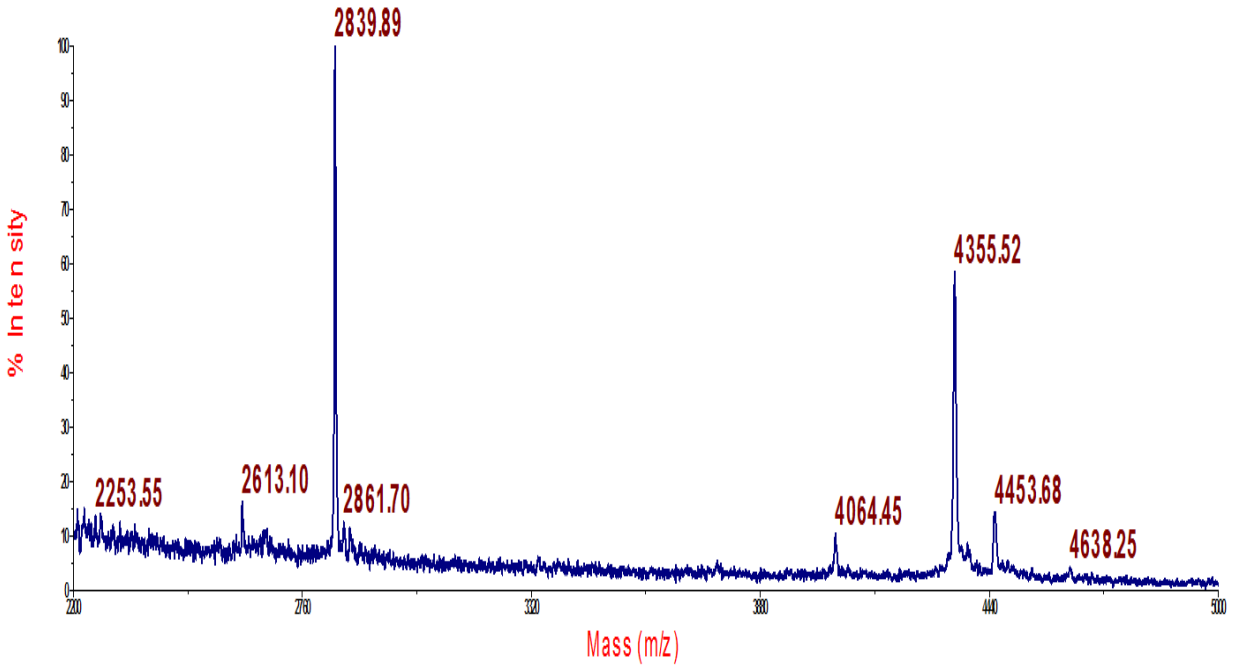


Figure 24 Enumeration of *L. monocytogenes* 7644 inoculated in fermented sausage