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Management of Innovation in the Agricultural and Food Systems of the Mediterranean Region

– XXV cycle –

DEVELOPMENT OF INNOVATIVE SYSTEMS FOR THE IDENTIFICATION AND MONITORING OF *LISTERIA MONOCYTOGENES* IN FRESH-CUT PRODUCTS

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SUMMARY

Abstract

The foodborne pathogen *Listeria monocytogenes* is the causative agent of listeriosis, a severe disease with high hospitalization and case fatality rates. *L. monocytogenes* can survive and grow over a wide range of environmental conditions. This allows the pathogen to overcome food preservation and safety barriers, and pose a potential risk to human health. For these reasons, a fast detection and quantification of the pathogens are key points to ensure the safety of the consumers. The official recommended methods to detect *L. monocytogenes* in foods are carried out by culture-dependent methods, while the Most Probable Number (MPN) method is generally used to enumerate *L. monocytogenes* at low concentrations. These conventional methods are laborious and may require 5-10 days to confirm a positive results, a time that goes beyond the shelf life of the product itself. In this temporal space, packaging and storage conditions of minimally processed vegetables and fruits may encourage the growth of this psychrotrophic pathogen making it necessary the development of alternative methods of monitoring and controlling of *L. monocytogenes* in fresh-cut products.

In the present work we integrated conventional MPN technique with qPCR, in order to keep the advantages of both methods, sensitivity of MPN and reliability and quickness of qPCR. Moreover, we developed a qPCR enrichment-based method for a fast and reliable detection of *L. monocytogenes* in minimally processed vegetables. In order to validate the proposed technique, the results were compared with conventional culture-dependent methods. Finally, probiotic *Lactobacillus* spp. were investigated for their antagonistic effect against *Listeria monocytogenes* on fresh-cut pineapple pieces.

By the proposed molecular-baesd approach we are able to quantify *L. monocytogenes* in artificially contaminated fresh-cut vegetables at an initial concentration of 1 cfu g^{-1} in 48 hours. QPCR alone allowed a limit of detection (LOD) of 10^1 CFU g^{-1} after 2 hours of enrichment in a selective medium. In addition, the occurrence of the pathogenic bacteria in mixed salad samples and fresh-cut melons was monitored in two production plant from the receipt of the raw materials to the early stages of shelf life. No sample was found to be contaminated

by *L. monocytogenes*. The antagonistic assays indicated that *Lactobacillus fermentum* and *Lactobacillus plantarum* were able to inhibit the growth of *L. monocytogenes* on pineapples pieces along conservation, being *L. plantarum* more effective than *L. fermentum*.

The reported results suggest that molecular methods could integrate conventional methods for a faster detection of foodborne pathogens in fresh-cut vegetables. In addition, we proposed two new probiotic strains that could be successfully applied during processing of fresh-cut pineapples, contributing at the same time to inducing a protective effect against a relevant foodborne pathogen, such as *L. monocytogenes*.

Keywords: *Listeria monocytogenes*, fresh-cut vegetables and fruits, MPN, qPCR, food safety, fresh-cut pineapples, probiotic.

Abstract (Italian)

Listeria monocytogenes è un patogeno di origine alimentare ed è l'agente eziologico della listeriosi, una grave affezione con elevato tasso di ospedalizzazione e di mortalità. *L. monocytogenes* può sopravvivere e crescere in un ampia gamma di condizioni ambientali. Ciò permette al patogeno di superare gli ostacoli imposti dalle esigenze di conservazione e sicurezza alimentare, rappresentando un rischio potenziale per la salute umana. Per questi motivi, il rapido rilevamento e la quantificazione del patogeno sono punti chiave per garantire la sicurezza dei consumatori. I metodi ufficiali che sono raccomandati per il rilevamento di *L. monocytogenes* negli alimenti sono considerati metodi coltura-dipendenti, mentre il metodo del numero più probabile (MPN) è generalmente usato per quantificare *L. monocytogenes* a basse concentrazioni. Questi metodi convenzionali sono complessi e possono richiedere 5-10 giorni per confermare un risultato positivo, un tempo che è superiore alla conservabilità del prodotto stesso. In questo spazio temporale, le condizioni di confezionamento e conservazione di frutta e verdura di IV gamma possono favorire la crescita di questo patogeno psicrotrofico, rendendo necessario lo sviluppo di metodi alternativi di monitoraggio e di controllo di *L. monocytogenes* nei prodotti minimamente processati.

Nel presente lavoro la tecnica MPN convenzionale è stata integrata con la real-time PCR quantitativa (qPCR) al fine di mantenere i vantaggi di entrambi i metodi: sensibilità del MPN e affidabilità e rapidità della qPCR. Inoltre, è stato sviluppato un metodo basato sulla qPCR dopo arricchimento selettivo per la rilevazione rapida e affidabile di *L. monocytogenes* negli ortaggi di IV gamma. Per validare la tecnica proposta, i risultati sono stati confrontati con i convenzionali metodi coltura-dipendenti. Infine, probiotici *Lactobacillus* spp. sono stati studiati per il loro effetto antagonista contro *Listeria monocytogenes* su pezzi di ananas fresh-cut.

Con l'approccio molecolare proposto è stato possibile quantificare la presenza di *L. monocytogenes* in prodotti di IV gamma contaminati artificialmente ad una concentrazione iniziale di 1 cfu g^{-1} in 48 ore. La tecnica qPCR ha consentito un limite di rilevabilità (LOD) di 10^1 CFU g^{-1} dopo 2 ore di arricchimento in terreno selettivo. Inoltre, la presenza di *L. monocytogenes* in campioni di insalata mista e

meloni di IV gamma è stata monitorata in due impianti di produzione dal momento del ricevimento delle materie prime alle prime fasi della *shelf-life*. Nessun campione era contaminato da *L. monocytogenes*. Gli esperimenti di antagonismo hanno mostrato che *Lactobacillus fermentum* e *Lactobacillus plantarum* erano in grado di inibire la crescita di *L. monocytogenes* su ananas di IV gamma durante il periodo di conservazione, essendo *L. plantarum* più efficace di *L. fermentum*.
I risultati riportati suggeriscono che i metodi molecolari potrebbero integrare i metodi convenzionali per la rilevazione rapida di agenti patogeni di origine alimentare nelle verdure di IV gamma. Inoltre, sono stati proposti due nuovi ceppi probiotici che potrebbero essere applicati con successo durante l'elaborazione di ananas di IV gamma, contribuendo allo stesso tempo ad indurre un effetto protettivo nei confronti di un importante patogeno alimentare quale *L. monocytogenes*.

Parole chiave: *Listeria monocytogenes*, prodotti di IV gamma, MPN, qPCR, sicurezza alimentare, ananas di IV gamma, probiotici.

SECTION 1:
INTRODUCTION

1. THE GENUS *LISTERIA*

1.1. Taxonomy of the genus *Listeria*

The genus *Listeria* contains ten species of low G+C content (36–42.5%) Gram-positive bacteria closely related to the genus *Bacillus*. The oldest known six species are *Listeria monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri* and *L. grayi*, while only recently *L. marthii*, *L. fleischmannii*, *L. weihenstephanensis* and *L. rocourtiae* have been described as novel species in the genus *Listeria* (Bertsch et al., 2013; Graves et al., 2010; Lang Halter et al., 2013; Leclercq et al., 2010). From a taxonomical point of view six species (*L. monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii*) are relatively closely related and usually referred to the genus *Listeria sensu stricto*, while the species *L. rocourtiae*, *L. weihenstephanensis*, *L. fleischmannii* and *L. grayi* are phylogenetically divergent from the genus *Listeria sensu stricto* (Bertsch et al., 2013; Lang Halter et al., 2013; Leclercq et al., 2010). The phylogeny of the genus Listeria *sensu stricto* supports that *Listeria innocua*, *Listeria marthii*, *Listeria monocytogenes* and *Listeria welshimeri* share a common ancestor, while *Listeria ivanovii* and *Listeria seeligeri* form another clade (Den Bakker et al., 2013). *Listeria ivanovii* has been divided into two subspecies, *Listeria ivanovii* subsp. *ivanovii* and *Listeria ivanovii* subsp. *londoniensis* (Den Bakker et al., 2010).

1.2. Morphology and growth conditions

Listeria spp. are small (0.4–0.5 µm in diameter and 1-2 µm in length), Gram-positive rod-shape bacteria unable to produce spores and capsules. Cells are found singly, or in short chains, or may be arranged in V and Y forms or in palisades. *Listeria* spp. are motile microorganism because able to produce among two to six peritrichous flagella. However, motility is observed in strains cultured below 30 °C and seems to depending by the temperature. Expression of the gene encoding for the flagellin protein (*flaA*) appears to be downregulated at 37°C and related to mechanism of the host cell invasion and virulence (Dons et al., 2004; Bigot et al., 2005).

All *Listeria* species grow well on most non-selective bacteriological media including blood agar base, nutrient, tryptose, and tryptose soy or brain heart infusion agars. Growth rate is enhanced by the presence of fermentable sugar, particularly glucose, blood or serum (Ludwig et al., 2009).

On nutrient agar, colonies are 0.2-0.8 mm in diameter, non-pigmented, round, translucent, low-convex with a smooth surface, after 24 h of incubation, achieving 5 mm in diameter after 5-10 days. Rough colonies may occasionally be observed probably due to bacteria auto-agglutination. Differences in virulence between rough and smooth colonies have been observed (Kämpfer et al. 1991). The growth of all *Listeria* species generates a characteristic sweet caramel or buttery smell due to the generation of butyric acid.

The range of temperatures over which *Listeria* spp. can grow is among 2 to 45 °C, with optimal growth at 30–37 °C. This microorganism do not survive heating at 60 °C for 30 min (Seeliger and Jones, 1986). *Listeria* spp. normally grows in a wide range of pH (4.4-9.6), with the optimal growth rate observed at neutral to slightly alkaline pH. However, it was observed that a pre-adaptation step of the cells to a non-lethal pH 5.5 for 1 h led to survival upon exposure to a lethal pH 3.5 (O'Driscoll et al. 1996). The adaptive acid tolerance response in which a short adaptive period at a non-lethal pH induces metabolic changes that allow the organism to survive a lethal pH involved a variety of regulatory responses, as recently reviewed by Smith et al., (2013).

Listeria can grow in media containing 10% (w/v) NaCl with survival at higher concentrations. Tolerance to high osmolarity is attributed mainly to its ability to accumulate compatible solutes, particularly glycine betaine (Ko et al., 1994). Recently, it was observed that salt stress phenotypes in *Listeria* spp. vary by genetic lineage and temperature (Bergholz et al., 2010). *Listeria* is one of the few foodborne pathogens that can grow at an activity water value below 0.93.

The cell wall contains a directly cross-linked peptidoglycan based on meso-diaminopimelic acid (meso-DAP), but not contain arabinose. Alanine and glutamic acid are also present. Mycolic acids are not present. In addition to N-acetylmuramic acid and N-acetylglucosamine, glucosamine also occurs as a component of the cell-

wall polysaccharide. Ribitol and lipo-teichoic acids are present for example in *Listeria monocytogenes*: these, together with flagella antigens, are responsible for the serological types. Mycolic acids are not present. The fatty acid compositions consist of mainly straight-chain saturated, anteiso-methyl-branched-chain types. Menaquinones are the sole respiratory quinones (Ludwig et al., 2009).

1.3. Metabolism and biochemical characters

Listeria spp. are aerobic, microaerophilic, facultatively anaerobic, catalase-positive and oxidase-negative. Catabolism of glucose proceeds by the Embden–Meyerhof pathway both anaerobically and aerobically. Anaerobically, only hexoses and pentoses support growth, the catabolism of glucose is homofermentative and lactate is produced. Under aerobic conditions maltose and lactose support growth of some strains, but sucrose does not, and *Listeria* spp. are able to produce lactic, acetic, isobutyric, and isovaleric acids. All *Listeria* spp. are methyl red and Voges-Proskauer test positive. Acid is also produced from amygdalin, cellobiose, fructose, mannose, salicin, maltose, dextrin, alpha-methyl-D-glucoside, and glycerol. Acid production from galactose, lactose, melezitose, sorbitol, starch, sucrose, and trehalose is variable. Acid is almost never produced from adonitol, arabinose, dulcitol, erythritol, glycogen, inositol, inulin, melibiose, raffinose, or sorbose. Phenylalanine-deaminase, ornithine, lysine, and arginine decarboxylases are not produced. *Listeria* spp. not utilized exogenous citrate, not produced H_2S and indole. Esculin and sodium hippurate are hydrolyzed. Urea, gelatin, casein, and milk are not hydrolyzed (Ludwig et al., 2009).

All *Listeria* species are phenotypically very similar, but can be distinguished by combinations of the sugar fermentation (acid production from D-xylose, L-rhamnose, alpha methyl- D-mannoside, and mannitol) and the hemolytic ability, usually evaluated by the CAMP test. *L. ivanovii*, *L. monocytogenes*, and *L. seeligeri* are hemolytic on agars plates containing sheep, cow, horse, rabbit or human blood. In contrast, *L. innocua*, *L. welshimeri*, and *L. grayi* are not hemolytic. Positive CAMP test *L. monocytogenes*, *L. seeligeri* and *L. ivanovii* can be discriminate depending of the enhanced zone of beta-hemolysis at the intersection of the

reference test strains *Staphyloccoccus aureus* and *Rhodococcus equi*. Thus, hemolysis of *L. monocytogenes* increases in the close area of *S. aureus* but not in that of *R. equi*, while the reverse is true for *L. ivanovii*. Hemolytic trait is strictly related to the virulence gene cluster, while non-hemolytic and a number of weakly hemolytic strains are not or are weakly pathogenic (Ludwig et al., 2009). The main biochemical features among *Listeria* spp. are reported in table 1.

Table 1. Biochemical features of *Listeria* spp.

	Listeria monocytogenes	*Listeria innocua*	*Listeria ivanovii*	*Listeria seeligeri*	*Listeria welshimeri*	*Listeria grayi*
Hemolysin	+	-	+	+	-	-
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-
L-Rhamnose	+	+/-	-	-	+/-	+/-
D-Mannitol	-	-	-	-	-	+
D-Xylose	-	-	+	+	+	-
α-Methyl-Mannoside	+	+	-	-	+	+

1.4. General chromosome features of *Listeria* species

The *Listeria* spp. genome encode approximately 2800 potential proteins, of which two thirds have an assigned gene function, leaving some 900 genes with unknown function. Although differences between the species are seen due to gene acquisition or gene deletion, very little rearrangement or inversion of genome segments is detected resulting in a highly conserved synteny in gene organization. This may be due to the generally low occurrence of transposons and insertion sequences in *Listeria* genomes (Rocourt and Buchrieser, 2007).

Extrachromosomal DNA has been detected in all species of *Listeria*, most of which carry out a plasmid larger than 20 MDa (Perez-Diaz et al., 1982; Kuenne et al., 2010). It was determined that 95% of the *L. monocytogenes* plasmid-positive strains were resistant towards cadmium versus only 13% of the plasmid negative strains (Lebrun et al., 1992).

1.5. *Listeria monocytogenes* serotypes

Listeria species can be differentiate at serotypes level because possess group-specific surface proteins, such as somatic (O) and flagellar (H) antigens. While there are 15 *Listeria* somatic (O) antigen subtypes (I–XV), flagellar (H) antigens comprise four subtypes (A–D), with the serotypes of individual *Listeria* strains being determined by their unique combinations of O and H antigens. Through examination of group-specific *Listeria* O and H antigens in slide agglutination, at least 12 serotypes (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7) have been recognized in *L. monocytogenes* (Liu, 2006). *Listeria monocytogenes* strains have been classified into three main evolutionary lines: (i) lineage I, consisting of serotypes 4b, 1 / 2b, 3c and 3b, (ii) lineage II, consisting of serotypes 1 / 2a, 1 / 2c and 3a and (iii) lineage III, containing serotypes 4a and 4c (Cheng et al., 2008). In addition it has been observed that *L. monocytogenes* serotypes 1/2a, 1/2b and 4b are responsible for 98 % of documented human listeriosis cases, whereas serotypes 4a and 4c are rarely associated with outbreaks of the disease. Furthermore, while *L. monocytogenes* serotype 4b strains are isolated mostly from epidemic outbreaks of listeriosis, serotypes 1/2a and 1/2b are linked to sporadic *L. monocytogenes* infection (Orsi et al., 2011).

2. HUMAN LISTERIOSIS

2.1. Pathogenesis of infection and virulence factors

L. monocytogenes is an intracellular pathogen which infects the human host by the ingestion of contaminated foods and is able to get across three tight physiological barriers: the intestinal, the blood-brain, and the feto-placental barriers. By crossing the intestinal barrier, the bacterium is absorbed from the intestinal lumen, traversing the epithelial cell layer, and if the immune system does not control the infection, the pathogen disseminates to the bloodstream and mesenteric lymph nodes. Once within the host, they parasitize macrophages and actively invade a range of non-phagocytic cells including epithelial cells, hepatocytes, fibroblasts, and cells of the endothelium and nervous tissue (Camejo et al., 2011).

The mechanism of the infection occurs in subsequent steps involving several virulence factors that are expressed under the positive control of PrfA (Pleiotropic Regulatory Factor Activator) (de las Heras et al., 2011). Outside a host cell, the transcriptional activator PrfA exists in a low-activity state, with correspondingly low levels of virulence gene expression, but once inside the host, PrfA becomes activated and induces the expression of gene products that are needed for host cell invasion. The core *prfA* regulon encompasses 10 genes that are directly regulated by *prfA*. Nonetheless, up to 145 additional putative *prfA* regulated genes have been investigated by microarray expression data or proteomic analyses (Milohanic et al., 2003; Port and Freitag, 2007; de las Heras et al., 2011).

Bacterial invasion of host cells is a process mediated by surface proteins, mainly the internalins InlA and InlB, able to bind specific host-cell receptors. In consequence of this interaction, the bacterium is phagocytized by the host cell and enclosed in a subcellular organelle called a phagolysosome. The low pH of this environment activates listeriolysin O (LLO), an exotoxin that can lyse the membrane of the organelle within 30 minutes allowing the bacterium to escape into the cytoplasm. All pathogenic strains of listeria produce listeriolysin O and the entry of the bacteria into the cytoplasm of the host cell is required for pathogenesis. In addition, two phospholipases namely phosphatidylinositol-specific phospholipase C (PI-PLC) and

phosphatidylcholine (PC-PLC) contribute to break down the phagosome. Once in the cytoplasm, the bacteria proliferate using nutrients from the host that are acquired through the bacterial hexose phosphate transporter (Hpt). Then, *L. monocytogenes* utilizes an actin-based motility system for the invasion of neighboring cells by a process called cell-to-cell spreading. ActA, is a surface protein responsible for the polymerization of actin filaments at one pole of the bacteria, forming a structure resembling a comet tail that enables bacterial propulsion and movement in the host cell cytosol. ActA is sufficient to promote bacterial intracellular motility in the absence of other Listeria factors. The bacteria enter adjacent cells and secrete LLO and the broad specificity phosphatidylcholine phospholipase C (PC-PLC) to escape from the double membraned secondary vacuoles that are formed as a result of cell-to-cell spread (Freitag et al., 2009). *Listeria monocytogenes* is thus able to spreads from cell to cell without directly contacting the extracellular environment. Therefore, host survival is dependent on the development of an effective adaptive immune response, which, if not provided, can permit bacteria to re-enter the bloodstream and possibly reach the brain or the placenta, causing potentially fatal systemic infections (Southwick and Purich, 1996).

2.2. Human listeriosis and clinical manifestations

Listeria monocytogenes is the causative agent of listeriosis, a highly fatal opportunistic foodborne disease. Pregnant women, neonates, the elderly, and debilitated or immunocompromised patients in general are predominantly affected, although the disease can also develop in health individuals. According to case reports, the incubation period is from 24 hours to 70 days.

2.3. Listeriosis in pregnancy

Listeriosis is 18 times more common in pregnancy than in the non-pregnant population, and 16-27% of all infections with *Listeria* occur in pregnant women (Lamont et al., 2011). Infection with *L. monocytogenes* can occur at all stages of pregnancy and it may result in fetal loss, stillbirth, premature delivery, or neonatal

infection (Mylonakis et al., 2002; Janakiraman, 2008). Among pregnant women diagnosed with listeriosis, the rate of fetal mortality is between 16 and 45%. Symptoms in women with listeriosis during pregnancy may be non-specific, and they often manifest as a mild illness (Benshushan et al., 2002; Delgado, 2008).

2.4. Neonatal listeriosis

In contrast, neonatal infection caused by *L. monocytogenes* is a serious and often fatal disease, which may manifest into two clinical forms: early and late onset. Early onset represent between 45 and 70% of neonatal listeriosis. Neonatal listeriosis may occur by vertical transmission *of L. monocytogenes* from mother to fetus, either by inhalation of infected amniotic fluid, transplacentally from the maternal circulation or by ascending colonization from the vagina resulting in illness at birth or shortly thereafter, usually occurring within the first week of life (Lamont et al., 2011). Neonatal listeriosis often presents with sepsis rather than meningitis. Signs and symptoms include respiratory distress, fever, and neurologic abnormalities. *Listeria monocytogenes* may be isolated from blood, cerebrospinal fluid, oropharyngeal secretions, placenta, amniotic fluid, urine, or external sites including conjuctiva, ear, nose, or throat.

The late-onset type may occur from one to several weeks after birth. Infants are usually born healthy and full term to mothers who have had uncomplicated pregnancies. Listeriosis presents as meningitis more frequently than in early-onset disease. Mortality rates for early- and late-onset disease are usually 20 to 60% (Lamont et al., 2011). The presumed source of infection in neonatal listeriosis is through transplacental transmission. Nonetheless, contaminated foods likely contribute to transmission through direct or indirect contact. Nosocomial transmission can also occurs (Schuchat et al., 1991).

2.5. Listeriosis in adults

In adults listeriosis represent a significant risk in some pathological or physiological conditions including malignancy, organ transplants, immunosuppressive therapy, infection with the human immunodeficiency virus (HIV), and advanced age. Sepsis

and central nervous system infections are the most frequently observed clinical presentations. Sepsis or bacteremia without a localized infection is most common in compromised hosts. The patient often appears severely ill with fever, nausea, vomiting and malaise. Sepsis may progress to disseminated intravascular coagulation, acute respiratory distress syndrome and multi-organ system failure. The clinical features of listerial sepsis are similar to other types of bacterial sepsis and its diagnosis is based on a positive blood culture (Lorber, 1996; Schlech, 2000). Clinical syndromes due to *L. monocytogenes* in central nervous system infections are meningitis, meningoencephalitis and abscess formation. *Listeria* is an important cause of bacterial meningitis in non-pregnant adults with a mortality rate of approximately 30% (Mylonakis et al., 1998). Clinical signs of listerial meningitis are usually characterized by high fever, nuchal rigidity, movement disorders such as tremor and/or ataxia, and seizures (Skopberg et al., 1992; Doganay, 2003). The most common non-meningitic form of central nervous system listeriosis is encephalitis, involving the brainstem, that is characterized by focal neurological findings in the hindbrain, including ataxia and multiple cranial nerve abnormalities, while fever may be absent. *L. monocytogenes* may also be present within brain abscesses in about 10% of cases when the central nervous system is involved. Abscesses are particularly likely to occur in the immunosuppressed population, and the subsequent mortality rate is quite high (Doganay, 2003; Painter and Slutsker, 2007). Listerial endocarditis has been found to preferentially infect left-sided valves and is often a source of systemic bacterial emboli. The mortality rate for listerial endocarditis is approximately 50% (Doganay, 2003).

Consumption of food contaminated with *L. monocytogenes* can result in febrile gastrointestinal disease presenting with nausea, vomiting and diarrhea. Subjects become within 24-48h of exposure to the contaminated food. The outbreaks have shown that rice salad, shrimp, chocolate milk, and corn salad are vehicles. This disease should be considered when stool cultures are negative in a patient with acute gastroenteritis. In a few instances, gastroenteritis leads to invasive listeriosis (Schlech, 2000). *L. monocytogenes* causes not only systemic disease, but also localized infections. Direct contact can provoke conjunctivitis, skin infection and

lymphadenitis. Listerial bacteremia can lead to the development of peritonitis, cholecystitis, hepatitis, pleuritis, splenic abscesses, pericarditis, osteomyelitis and endophthalmitis. This localized infections can be seen as the result of septic emboli with listerial endocarditis. Patients having localized listerial infection usually suffer from an underlying disease (Vázquez-Boland et al., 2001).

2.6. Trends and sources of listeriosis in European Union

EFSA is responsible for examining the data on zoonoses, antimicrobial resistance and food-borne outbreaks submitted by Member States in accordance with Directive 2003/99/EC. The most recent summary report on zoonosis events analyzing data from 2012 between the Member States.

In 2012, EFSA reported 1,642 confirmed human cases of listeriosis between 26 Member States, a 10.5 % increase compared with 2011. The incidence rate was 0.41 cases per 100,000 population with the highest notification rates observed in Finland, Spain and Denmark (1.13, 0.93 and 0.90 cases per 100,000 population, respectively). The vast majority of cases were reported to be domestically acquired. Statistically increasing trends were also observed in Finland, Germany, the Netherlands and Poland. No country-specific decreasing trends were observed (EFSA, 2014).

A total of 198 deaths due to listeriosis were reported by 18 Member States in 2012, which was the highest number of fatal cases reported since 2006. Fifteen Member States reported one or more fatal cases with France reporting the highest number, 63 cases. The EU case fatality rate was 17.8 % among the 1,112 confirmed cases. The most common serotypes in 2012 were 1/2a (46.8 %) and 4b (41.7 %), followed by 1/2b (8.5 %), 1/2c (2.7 %) and 3a (0.3 %).

Five strong-evidence food-borne outbreaks caused by *L. monocytogenes* were reported by three Member States; four were general and one was a household outbreak. The outbreaks resulted in 55 cases, 47 hospitalisations and nine deaths, i.e. 37.5 % of all deaths due to reported strong-evidence food-borne outbreaks in 2012. Mixed food (sandwiches), bakery products (pork pies), bovine meat and products thereof (pressed beef), cheese and other or mixed red meat and products

thereof (meat jelly), but not fresh-cut vegetables were the implicated foods (EFSA, 2014).

The highest notification rates of listeriosis were reported in persons aged below one and those aged 65 years and above. In the latter group, the rates increased by age. In those aged below one year, 79 % of the cases were reported as related to transmission during pregnancy. Major differences in notification rates were also observed in terms of gender. Female cases dominated in the age groups 15-24 and 25-44 years and 71.3 % of these cases were related to pregnancy. Higher incidence rates were observed in male cases compared to female cases in all age groups above 45 years. In these age groups, the male-to-female rate ratio increased by age and in the oldest age group, 85 years or above, the male-to-female rate ratio was 1.7 (EFSA, 2014).

Listeria monocytogenes prevalence at point retail and plant was analyzed in RTE foods in which the pathogen was detected either by qualitative (absence or presence, using detection methods) and/or by quantitative investigations (determination of *L. monocytogenes* counts using enumeration methods). *L. monocytogenes* was rarely detected above the legal safety limit from ready-to-eat foods at point of retail. In 2012, only 1 of the 2,285 units of RTE salads tested by enumeration method was found to contain *L. monocytogenes* at a level above 100 cfu/g. However, there were no findings of *L. monocytogenes* in the relatively few tested pre-cut vegetables and fruits.

2.7. Listeriosis outbreaks related to contaminated vegetables products

CDC (Centers for Disease Control and Prevention), estimates that approximately 1600 illnesses and 260 deaths due to listeriosis occur annually in the United States, with an average annual incidence of 0.29 cases per 100,000 population for 2009-2011 (CDC, 2013). In the USDA–FSIS/FDA risk assessment, vegetables were classified in the moderate risk category, due largely to the high number of annual servings and moderate frequency of contamination by *L. monocytogenes* (about 2 to 5% of samples) (Norton and Braden, 2007). Compared to other food categories, few invasive listeriosis outbreaks have been linked to consumption of contaminated

vegetables or fruit products. In table 2 some of the most important outbreaks linked to the consumption of fresh vegetables are summarized.

A large coleslaw-associated outbreak that occurred in the Canadian Maritime Provinces which resulting in 18 fatalities was among the first to provide convincing evidence for transmission of *L. monocytogenes* by food (Schlech et al., 1983). In addition outbreaks likely associated with unopened packages of raw vegetables occurred among hospitalized patients (Ho et al., 1986). Similarly, more recently sporadic cases of listeriosis in England and Wales were associated with the consumption of pre-packed mixed salad vegetables and mixed salads (Gillespie et al., 2010; Little et al., 2010). In the USA, cases of hospital-acquired listeriosis have been recently caused by contaminated diced celery (Gaul et al., 2008). However, the largest multistate listeriosis outbreak in U.S. history occurred in 2011, when 147 illnesses, 33 deaths, and 1 miscarriage occurred among residents of 28 states; the outbreak was associated with consumption of cantaloupe from a single farm and the company (Carol's Cuts LLC, Kansas, US) recalled 4,800 individual packages of fresh cut cantaloupe and cut mixed fruit containing cantaloupe from the market (CDC, 2012).

Table 2. Listeriosis outbreaks linked to consumption of fresh vegetables.

Country	**Year**	**Vehicle**	**Reference**
Canada	1979	Coleslaw	Schlech et al., 1983
USA	1981	Celery, lettuce	Ho et al., 1986
England Wales	2004-2007	Several foods including prepacked mix salads	Little et al., 2010
England	2005-2008	Several foods including mix salads	Gillespie et al., 2010
USA multistate	2008	Sprout	Cartwright et al., 2013
Texas	2010	Diced celery	Gaul et al., 2013
USA multistate	2011	Cantaloupe melons	CDC 2012
Midlands, England	2011	Prepacked sandwiches and salads	Coetzee et al., 2011

3. *L. MONOCYTOGENES* AND FRESH-CUT PRODUCTS: A FOOD SAFETY CONCERN

3.1. EU legislation and compliance with microbiological criteria

For a healthy human population, foods in which the levels do not exceed 100 cfu/g are considered to pose a negligible risk. Therefore, the EU microbiological criterion for *L. monocytogenes* is set as ≤100 cfu/g for RTE products on the market.

EU legislation (Regulation (EC) No 2073/2005) lays down food safety criteria for *L. monocytogenes* in RTE foods. This regulation came into force in January 2006.

Regulation (EC) No 2073/2005 covers primarily RTE food products, and requires the following:

- in RTE products intended for infants and for special medical purposes *L. monocytogenes* must not be present in 25 g;
- *L. monocytogenes* must not be present in levels exceeding 100 cfu/g during the shelf-life of other RTE products;
- in RTE foods that are able to support the growth of the bacterium, *L. monocytogenes* may not be present in 25 g at the time of leaving the production plant. However, if the producer can demonstrate that the product will not exceed the limit of 100 cfu/g throughout its shelf-life, this criterion does not apply;
- in the case of RTE foods that are able to support the growth of *L. monocytogenes*, the microbiological criterion to be applied depends on the stage in the food chain and whether the producer has demonstrated that *L. monocytogenes* will not multiply to levels exceeding 100 cfu/g throughout the shelf-life.

3.2. *L. monocytogenes* and fresh-cut vegetables and fruits

L. monocytogenes is associated with significant food safety control problems due to its wide distribution in nature and its capacity to survive and grow on the food products despite frequent exposure to harsh environmental conditions associated with food processing and preservation measures. In addition, *L. monocytogenes*

may persist in food processing industry equipment and premises due to its ability to form biofilms. These structures confer protection to bacterial cells and decrease the efficiency of cleaning and disinfection procedures. This evidence suggests that *L. monocytogenes* biofilms represent a threat to food safety, as bacteria can be transferred to food products when they come into contact with biofilms due to cross contamination of the pathogen during processing phases (Møretrø and Langsrud, 2004; Gandhi and Chikindas, 2007). For this reasons it appears critical to detect and remove *L. monocytogenes* biofilms in food processing environments in order to improve food safety.

3.3. Survival under adverse environmental conditions

L. monocytogenes is psychrotrophic, able to multiply down to a few degrees below 0 °C. It can grow between pH 4.6 and 9.5 and at a water activity as low as 0.92 (Buchanan et al., 2004). In minimally processed vegetables and fruits particularly insidious is the ability of *L. monocytogenes* to survive and grow over a wide range of temperatures (2–45 °C) (Gandhi and Chikindas, 2007). In agree, several studies reported an increase of *L. monocytogenes* concentrations on fresh-cut vegetables stored under different conditions (Sant'Ana et al., 2012a; Likotrafiti et al., 2013; Vandamm et al., 2013).

In vegetables fresh-cut, the acid stress challenges faced by *L. monocytogenes* cells include organic acids used as food preservatives and decontaminants. However, adaptation to acidic environments allow to this pathogen to contaminate unfriendly matrices such as fresh-cut fruits. Accordingly, growth of *L. monocytogenes* was reported on several minimally processed fruits characterized by a low pH such as apples (Alegre et al., 2010a), peaches (Alegre et al., 2010b), and oranges (Caggia et al., 2009).

Among others stress, the high level of sugars and sodium salts typically used as food preservers threaten bacterial cells with dehydration, which leads to increased intracellular solute concentrations and disruption of various biological functions. Furthermore, in food environments oxidative stress might develop due to atmospheric modification as well as by chemical reagents applied as detergents and

disinfectants. Reactive oxygen species are also produced as byproducts of metabolism and can accumulate due to respiratory chain impairment or metabolic alterations encountered in other stress situations. Finally, environmental *L. monocytogenes* strains inhabiting fresh-cut vegetables and processing plants may frequently encounter sub-lethal alkaline stress associated with detergents and sanitizing agents applied as food hygiene measures (Soni et al., 2011).

L. monocytogenes responds to and survives in the harsh food environments by utilizing a number of stress adaptation mechanisms involving complex regulatory systems under the control of the general stress sigma factor (σ^B). Exposure to stress conditions induces change in the membrane composition, induction of cold stress proteins and proteases, induction of the acid tolerance response, accumulation of compatible solutes such as glycine betaine as cryoprotectants or osmoprotectants (Gandhi and Chikindas, 2007; Soni et al., 2011). To cope against oxidative stress, molecular detoxification of reactive oxygen species, as well as protein, membrane, and nucleic acid damage repair mechanisms must be activated (Soni et al., 2011).

In addition, antibiotic resistance in the foodborne pathogen *Listeria* is emerging in recent years (Lungu et al., 2011) and the spread of resistance among foodborne microorganisms to sanitizers and disinfectants commonly used by the food industry are also becoming a concern (Gandhi and Chikindas, 2007). In a recent survey *L. monocytogenes* strains isolated from different RTE products, were submitted to susceptibility test, showing that 83.8% of the strains were susceptible to 15 antimicrobials; two were penicillin-resistant, and one was multidrug-resistant to kanamycin, tetracycline, sulfamethoxazole, rifampin, gentamycin, penicillin, and ampicillin (Chen et al., 2014). This results suggested that minimally processed foods may serve as potential vehicles for transmission of virulent *L. monocytogenes*.

3.4. Biofilm formation

Biofilms are defined as communities of microorganisms that attach to surfaces and are a prevalent mode of growth for microorganisms in nature. Currently, it is widely accepted that biofilms consist in microbial sessile communities characterized by

cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances, mainly polysaccharides, and with an altered phenotype (particularly, growth rate and gene transcription) compared to planktonic cells (Donlan and Costerton, 2002). Biofilm formation can be divided into key steps in which reversible attachment of freely moving bacteria to a surface is followed by irreversible binding to the surface, growth of microcolonies and production of a polymer matrix. The next step is the maturation of the biofilm in a three- dimensional structure often showing water filled channels and tower-like or mushroom-like structures. Finally, some bacteria detach from the biofilm and are dispersed in the environment, thus allowing colonization of other surfaces (reviewed by Srey et al., 2013). It has been suggested that biofilm may represent the default mode of growth for several bacterial species in natural environments as they provide defense from chemical and physical stresses and represent a mechanism to stably colonize a favorable niche, exploiting the benefits of gene transfer and division of metabolic burden (Jefferson, 2004). Cells growing in biofilms show several differences compared to planktonic cell. Biofilms generally show slower growth rate, variation in the transcriptome (Donlan and Costerton, 2002), increased conjugation rate (Hausner and Wuertz, 1999) and, most importantly, an enhanced resistance to biocides and antibiotics (Robbins et al., 2005).

Biofilms can occur on a wide variety of surfaces, including industrial equipment and food processing surfaces, such as floor drains, conveyer belts, rubber, plastic, glass and stainless steel equipment and other food-contact materials (Di Bonaventura et al., 2008; Shi and Zhu, 2009; Van Houdt and Michiels, 2010). Furthermore, it must be considered that some factors typically occurring in the food processing such as moisture loss, deposits of soil or debris on the surfaces, could assist the biofilm formation by microorganisms proceedings from both raw materials and *in situ* contaminations.

Undoubtedly, the risk assessment and reduction related to the occurrence of *L. monocytogenes* biofilms may constitute an important expense for the enterprises. Therefore, an optimized management of the food-processing plant should consider

the prevention strategies as the most effective approach. In particular, the proper design of food contact equipment is often the most recommended policy to counteract the biofilm formation (Botticella et al., 2013). Materials for industrial installations should always be preferred with smooth rather than irregular surfaces. Indeed, roughened materials expose a greater surface area to the microorganisms, being the depressions the more favorable sites for colonization (Chaturongkasumrit et al., 2011; Myszka and Czaczyk, 2011; Srey et al., 2013). For the same reason, food contact materials should be resistant to corrosion and damage in order to avoid cracks or scratches. Moreover, the installations should be free of sharp edges and overall its surfaces always easily cleanable, allowing the "clean in place", a system including jetting and spraying of the surfaces with an increased turbulence and flow velocity without the manual involvement of the operator (Srey et al., 2013). A good cleaning process should remove any food residues and other compounds that may promote bacteria proliferation and biofilm formation (Simões et al., 2010). In addition, the cleaning flush should be carried out in a way that can break-up or dissolve the exopolysaccharide matrix associated with the biofilms so that disinfectants can gain direct access to the bacteria cells (Srey et al., 2013). Gião and Keevil, (2012) demonstrated that water flow does not have the same efficiency in removing cells from different material surfaces, emphasizing the need to optimize cleaning and sampling procedures by considering the conditions in which cells attach to surfaces and the physico-chemical characteristics of the surfaces.

It is well known that biofilm formation is strongly affected by the hydrophobic or hydrophilic interactions between microbial cell charge and contact surfaces so that great care should be taken in the choice of materials for installations. Materials most commonly used in the food industry can be hydrophilic, such as stainless steel and glass, or hydrophobic, for example polymeric materials (Blackman and Frank, 1996; Chmielewski and Frank, 2003). Several authors are presently investigating relationships between biofilms and synthetic surfaces in order to provide important insights that could lead to new strategies to remediate and avoid listerial biofilm formation in the food industry. However, probably depending on the several factors involved in biofilm formation and different methods applied, their

conclusion are often divergent. Thus, Rodríguez and co-authors (2008), suggested that *Listeria* biofilms may adhere more tightly to hydrophobic than hydrophilic surfaces when measured at a cellular level. In contrast, Di Bonaventura et al., (2008), found that biofilm levels of *L. monocytogenes* were significantly higher on glass at 4, 12 and 22 °C, compared to polystyrene and stainless steel, while at 37 °C, the same strain produced biofilm at significantly higher levels on glass and stainless steel, compared to polystyrene. The same authors reported a positive correlation between hydrophobicity and heat suggesting that biofilm formation is significantly influenced by temperature, probably due to modification of the cell surface hydrophobicity (Di Bonaventura et al., 2008).

The most frequently employed chemical agents for sanitation procedures are strong oxidizing agents with a broad antimicrobial spectrum: hypochlorous acid, chlorine, iodine, ozone, hydrogen peroxide, peroxyacetic acid, quaternary ammonium chloride and anionic acids (Grinstead, 2009; Van Houdt and Michiels, 2010; da Silva and De Martinis, 2013). The effectiveness of these methods on *L. monocytogenes* biofilm eradication was studied and compared at different experimental conditions in order to establish the best treatment for specific claims (Ölmez and Temur, 2010; Charalambia-Eirini et al., 2011; Vaid et al., 2012). In alternative to conventional chemical-based strategies, physical methods such as irradiation or ultrasound have also been reported as effective techniques against both biofilm and planktonic cells (Van Houdt and Michiels, 2010).

However, in the last years, the increasing consumers attitude to avoid chemicals and to prefer environment friendly treatments, is addressing new green emerging strategy. Recently, rhamnolipid and surfactin, biosurfactants, surface-active compounds from microbial origin were investigated to prevent the adhesion of *L. monocytogenes* on different pre-conditioned surfaces such as stainless steel, polypropylene, polystyrene (Araújo et al., 2011). In another study, Zezzi do Valle Gomes and Nitschke, (2012) demonstrated that the pre-conditioning with the biosurfactants rhamnolipid and surfactin can delay the adhesion of food pathogenic bacteria even reducing the hydrophobicity of a polystyrene surface. The same biosurfactants also showed an interesting potential as agents to disrupt pre-formed

biofilms of *L. monocytogenes,* being surfactin more efficient than rhamnolipids. With a similar approach, Borges and coauthors (2013), demonstrated that isothiocyanates are able to inhibit planktonic bacterial growth, cell motility and to change cell surface properties, also affecting the biofilm formation from *L. monocytogenes*. Orgaz et al. (2011), have recently investigated the effect of chitosan on already established biofilms suggesting its usage in solution or as surface coating.

Another emerging approach is the utilization of essential oils as anti-biofilms agents. In another section of this work it is reported on their antimicrobial activity and potential applications in foods. The effect of various essential oils and their individual constituents on biofilms formed by *L. monocytogenes* has been investigated and several authors suggest them to be good candidates for further development of eco-friendly disinfectants (Sandasi et al., 2008; De Oliveira et al., 2010a; Leonard et al., 2010; De Oliveira et al., 2012; Jadhav et al., 2013).

In the field of the green-based biofilm control strategies a valuable alternative was recently proposed by Woo and Ahn (2013) by using a probiotic approach. This authors found that co-culture with *Lactobacillus paracasei* and *Lactobacillus rhamnosus* effectively inhibited by more than 3 log the biofilm formation of *L. monocytogenes* through mechanisms of competition, exclusion and displacement, suggesting that probiotic strains can be used as alternative way to successfully reduce the biofilm formation.

In addition, some authors have suggested that antimicrobial peptides or bacteriocin-producing strains may also contribute to control the initial adhesion and biofilm formation by *L. monocytogenes* on abiotic surfaces (Guerrieri et al., 2009; Winkelströter et al., 2011). The application of enterocin AS-48 produced by *Enterococcus faecalis* and *Enterococcus faecium* strains to polystyrene microtiter plates synergistically improved the bactericidal effects of biocides against planktonic and sessile *L. monocytogenes*, avoiding the biofilm formation for at least 24 h at a bacteriocin concentration of 25 mg/ml (Caballero Gómez et al., 2012).

4. DETECTION AND CONTROL OF *LISTERIA MONOCYTOGENES* IN FRESH CUT VEGETABLES

4.1. Culture methods

Historically, a cold enrichment at refrigeration temperatures was the first successful attempt to overcome the difficulty of identifying and isolating *L. monocytogenes* from contaminated matrices (Gray et al., 1948), by exploiting its psychrotrophic nature and simultaneously suppressing growth of indigenous non-psychrotrophic microorganisms. However, this technique would often require from several months to several weeks for completion. Therefore, although incubation at 4 °C is partially selective for growth of *L. monocytogenes*, several investigators began adding selective agents to various non-selective enrichment broths in an effort to inhibit non-*Listeria* microbes. Enrichment at elevated temperatures is usually performed at 30 or 37 °C, although it was observed that *Listeria* spp. were more susceptible to commonly used *Listeria* selective agents (i.e., ceftazidime, fosfomycin, potassium tellurite, lithium chloride, nalidixic acid, acriflavine, potassium thiocyanate, thallous acetate and polymyxin B) when incubated at 37 rather than 30 °C.

Based on the resistance of *L. monocytogenes* to various selective agents, including chemicals, antimicrobials, and dyes various selective enrichment media have been optimized. However, the addition of selective agents may partially or completely inhibit growth of this pathogen, particularly when the organism is sub-lethally injured. For this reason, oft enrichment approaches have been developed that include primary or secondary selective enrichments.

Fraser broth is an advantageous medium for detecting *Listeria* spp. in enriched food and environmental samples which contains lithium chloride (3.0 g/L) and ferric ammonium citrate (0.5 g/L). The occurrence of presumptive *Listeria* spp. is easily detectable by the turning to black from esculin hydrolysis, within 48 h of incubation. Half Fraser broth is recommended for the primary enrichment.

Another selective media currently recommended by the USDA–FSIS is the University of Vermont Medium (UVM) broth that contain 20 mg/L nalidixic acid.

Other frequently recommended (FDA, ISO and USDA) media for the isolation of *Listeria* spp. are PALCAM agar (Polymyxin, Acriflavine, Lithium Chlorid, Ceftazidime, Aesculin, Mannitol) and Oxford Agar (OXA). It is recommended that PALCAM agar plates be incubated for 40 to 48 h at 30 °C under microaerobic conditions (5% oxygen, 7.5% carbon dioxide, 7.5% hydrogen, and 80% nitrogen). This medium is the basis for the Netherlands Government Food Inspection Service (NGFIS) method for Listeria detection and isolation. Oxford Agar (OXA) contains several selective agents including colistin sulfate (20 mg/L), fosfomycin (10 mg/L), cefotetan (2 mg/L), cycloheximide (400 mg/L), lithium chloride (15 g/L), and acriflavine (5 mg/L). A modified formulation by incorporating moxalactam is known as Modified Oxford Agar (MOX). However, PALCAM and OXFORD do not distinguish between pathogenic and non-pathogenic Listeria spp..

Chromogenic media are based on the detection of essential determinants of pathogenicity of *Listeria* spp.. The recovery of pathogenic strains with Listeria agar according to Ottaviani and Agosti (ALOA) detects the phosphatidylinositol-specific phospholipase C (PI-PLC), encoded by the virulence gene *plcA*, specifically for *L. monocytogenes* and *L. ivanovii* forming an opaque halo around the colonies in parallel with ß-glucosidase activity. Since the halo size increases rapidly so that negative colonies cannot be distinguished from positives, it is important to check ALOA plates after 24 h of incubation.

Similar commercialized media are BCM® Listeria (Biosynth, Switzerland), OCLA® (Oxoid, UK), CHROMagar® (BD e Diagnostic Systems, USA) and OAA® (bioMérieux, France). Rapid L'mono® (Bio-Rad Laboratories, USA), based on xylose fermentation by *L. ivanovii* (yellow halo), distinguishes non haemolytic from haemolytic *Listeria* through cleavage of another substrate for PI-PLC, 5-bromo-4-chloro-3-indolyl-myo-inositol-1-phosphat (X-IP), which results in blue colonies (Zunabovic et al., 2011).

4.2. Reccomended method for the detection of *L. monocytogenes* in food

The most usually recommended methods to detect *L. monocytogenes* in foods are those developed by the US Department of Agriculture–Food Safety and Inspection

Service (USDA–FSIS), the Federal Drug Administration (FDA), the Netherlands Government Food Inspection Service (NGFIS) and the ISO 11290:1 (anonymus, 1996). A schematic representation of the all methods is reported in figure 1. Basically, testing consists of three steps: enrichment, isolation, and confirmation. After enrichment in selective media, the culture is tested on different selective media to determine if the physiology is typical of *Listeria* spp.. If these criteria are satisfied, the culture is then tested for its biochemical aptitudes, mainly negative mannitol degradation and the ability to induce haemolysis on blood agar. Positive cultures must be further tested for negative xylose and positive rhamnose utilization before concluding that the sample is *L. monocytogenes* rather than one of the other *Listeria* species (Churchill et al., 2006).

The FDA method, originally developed by Lovett and Hitchins, (1989), is the most frequently used procedure in the United States for detecting and enumerating *L. monocytogenes* in food products such as seafood, dairy, fruit and vegetables. The standard FDA methodology include an enrichment step for 48 h at 30 °C in Listeria Enrichment broth (LEB, FDA BAM formulation) containing the selective agents acriflavin, naladixic acid and the antifungal agent cycloheximide. A previous step of 4 h at 30 °C without selective agents is recommended to recover injured cells. Enriched cultures are then plated onto selective agar (Oxford, PALCAM, MOX) (Fig. 1A).

The USDA-FSIS protocol is an alternative methods widely used to detect *L. monocytogenes* in meat and poultry. This two-stage enrichment method uses a modification of the UVM containing acriflavin and naladixic acid for primary enrichment, followed by secondary enrichment in Fraser broth and plating onto Modified Oxford (MOX) agar containing the selective agents moxalactam and colistin sulphate (Fig.1 B).

The ISO 11290:1 1996 is the standard European method for the detection of *L. monocytogenes* in food and environment samples. This protocol is based on two-stage enrichment process. The food sample is first enriched in half Fraser broth for 24 h, containing only half the concentration of selective agents, to encourage the growth of damaged cells. Then, an aliquot is transferred to full strength Fraser broth

for further enrichment (48 h at 37 °C). Fraser broth also contains the selective agents acriflavin and naladixic acid as well as esculin, which allows detection of β-D-D-glucosidase activity by Listeria, causing a blackening of the medium. Both the primary and secondary enriched broth are plated on Oxford and PALCAM agars or Agar Listeria Ottavani and Agosti (ALOA) that allow *L. monocytogenes* to be distinguished from other *Listeria* spp. (Fig. 1C).

A detection method widely used in Europe is the Netherlands Government Food Inspection Service (NGFIS), a method developed by Netten et al., (1989). This include growth of *Listeria* in a nutrient broth for 48 h followed by plating on PALCAM agar (Fig. 1D).

Fig. 1A

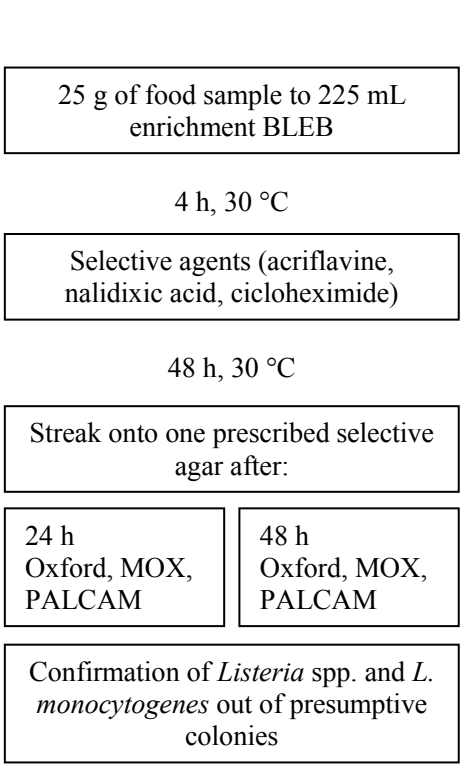


Fig. 1B

USDA-FSIS

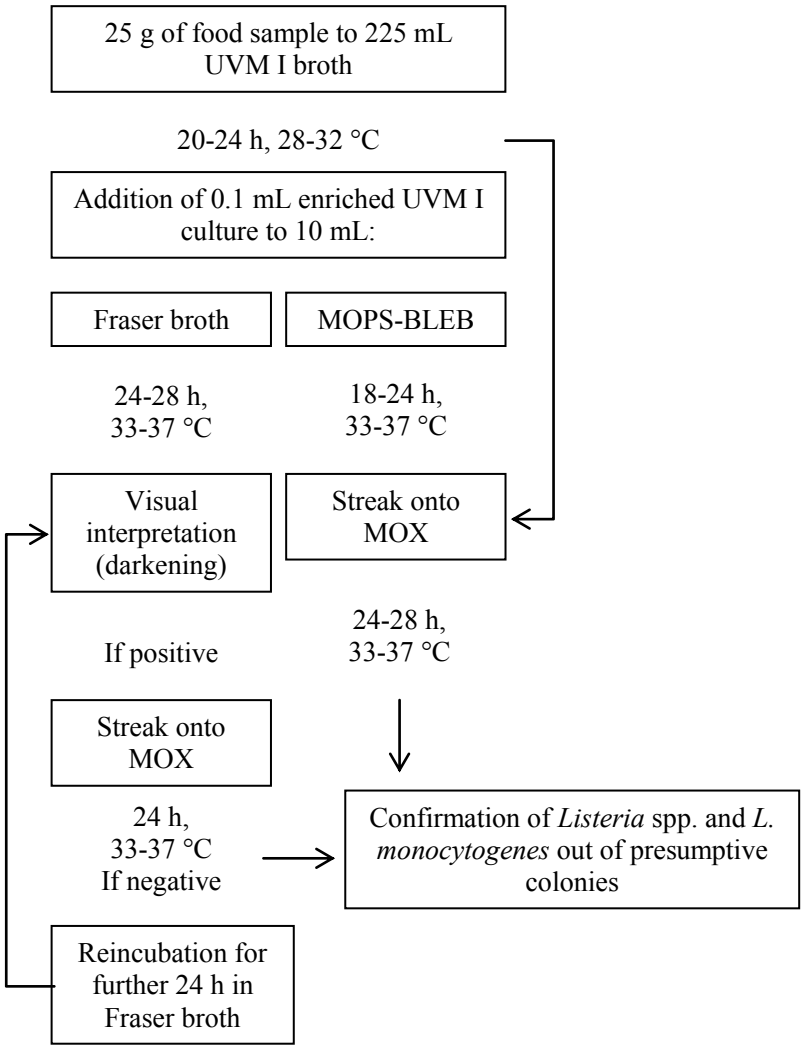


Fig. 1C

ISO 11290-1:1996

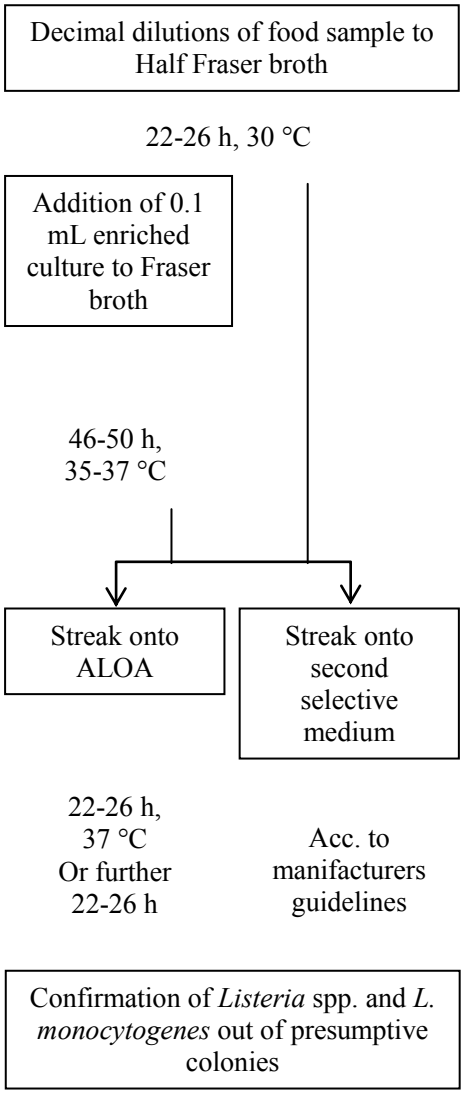


Fig. 1D

NGFIS

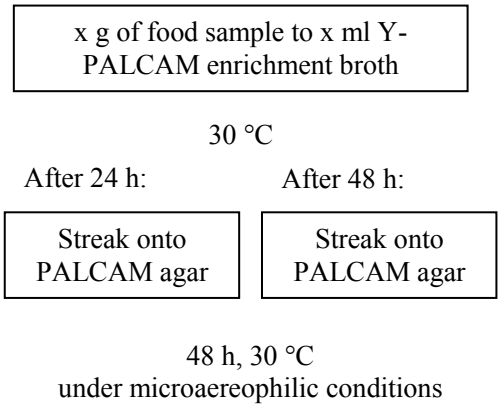


Figure 1. Survey on official methods for the detection of *Listeria monocytogenes* adapted by Zunabovic et al., (2011).

4.3. Enumeration of *L. monocytogenes* by the Most Probable Number (MPN)

MPN is a procedure to estimate the population density of viable microorganisms in sample. MPN values are particularly useful when low concentrations of organisms (<100/g) are encountered and therefore used to determine the estimated number of pathogens in food samples. MPN is based upon the application of the theory of probability to the numbers of observed positive growth responses to a standard dilution series of sample inoculum placed into a set number of culture media tubes. However, while plate counts are direct counts of living organisms, the MPN values are only estimates. Therefore, the procedure does not lead to a single maximum likelihood estimate but, rather, to an interval estimate. Positive growth response after incubation may be indicated by such observations as gas production in fermentation tubes or visible turbidity in broth tubes, depending upon the type of media employed. For example, *L. monocytogenes* positive tubes are indicated by the turning of culture medium to black due to aesculin hydrolysis. Then, sample should be diluted in such a manner that higher dilutions of the sample will result in

fewer positive culture tubes in the series. The number of sample dilutions to be prepared is generally based on the expected population contained within the sample. If particularly high microbial populations are expected, the sample must be diluted to a range where the MPN can be obtained. Most reliable results occur when all tubes at the lower dilution are positive and all tubes at the higher dilution are negative (USDA, 2008). Although this method is very useful, it is laborious and requires large quantities of enrichment tubes, and multiple agar media be streaked for isolation for each tube.

4.4. Immunoassays-based techniques

Immunoassays methods are based on the natural binding affinity of antibodies to antigens and they are considered attractive approaches to detect *L. monocytogenes* in food, because fast and low cost.

ELISA (enzyme-linked immunosorbent assays) allow to immobilize anti-Listeria antibodies to a microtitre well for antigen capture in combination with a secondary antibodies linked to an enzyme to detect the sequestered antigen. Species-specific immunoanalysis detection of *L. monocytogenes* is based on structural components, such as flagella, the listeriolysin O (LLO) toxin, the protein p60 (encoded by the *iap* gene).

Since ELISA combine easy and fast results is wide used for *Listeria* detection in the food industry (Ky et al., 2004; Kim et al., 2007). Indeed, several kits allowing a result within 30 h of sample receipt are commercially available. Recently, an indirect ELISA assay was developed to detect the presence of *Listeria monocytogenes* and *Escherichia coli* O157 in fresh-cut vegetables with a detection limit lower than 10^3 CFU g^{-1} and requiring a time from 1 to 7 hours (Cavaiuolo et al., 2013). Nano-encapsulation of labelled antibody was realized to improve the detection limit and accelerate the assay performance a conventional ELISA (Oaew et al., 2012). Phage display biopanning is a technique that has been used in an attempt to generate alternative binders to *L. monocytogenes* (Paoli et al. 2007; and Nanduri et al. 2007). Carnazza et al. (2007) described the production of phage display-derived peptide binders to *L. monocytogenes* but no data are presented on

cross-reactivity of their binders with other *Listeria* spp. or relevant foodborne bacteria to support the specificity claim. Recently, Morton et al., (2013) improve this approach in order to produce *L. monocytogenes*-specific binders with the ability to distinguish pathogenic *L. monocytogenes* from other *Listeria* species.

However, despite advances in the field, immuno-based methods also have some drawbacks when compared to the detection by molecular-based approaches. The main weaknesses concern both specificity and sensitivity. For example, cross reactivity can occur in samples contaminated with other closely-related species reducing the specificity of the method. Gasanov et al. (2005) reported that culture based methods have a sensitivity of 10^4 cells/mL while immunological assays have a sensitivity of 10^5 cells/mL for an enriched sample. Also, the reaction sensitivity can range depending from the antigenic expression under different cell environments (Brehm-Stecher and Johnson, 2007). In addition, sensitivity and specificity have to be tested extensively during immune assays because antibodies produced from different animals can differ in their efficiency. Therefore, antibody preparation is very time-consuming. Furthermore, there can also be a loss of target molecule during processing due to the reduced half-life of the proteins or because their concentration can vary under different environmental conditions.

4.5. Immunomagnetic separation

Among immune assays methods, immunomagnetic separation (IMS) use paramagnetic beads coated with specific antibodies to extract and concentrate the target organism from the samples, thus reducing the time required for enrichment and improving the sensitivity of subsequent assays (Paoli et al., 2007). Therefore, in the last years immunomagnetic separation is an attractive approach to capture cells that may be presumptively identified by plating them on selective or differential media (Wadud et al., 2010), or their identity may be confirmed by PCR (Yang et al., 2007; Yamg et al., 2013) or detected by using a biosensor (Mendonça et al., 2012).

4.6. Polymerase Chain Reaction (PCR)

In the last years, a considerable number of detection methods using faster molecular tools have been developed. These techniques involve the extraction of microbial DNA from food matrices as target for the selective amplification reaction, called Polymerase Chain Reaction (PCR), of a fragment of DNA specific for the microbial species. Conventional PCR is also known as end point PCR because the amplification product is observed in an agarose gel at the end of the reaction.

The PCR method circumvents the problem that some cells do not grow in the selective media. Some authors found that PCR detection of *L. monocytogenes* is more sensitive than culture-based methods for detecting the pathogen in contaminated food samples (Aznar and Alarcón, 2002; Shearer et al., 2001). The authors also claim that PCR method avoids the problem that some cells do not grow in the selective media. In addition, some of the false–negatives reported for culturing methods are due to colorimetric changes to differentiate between species, and these methods are influenced by the subjectivity of the observer (Aznar and Alarcón, 2002; Shearer et al., 2001).

Nowadays, advances in sequencing provide a greater availability of known LAB genomes allowing an increasing availability of target sequences to discriminate *Listeria monocytogenes* and *Listeria* spp.. The most reported primers are designed targeting genes encoding the 16S rRNA or virulence factors.

Aznar and Alarcon, (2002) reported the most common target genes to detect *L. monocytogenes* by PCR, including *hly* (the mainly used), *inlA*, *inlB*, *iap*, intergenic spacer regions, 16S and 23S rRNA, genes encoding invasion-associated protein p60, aminopeptidase C, phospholipase C protein, a fibronectin-binding protein and dth-18 delayed-type hypersensitivity protein. Rossmanith and coauthors, (2006) detect *L. monocytogenes* in artificially and naturally contaminated food samples by using as target the *prfA* gene.

A further survey of the target genes used in the last years to identify *L. monocytogenes* was summarized by Liu (2006).

4.7. Quantitative Real Time PCR (qPCR)

In contrast to PCR, real-time PCR, is a molecular-based method that allow to monitor the amplicon as it accumulates (in real time). This is possible by monitoring the change in fluorescence signal which in turn in direct proportion on the amount of the generated PCR product. For this reason, real time PCR is also known as quantitative real time PCR (qPCR). The progress of a real-time PCR is monitored by an instrument that combines thermal cycling with fluorescent dye scanning capability. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction.

The main advantages of qPCR include:

- ability to monitor the progress of the PCR reaction as it occurs in real time;
- ability to precisely measure the amount of amplicon at each cycle, which allows highly accurate quantification of the amount of starting material in samples;
- an increased dynamic range of detection covering 6 to 8 orders of magnitude.;
- amplification and detection occurs in a single tube, eliminating post-PCR manipulations;
- possible automation.

Fluorescent reporters used in real-time PCR can be either a non-specific double-stranded DNA (dsDNA)-binding dyes (SYBR Green), or dye molecules attached to PCR primers or probes that hybridize with PCR product during amplification.

SYBR Green is an asymmetrical cyanine dye binding the DNA, resulting in a DNA-dye-complex that absorbs blue light (λmax = 497 nm) and emits green light (λmax = 520 nm).

The key advantages of this technique include the easiness to design specific primers for the target sequence of interest and the high sensitivity, because SYBR Green is able to produce more than 1 reporter per amplicon. Furthermore, it is an inexpensive method with respect to the TaqMan detection because dye-labeled oligonucleotides are not required. Finally, a melt curve assess the specificity of

PCR. However, SYBR green detects all double-stranded DNA including primer-dimer products that can influence the fluorescence ability. In addition, SYBR Green not allow to perform multiplex qPCR assays.

The most widely used probes are the TaqMan probes (also known as hydrolysis probes) which depend upon the 5'→3' exonuclease activity of Taq Polymerase. In this technology, as long as the reporter and quencher dye are on the same oligonucleotide (an oligonucleotide other than the primer pair) the fluorescence is quenched; the probe then anneals to the DNA strand close to one of the primers. During extension of the primer, the polymerase cleaves the reporter dye using its exonuclease activity. Once released the reporter dye is no longer quenched and fluorescence can thus be measured.

The main advantage of the TaqMan probes is the very high specificity, because the method combines either the specificity of primers and TaqMan probes, resulting in the absence of aspecific PCR products. Since it is possible use differently labeled probes, for example FAM-labeled probe for nuclear target sequence and VIC-labeled probe for mitochondrial target, multiplex qPCR can be assayed to simultaneously amplify and detect different target sequences in the same reaction tube. Nonetheless, some disadvantages of TaqMan probes relative to SYBR Green are mainly related to the difficult to design oligonucleotides (primers and probes) for efficient amplification and efficient probe hydrolysis . In addition, TaqMan probes are more expensive due to the cost of dual-labeled oligonucleotides.

Recently, a combination of four qualitative SYBR®Green qPCR screening assays targeting the *iap*, *prs* and *hlyA* genes was developed to discriminate *Listeria* genus (except *Listeria grayi*) and *Listeria monocytogenes*, that allow to use the same polymerase chain reaction programme (Barbau-Piednoir et al., 2012). Q-PCR with high-resolution melting curve analysis was developed and assessed for rapid identification of six *Listeria* species by using as target the *ssrA* gene (Jin et al., 2012). The *iap* gene was used as a target for amplification of a 175-bp (*L. monocytogenes*) and a 309-bp (*L. innocua*) fragment (Hein et al., 2001). Rodríguez-Lazaro et al. (2005) developed a quantitative PCR (qPCR) using the *hly* gene as

target and showed 100% sensitivity in detecting *L. monocytogenes* from non-*Listeria* and other *Listeria* species.

4.8. Multiplex PCR

Multiplex PCR is a variation of the traditional PCR that use multiple sets of primers to amplify a number of genes or gene fragments simultaneously. Thus, it is important that the multiplex PCR assay developed detects all the species efficiently with significant sensitivity. The sensitivity should be such that all the pathogens are detected in the standard limits of detection for approved methods of isolation. Multiplex PCR reactions allow to detect different *Listeria* species in one single run, by using primers binding highly conserved *Listeria* 23S rRNA and *hlyA* (Churchill et al., 2006). Multiplex PCR targeting *inlA*, *inlC* together with *intJ* improved species-specific detection facilitating the simultaneous confirmation of *L. monocytogenes* and other *Listeria* species (Liu et al., 2007). Nabi and coauthors (2011) are able to discriminate *L. monocytogenes* from other *Listeria* spp. in a screening a total of 48 samples of ready to-eat salads. Recently a multiplex PCR method was optimized for rapid and simultaneous detection of six *Listeria* species including *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri* and *L. welshimeri* to identify specific *Listeria* species in processed foods (Ryu et al., 2013). A list of primers for multiplex PCR detection was summarized by Settani and Corsetti (2007).

4.9. Genetic subtyping

Several molecular-based methods are used for further verification after a food sample is suspected of harbouring a pathogen. These methods include RFLP (Restriction Fragment Length Polymorphism), RAPD (Randomly Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), ribotyping and PFGE (Pulsed-Field Gel Electrophoresis) MLST (Multi Locus Sequence Typing) MVLST (Multi Virulence Locus Sequence Typing). Subtyping approach provide important information from epidemiological and source-tracking points of view and it is needed to identify the precise strains responsible for causing

disease, or that contaminate a certain type of food (for a comprehensive review see Churchill et al., 2006; Liu, 2006; Zunabovic et al., 2012).

4.10. Drawbacks of PCR-based methods

The main drawbacks of the molecular approaches concern their possibilities to produce both positive and false-negative results. False-positive results are mainly related to the failure to distinguish between DNA from dead or live cells (reviewed by Elizaquível et al., 2014). This is particularly significant for processed food or foods subjected to long-time storage due to the relatively long persistence of DNA after cell death. In the past, live cells were detected by amplification of mRNA, which is a direct indicator of active bacterial metabolism. However, this approach often give false-negative results due to RNA instability or degradation during sample handling. In the last years, the detection of viable cells was introduced consisting in coupling PCR or qPCR with nucleic acid intercalating dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA) (Nocker et al., 2006; Justé et al., 2008, Elizaquível et al., 2012). Live cells with intact membranes are distinguished by their ability to exclude DNA-binding dyes that easily penetrate dead or membrane-compromised cells. Cells exposed to EMA or PMA for 5 min allow the dye to penetrate dead cells with compromised cell walls/membranes and to covalent bind to their DNA, resulting in the inhibition of PCR amplification of DNA from dead cells (Nocker et al., 2006).

Foodborne pathogens are usually present at very low levels and have to be concentrated or enriched to enhance their detection (Sánchez et al., 2012). Therefore, false-negative results can occur due to the difficulty to extract low concentration of the target microbial DNA from food samples. In addition, foods contain a number of components that can interfere with the PCR reaction including proteins, fats, polyphenolic compounds, polysaccharides, humic acids. Additionally, certain components of selective media used to enrich for *Listeria* may also have inhibitory activity, including acriflavin, bile salts, esculin, and ferric ammonium citrate (Pinto et al., 2007). The inhibitor compounds can interfere with the reaction at several levels, for example target or primer-degrading nucleases, and competitors

of Mg^{2+}, leading to decreasing and even to complete inhibe the DNA polymerase activity. PCR inhibitors could be not completely removed during classical extraction protocols remaining as contaminants in the final DNA preparations. Therefore, DNA extraction has been highlighted as a limitation of culture-independent methods (Cankar et al., 2006). However, several protocol and commercial DNA extraction kit have been optimized depending from the food matrix (Amagliani et al., 2007; Pinto et al., 2007; Elizaquível et al., 2008; Jara et al., 2008).

With the aim to verify if a negative PCR result is due to the occurrence of inhibitors, an Internal Amplification Control (IAC) DNA fragment can be added to the DNA extracted from the sample. Thus, if no amplification is observed for the IAC, it is logical to assume the presence of PCR inhibitors. In contrast, if the IAC is amplified but a negative result for the target fragment is obtained, it is evident that the sample did not contain the target DNA (Rodriguez- Lazaro et al., 2005). In addition, an enrichment step would dilute dead cells and allow the living cells to grow and increase their relative population with respect to dead cells, improving detection rates and avoiding false positives due to the presence of dead cells, and false negatives due to the low concentration in the initial sample (Elizaquível et al., 2011).

4.11. Detection and quantification of *L. monocytogenes* in fresh-cut products

The combination of microbiological enrichment and molecular pathogen detection is the most promising solution to add up the advantages of both methods. So the detection limit of molecular approaches such as qPCR is far exceeded after successful enrichment. Short enrichments if performed in an appropriate way are mostly sufficient to provide enough DNA for detection, and the confirmation of the classification of the pathogen is made during the detection step. The major disadvantage is the missing quantification because of the enrichment prior to qPCR. Therefore, these methods can provide only qualitative results (Rossmanith and Wagner, 2010). Thus, the most recent advances in the field are focused on the reduction of the limit of detection end the corresponding time for the analysis. The

performances of these methods were, in some cases, successfully compared with the official protocol or with diagnostic commercial kits. However, although quantitative PCR methods are increasingly being used to detect bacterial pathogens in food, detection limits rarely exceed 10^2-10^3 CFU/g (Elizaquível and Aznar, 2008). As previously reported, lower detection values below 10 CFU/ml, are only achieved after an enrichment step (Elizaquível and Aznar, 2008).

In a recent study, real-time PCR assay applied to natural and artificially contaminated culture enriched foods including vegetables enabled detection of 1–5 CFU *L. monocytogenes* per 25 g/ml of food sample in 30 h. This assay was also compared to the commercial Roche Diagnostics 'LightCycler foodproof *Listeria monocytogenes* Detection Kit'and the results were found to be in congruence for both the methods (O' Grady et al., 2008). Similarly, a two-step enrichment involving a 24-h incubation in half-Fraser broth followed by a 6-h subculture in Fraser broth was used, followed by cell lysis and real-time PCR by using a TaqMan probe always confirmed the results obtained by the standard procedure EN ISO 11290-1 (Oravcová et al., 2007). Kotzekidou (2013) showed that a real-time PCR methods using commercial kits for pathogen detection (*L. monocytogenes*, Salmonella spp. and *E. coli* O157:H7) in raw food and ready-to-eat products have the potential to be of benefit in routine food testing as they are easy to use. However, 52 h for *L. monocytogenes* are needed to perform the complete according to the manufacturer's instructions.

Recently, Garrido et al., (20013 a; b) suggest the use of one single enrichment broth during 24 h for the simultaneous and reliable screening of Salmonella spp. and *L. monocytogenes* in food and environmental samples. In a study performed by Bhagwat (2003), a modified Association of Official Analytical Chemists (AOAC)-approved PCR method based on a two-steps of enrichment was used for the real-time detection of three pathogens simultaneously from artificially inoculated fresh-cut vegetables. This method allows to detect 1–10 cells/ml of *E. coli* O157 and Salmonella spp.. However, the sensitivity was much less for *L. monocytogenes* at 100–1000 cells/ml because hindered by interference from vegetable compounds. With another approach, Wadud et al., (2011) evaluate the performance of the

ALOA chromogenic media in combination with immunomagnetic separation for the detection of *Listeria monocytogenes* in a variety of ready-to-eat food products including vegetables. IMS–ALOA method was found to be equivalent to Health Canada's reference culture method as well as comparable to BAX-PCR method in terms of the sensitivity. The method was able to detect *L. monocytogenes* at levels near or below 1 cfu/25 g regulatory limit in ready-to-eat food matrices after 24 h enrichment, with a turnaround time of 3 days compared to 7–8 days for culture method.

Multi-pathogen real-time assays can prove to be very efficient, time saving and cost-effective. Elizaquível and coauthors (2011) revealed the presence of three pathogens mostly at levels between 10^2 and 10^4 cells/g. Besides quantification, the qPCR results (presence/absence) were compared with those of the standard mini-VIDAS system. The authors found that qPCR outperformed the mini-VIDAS procedures, in terms of both time and accuracy, suggesting qPCR to be useful as a rapid diagnostic test for the direct detection of pathogens in food, without the need for enrichment steps. Similarly, Sánchez et al., (2012) were able to recover (by precipitation with polyethylene glycol), detect and quantify enteric viruses and foodborne pathogenic bacteria including *L. monocytogenes* within 5 h, for the detection of both types of foodborne pathogens in fresh-cut vegetables. In an attempt to reduce time and cost of the analysis, multiplex PCR was used for the simultaneous detection of different foodborne pathogens. For instance, Bhagwat (2003) used multiplex PCR to simultaneously detect *L. monocytogenes*, *S. typhimurium*, and *E. coli* O157:H7 in artificially contaminated products. Likewise, four major food-borne pathogens including *L. monocytogenes* in ready-to-eat vegetables (fresh-cut lettuce, broccoli) and fruits were detected with a sensitivity of the assay of 10^4 cfu/PCR reaction (Wang and Slavik, 2005). A similar approach was reported for the detection of *Escherichia coli* O175:H7, *Salmonella* spp., and *Listeria monocytogenes* in a complex food matrix (Germini et al., 2009).

Enumeration of *Listeria monocytogenes* in foods is generally performed by the Most Probable Number method, which requires replicated dilution series of food in selective enrichment broth followed by plating on selective agar plates and various

biochemical assays for confirmation. Although MPN method has the advantage of enabling detection of the target pathogen even when it is present in low numbers in food matrices, it is laborious and may require 7-12 days for confirmation of results, a time that goes beyond the shelf life of the product itself. However, it is worthy to note that the MPN-PCR method is an effective tool to simultaneously detect the occurrence of food-borne pathogens quantitatively and qualitatively.

Of late, PCR coupled with the MPN method has been used to detect and quantify the presence of *L. monocytogenes* in ready-to-eat foods from local markets (Cordano and Jacquet, 2009; Marion et al., 2012). Ponniah et al., (2012) analyzed a total of 140 raw and ready-to-eat food samples by plating on selective PALCAM media and the suspected colonies were confirmed by MPN-PCR technique. With a similar approach, some studies evaluate the MPN method combined with qPCR for enumeration of *L. monocytogenes* in naturally and artificially contaminated minimally processed leafy vegetables (de Martinis et al., 2007; de Oliveira et al., 2010b). In these works, the authors found that qPCR was fast and easy to perform, with MPN results obtained in ca. 48 h for qPCR in comparison to 7 days for conventional method. In table 4 it is summarized a list of the methods proposed in the last years to detect some foodborne pathogens including *L. monocytogenes* in fresh-cut vegetables.

Table 4. Methods to detect *L. monocytogenes* (alone or with other foodborne pathogens) in fresh-cut vegetables. Limit of Detection (LOD), Limit of Quantitation (LOQ), time required for the analysis and tipology of the samples are reported.

Foodborne pathogen	Method	*Gene target*	LOD/ LOQ	Time for analysis	Samples analyzed	Reference
E. coli O157:H7 *L. monocytogenes* Salmonella	Multiplex qPCR	*prfA*	5 cfu 25 g^{-1} (LOD)	30 h	Natural samples	Garrido et al., 2013a
L. monocytogenes Salmonella	Multiplex qPCR	*hlyA*	5 cfu 25 g^{-1} (LOD)	24 h	Natural samples (n=95)	Garrido et al., 2013b
L. monocytogenes	Commercial real-time molecular beaconPCR		10^0 cfu g^{-1} (LOD)	52 h	Raw ingredients and RTE products (n=356)	Kotzekidou, 2013
E. coli O157:H7 *L. monocytogenes* Salmonella	PMA qPCR ISO 11290	*hlyA*	10^2-10^3 cfu g^{-1} (LOQ)	Without enrich.	Spinach and mixed salad artificially contaminated	Elizaquível et al., 2012
E. coli O157:H7 *L. monocytogenes* Salmonella Virus A Norovirus	Concentr. by PEG - qPCR	*hlyA*	10^2-10^3 cfu g^{-1} (LOQ)	Without enrich. 5 h	Parsley, spinach and mix salads	Sánchez et al., 2012
E. coli O157:H7 *L. monocytogenes* Salmonella	qPCR mini-VIDAS	*hlyA*	10^2 cfu g^{-1} (LOQ)	Without enrich. 5h	Not spiked food (n=99)	Elizaquível et al., 2011
L. monocytogenes	MPN-qPCR (SYBR Green)	16S	10^0 cfu g^{-1} (LOD)	48 h	vegetables samples (n = 162)	de Oliveira et al., 2010b
L. monocytogenes	qPCR	*ssrA*	1–5 cfu 25 g^{-1} (LOD)	30 h	Artificially inoculated vegetables	O'Grady et al., 2008
L. monocytogenes	qPCR ISO 11290	*actA*	10^0 cfu sample (LOD)	24 h Half Fraser + 6 h Fraser	Artificially contaminated (n=144) (44 positive)	Oravcová et al., 2007
E. coli O157:H7 *L. monocytogenes* Salmonella	qPCR		10^3 cfu mL^{-1} (LOD)	6 h + 4 h UPB	Fresh vegetables and pre-packed mixed-salad artificially contaminated	Bhagwat, 2003

4.12. Prevalence of *L. monocytogenes* in fresh-cut vegetables sold at retail market

Several surveys aimed at gathering data for risk assessments of *L. monocytogenes* in RTE vegetables in order to provide information to the health authorities on the presence of the pathogen in vegetable salad samples and to ascertain the risk of these products for consumers. In the last years, prevalence of *L. monocytogenes* in commercialized fresh-cut vegetables is worldwide reported by the scientific literature (Table 5).

A recent study evaluated the occurrence and counts of *L. monocytogenes* in 512 packages of ready-to-eat in the market in São Paulo, Brazil, containing at least one of the most consumed vegetables in the country (lettuce, carrot, collard green and cabbage). *L. monocytogenes* was detected in 3.1% of the samples (Sant'Ana et al., 2012b).

Between 2000 and 2005, 717 samples of three types of salads were analysed in Santiago (Chile) and *L. monocytogenes* isolates were found in 88 out of 347 (25.4%) samples of frozen vegetable salads and in 22 out of 216 (10.2%) freshly supermarkets prepared, cooked or raw ready-to-eat vegetable salads. In contrast, no *Listeria* spp. was isolated from 154 samples of raw minimally processed salads industrially prepared (Cordano and Jacquet, 2009).

Awaisheh (2009) investigate the incidence and contamination levels of different *Listeria monocytogenes* serovars and *Listeria* spp. in 360 samples of common ready-to-eat food products in Jordan. In this study, the presence of *L. monocytogenes* was determined using EN ISO protocol and confirmed by PCR technique. Five *Listeria* sp.: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. welshimeri* and *L. seeligeri* were isolated. *L. monocytogenes* was found in 6 RTE vegetables samples.

Prevalence of *Listeria monocytogenes* in 200 samples of ready to eat (RTE) foods of animal and vegetal origin including pre-packaged mixed salads collected from different outlets and processing plants in Sardinia revealed a contamination range of 22 and 9.5% for *Listeria* spp. and *L. monocytogenes*, respectively (Meloni et al., 2009).

The incidence of *Listeria monocytogenes* on persimmon (Diospyros kaki) surface was evaluated during a 5-month-period of two seasons periods (years 2005 and 2006). The fruits were collected in wholesale and street markets and retail in Sao Paulo and Campinas City, Brazil. A total of 582 fruits were analyzed using the Bax® System which is based on the Polymerase Chain Reaction. The incidence survey showed the absence of *L. monocytogenes* (Uchima et al., 2008).

In another survey in the same city Maistro et al., (2012) by using traditional and/or alternative methods detected *L. monocytogenes* on 2 samples of minimally processed vegetables (watercress and escarole).

Ponniah et al., (2010) examined a wide range of commercially available vegetables (n = 306) that are consumed in the minimally processed state in Malaysia by using the most probable number–polymerase chain reaction (MPN–PCR) method. It was found that *Listeria* spp. and *L. monocytogenes* could be detected in 33.3% and 22.5% of the vegetables respectively, being more frequently detected in Japanese parsley (31.3%) and yardlong bean (27.2%).

High contamination rate of *L. monocytogenes* in salads and vegetables from hypermarkets and streetside hawker stalls in Malaysia was of great concern due to emerging fresh produce-borne *L. monocytogenes* globally. Out of 396 samples, *Listeria* spp. was detected in 71 (17.9%) samples in which 45 (11.4%) were positive for *L. monocytogenes*. Among the studied RTE foods, salads and vegetables had the highest prevalence (14.7%) of *L. monocytogenes*, followed by chicken and chicken products (13.2%), beverages (10%), eggs and egg products (9.5%), beef and beef products (6.7%), lunch boxes (6.7%) and seafood and seafood products (6.7%) (Jamali et al., 2013), suggesting that consumption of RTE foods is a potential risk of listeriosis in this region. In another study performed between 1998 and 2007 the food-control laboratories in Israel tested 10,413 samples from five groups of RTE foods: salads/dips, dairy, fish, poultry and meat. A total of 1260 isolates of *Listeria monocytogenes* were identified with the highest level registered in the poultry group (27%) followed by salads/dips (9.2%) (Vasilev et al., 2010).

Recently, the prevalence of *L. monocytogenes* in various RTE food categories with special reference to ready-to-eat (RTE) fruits and vegetables, was investigated in Estonia between 2008-2010.

Table 5. Prevalence of *L. monocytogenes* in fresh-cut vegetables at retail markets.

Method of detection	Analyzed samples	Positive vegetable samples	Country	Reference
ISO 11290	396	14.7%	Malaysia	Jamali et al., 2013
ISO 11290	21,574	18.5%	Estonia	Kramarenko et al., 2013
ISO 11290 / qPCR	1032	0%	Greece	Kotzekidou, 2013
Vidas, Vip, Reveal, and traditional	172	2 (watercress and escarole)	São Paulo (Brazil)	Maistro et al., 2012
qPCR	512	3.1%	São Paulo (Brazil)	Sant'Ana et al., 2012b
ISO 11290	151	0.66% (salads)	Lisboa, Portugal	Santos et al., 2012
MPN/PCR	306	22.5%	Malaysia	Ponniah et al., 2010
ISO 11290	10,413	9.2%	Israel	Vasilev et al., 2010
ENI-ISO/PCR	360	6	Jordan	Awaisheh, 2009
MPN/PCR	717	25.4% (frozen salads) 10.2% (include fresh salads)	Santiago (Chile)	Cordano and Jacquet, 2009
ENI-ISO/PCR	200	9.5% pre-packaged mixed salads	Sardinia (Italy)	Meloni et al., 2009
Bax® System	582	(persimmon)	São Paulo (Brazil)	Uchima et al., 2008

A total of 554 (2.6%) of 21,574 food samples were positive for *L. monocytogenes.* Among RTE raw mixed salads (18.5%) were the most contaminated foods (Kramarenko et al., 2013). An assessment conducted in Lisbon retail markets, from 2007 to August in order to determine the microbial quality and the incidence of the major food borne pathogens in minimally processed salads commercialized in Portugal, revealed a low incidence of *L. monocytogenes* (0.66%) (Santos et al., 2012).

4.13. Emerging green strategies to control *L. monocytogenes* in fresh-cut vegetables and fruits

Fresh cut vegetables and fruits continues to rise in the market due to their freshness, economic handling and attractive presentation. They constitute a suitable meal for today's lifestyles, because they do not need preparation and provide a great variety of vitamins, minerals and other phytochemicals, which are important in human health (Little and Gillespie, 2008). Minimal food processing techniques constitute non-thermal technologies with guarantee of food preservation and safe standards as well as maintaining, as much as possible, the fresh-like characteristics of fruits and vegetables. Minimally processed products aim to extend the product shelf-life of 5–7 days at 4 °C, while ensuring food safety and maintaining nutritional and sensory quality (Ramos et al., 2013). When fruits and vegetables are minimally processed, they are submitted to unit operations that include selection, cleaning, washing, trimming, peeling, cutting and shredding, sanitizing and packing. As these operations do not assure the absence of microorganisms, minimally processed fruits and vegetables, require refrigeration as a primary means of preservation (Froder et al., 2007). Since refrigeration is the main hurdle to ensure the shelf life of fresh-cut vegetables and fruits, the ability of *L. monocytogenes* to survive and grow at refrigeration temperatures (2–4 °C) are two of the many factors that make the control of this foodborne pathogen difficult. Recently, Sant'Ana et al., (2012a) tried to determine the potential growth of *L. monocytogenes* and *Salmonella* in nine different types of fresh-cut vegetables (escarole, collard green, spinach, watercress, arugula, grated carrot, green salad, and mix for yakisoba) stored at refrigeration (7

°C) and abuse temperature (15 °C). *L. monocytogenes* was able to grow in more storage conditions and vegetables than *Salmonella*, indicating that this pathogen may grow and reach high populations in RTE vegetables depending on storage conditions. Similarly, *L. monocytogenes* was able to grow at different temperatures under different storage conditions was tested on commercial fresh-cut celery inoculated onto either freshly cut or outer uncut surfaces and stored in either sealed polyethylene bags or closed containers (Vandamm et al., 2013).

Over the last years, a number of strategies to minimize the microbial load of raw products including foodborne pathogens such as *L. monocytogenes*, and to control their growth during the shelf life have been explored. A variety of chemicals (including chlorine, hydrogen peroxide, organic acids and ozone) have been used to reduce initial bacterial populations on minimally processed produce (for a comprehensive review see Ramos et al., 2013). Chlorine is the most widely used sanitizer in the fresh produce industry. However, several studies indicate that chlorine concentrations traditionally used (50-200 ppm) are not effective in reducing pathogen load on fresh- cut produce (Goodburn and Wallace, 2013). Moreover, a prolonged exposure to chlorine vapor may cause irritation to the skin and respiratory tract of the workers, may affect the quality of foods and also adversely affect the environment (Beuchat, 1998). It is also known that the reaction of chlorine with organic matter results in the formation of carcinogenic products (trihalomethanes) for consumers (Gómez-López et al., 2013).

Alternatively, some physical methods are also available for reducing the microbiological load of fresh-cut products such as modified atmosphere packaging, irradiation, ultraviolet light, pulsed light, high pressure processing, ultrasound and cold plasma. Oliveira et al., (2010) found that the composition of the storage atmosphere within the packaging of shredded 'Romaine' lettuce had no significant effect on the survival and growth of different pathogens including *L. monocytogenes* at refrigeration temperatures. Ultraviolet (UV) light irradiation has demonstrated positive effects as a postharvest treatment in prolonging the storage life of fresh produce and as a non-thermal method of inactivation of pathogenic microorganisms. Results of a study performed by Gamage et al., (2013) show that

UV-C irradiation was able to restrict the growth of *L. monocytogenes* in the samples inoculated 24 h post treatment, with no significant differences in colour and weight loss of the broccoli compared to the control treatments. These results imply UV-C treatment may be effective not just in controlling pre-existing pathogens but also reducing the risk of post-harvest contamination. Accordingly, the use of double-sided UV-C radiation, at low doses, was effective in keeping *L. monocytogenes* at low levels during the storage period, without affecting the sensory quality of fresh-cut baby spinach leaves (Escalona et al., 2010). However, some limitations of these methods are the acceptance by consumers, increase produce stress and respiration rate that can induce a lignification-like process, high levels of CO_2 with a consequent development of off-flavors and potential stimulation of pathogens growth, low penetration depth, color changes, expensive equipment (Ramos et al., 2013).

Currently there is an increasing consumer pressure for replacing chemically synthesized antimicrobial by natural alternatives in order to ensure food safety (Xu et al., 2007). The development and application of more natural sanitizers with a broad spectrum antimicrobial activity and no toxicity for human in minimally processed foods providing enhanced sensory quality and extended shelf-life is of interest to catering industry and consumers (Lucera et al., 2012). Examples of suggested green strategies are reported in table 3.

This trend prompts a particular attention in the use of essential oils as antimicrobial compounds to be applied in minimally processed food approaches. The incorporation of antimicrobial agents in coatings is emerging as a promising technology, as it establishes contact with food and inhibits the growth of microorganisms present on the surface. The effect of bioactive films was investigated on fresh-cut broccoli artificially inoculated with foodborne pathogens. Biofilms were supplemented with two antimicrobial formulations containing rosemary and extract of Asian or Italian spice essential oil, respectively. In this experiment, bioactive films showed a significant reduction and a good capacity to control the growth of *L. monocytogenes* and *E. coli* at short-term storage (4 days), demonstrating the high antimicrobial potential of both types of films via the

diffusion of antimicrobial volatiles on pathogens bacteria in pre-cut vegetables (Takala et al., 2013). Similarly, the enrichment with bioactive compounds improved the antimicrobial action of chitosan and this strategy was proposed as a good alternative for controlling not only the mesophilic microorganisms present in broccoli but also the survival of *E. coli* and *L. monocytogenes*, without introducing deleterious effects on the sensory attributes of broccoli (Alvarez et al., 2013).

A mixture of origanum and rosmarinus extracts reduced the level of the autochthonous microflora and artificially inoculated bacteria on fresh-cut vegetables suggesting a synergetic effect of the essential oils (De Azeredo et al., 2011). The incorporation of the essential oil from cinnamon leaves in an edible film showed an increase of the antioxidant status and a reduction of the growth of different foodborne pathogens on fresh-cut peach (Ayala-Zavala et al., 2013). Scollard et al., (2009) suggested that the antilisterial properties of essential oils (thyme, oregano, and rosemary) could be enhanced in combination with high CO_2 atmospheres, when tested on vegetable matrices.

Bacteriophage prophylaxis is another possible alternative to reduce the contamination of foodborne pathogens in a more environmental friendly way than chemical approaches. Phages are bacterial viruses that invade specific bacterial cells, disrupt bacterial metabolism, and cause the bacterium to lyse without compromising the viability of other flora. Promising results using phage biocontrol have been reported for several pathogens, including *L. monocytogenes* (Guenther et al., 2009). Currently, bacteriophage preparations employing phage P100 is approved by FDA and USDA as ingredients for "food in general" in order to control *L. monocytogenes* contamination and commercialized as Listex P100 (FDA, 2006). Listex P100 is characterized by its broad spectrum towards *L. monocytogenes* strains, and therefore it can be used as a processing aid with all food products susceptible to *L. monocytogenes*. The efficacy of the bacteriophage Listex P100 to control *L. monocytogenes* growth on melon, pear and apple products (juices and slices) stored at 10 °C was recently investigated (Oliveira et al., 2014). In this study, the authors observed that phage treatment was more effective on melon and pear slices with a reduction of about 1.50 and 1.00 log cfu plug^{-1} respectively,

while, no effect on apple products was observed. Thus the authors propose the application of Listex P100 in order to avoid pathogen growth on fresh-cut and in fruit juices with high pH during storage at 10 °C, while the combination with other technologies could be required to improve the phage application on high acidity fruits (Oliveira et al., 2014).

Among the green strategy, biopreservation is a promising innovative way of extending the shelf-life of fresh fruits and vegetables, and reducing microbial hazards. Biopreservation consist on extension of the shelf-life and improvement of food safety using microorganisms and/or their metabolites. Some particular microorganisms that have been studied as possessing an antagonistic effect on pathogens are the lactic acid bacteria (LAB). This bacterial group is naturally present in food products and some studies suggested that when LAB are applied to produce surfaces they are strong competitors for physical space and nutrients, and/or may produce a wide range of antimicrobial metabolites such as organic acids, hydrogen peroxide, diacetyl and bacteriocins that negatively affect pathogens (Sagong et al., 2011). Bacteriocins are "generally recognized as safe" (GRAS) and they have been commonly employed in combination with other food additives as protective agents in fresh-cut produce. Lactic acid bacteria (LAB) bacteriocin-producing are to promote the microbial stability of both fermented and non-fermented vegetable food products using bacteriocinogenic strains as starter cultures, protective cultures or co-cultures and the employment of pure bacteriocins as food additives (Settanni and Corsetti, 2008).

LAB, including *Leuconostoc* spp., *Lactobacillus plantarum*, *Weissella* spp. and *Lactococcus lactis* have been isolated from fresh fruits and vegetables in a survey from commercial products in Spain and were reported to be inhibitory of *L. monocytogenes* without causing adverse effects on the fresh product (Trias et al., 2008a). The use of *Leuconostoc* strains as bioprotective agents was further investigated providing positive and encouraging results with different *in vivo* inhibitory range towards *L. monocytogenes* in Golden Delicious apple and Iceberg lettuce leaves. Moreover, two *Leuconostoc* strains produced Class IIa bacteriocins with high anti-listerial activity.

Table 3. Emerging green strategies recently adopted to control *L. monocytogenes* in fresh-cut vegetables and fruits.

Fresh-cut product	Microorganism	Strategy	Reference
Broccoli	*L. monocytogenes* *E. coli* *Salmonella typhimurium*	Bioactive films (organic acids mixture + rosemary extract + Asian or Italian spice essential oil)	Takala et al., 2013
Peach	*L. monocytogenes* *E. coli O157:H7* *Staphylococcus aureus*	Edible films (cinnamon leaf oil)	Ayala-Zavala et al., 2013
Broccoli	*L. monocytogenes,* *E. coli*	Chitosan enriched with bioactive compounds (tea tree, rosemary, pollen, propolis, pomegranate and resveratrol presented)	Alvarez et al., 2013
Iceberg lettuce, beet and rocket	*L. monocytogenes,* *Yersinia enterocolitica* *Aeromonas hydrophilla* *Pseudomonas fluorescens*	Essential oils (origanum vulgare and rosmarinus officinalis)	De Azeredo et al., 2011
Vegetable matrices	*L. monocytogenes*	Essential oils (thyme, oregano, and rosemary; different storage atmospheres and temperatures)	Scollard et al., 2009
Apples, lettuce	*L. monocytogenes*	Bacteriocins (*Leuconostoc* spp. produced bacteriocins belonged to the Class IIa)	Trias et al., 2008b
Lettuce	*L. monocytogenes*	Bacteriocins	Allende et al., 2007
Apples, lettuce	*L. monocytogenes,* *E. coli* *S. aureus* *Salmonella typhimurium* *Pseudomonas aeruginosa*	Antagonism (*Leuconostoc* spp. *Lactobacillus plantarum*, *Weissella* spp. *Lactococcus lactis*)	Trias et al., 2008a
Apples and peaches	*L. monocytogenes* *Salmonella*	Antagonism *Pseudomonas graminis*	Alegre et al., 2013a

The application of these strains in fresh fruits and vegetables did not cause sensory modifications (Trias et al., 2008b). Recently, Alegre et al., (2013a, 2013b) suggest the employment of a strain of *Pseudomonas graminis* from apple origin to avoid pathogens growth on minimally proceesed apples during storage when use as part of a hurdle technology in combination with disinfection techniques, low storagetemperature and modified atmosphere.

SECTION 2:
EXPERIMENTAL

5. AIMS OF THE STUDY

The main objective of this study was to increase the microbial safety of fresh-cut vegetables and fruits through a two-pronged approach:

1. by developing a reliable molecular method for a fast detection and quantification of *Listeria monocytogene* in fresh-cut vegetables and fruits.
   - 1.1 optimization of a protocol with high sensitivity and specificity to detect and quantify *L. monocytogenes* by qPCR;
   - 1.2 optimization of a fast, easy and cheap method to extract the microbial DNA from vegetables matrices;
   - 1.3 development of the MPN-qPCR method to quantify *L. monocytogenes* in artificially contaminated fresh-cut vegetables and its validation by conventional methods;
   - 1.4 development of a qPCR method to detect *L. monocytogenes* in artificially contaminated fresh-cut vegetables after a brief enrichment step and its validation by conventional methods;
   - 1.5 application of the proposed method to real samples;
   - 1.6 demonstration of the developed method by involving fresh-cut companies.

2. by investigating the antagonistic effect of probiotic lactic acid bacteria on fresh-cut pineapples artificially contaminated with *L. monocytogenes*.
   - 2.1 monitoring of *L. monocytogenes* and probiotic LAB populations during the shelf- life;
   - 2.2 monitoring the nutritional and sensorial quality of the fresh-cut pineapples during the shelf life.

6. A FAST, RELIABLE AND SENSITIVE METHOD FOR DETECTION AND QUANTIFICATION OF *LISTERIA MONOCYTOGENES* IN READY-TO-EAT FRESH CUT PRODUCTS BY MPN-QPCR

6.1. ABSTRACT

In the present work we developed a MPN quantitative real-time PCR (MPN-qPCR) method for a fast and reliable detection and quantification of *Listeria monocytogenes* in minimally processed vegetables. In order to validate the proposed technique, the results were compared with conventional MPN followed by phenotypic and biochemical assays methods. When *L. monocytogenes* was artificially inoculated in fresh-cut vegetables, a concentration of as low as 1 CFU g^{-1} could be assessed in 48 hours for the pathogen. QPCR alone allowed a limit of detection (LOD) of 10^1 CFU g^{-1} after 2 hours of enrichment for *L. monocytogenes*. In addition, the occurrence of the pathogenic bacteria in mixed salad samples and fresh-cut melons was monitored in two production plant from the receipt of the raw materials to the early stages of shelf life. No sample was found to be contaminated by *L. monocytogenes*.

Key words: *Listeria monocytogenes*, fresh-cut vegetables, MPN-qPCR

6.2. INTRODUCTION

Ready-to-eat (RTE) fresh vegetables and fruits have become an established product in worldwide markets whose acceptance considerably increased in the last years. However, the growing popularity of this high quality fresh food products poses many safety concern. Indeed, fresh-cut vegetables are a potential vehicle of transmission of food-borne pathogens being *Listeria monocytogenes* among the most hazardous. Generally, infection with *L. monocytogenes* may cause mild febrile gastroenteritis, but in susceptible individuals, such as young, old, pregnant and immune-compromised, invasive listeriosis can result in more serious diseases including meningitis, septicaemia, preterm birth, miscarriage, and rather high mortality rate (Painter and Slutsker, 2007). In the last years, outbreaks caused by the consumption of fresh vegetables contaminated with *L. monocytogenes* (Gillespie et al., 2010; Little et al., 2010; Gaul et al., 2013) have become increasingly recognized in developed countries. From a technological point of view, fresh-cut vegetables are considered "minimally processed food", hence characterized by the absence of treatment to break down the microbial load, with the exception of washing with chlorinated water and the compliance of the cold chain (Olaimat and Holley, 2012). Fresh-cut contamination can occur at pre- and post-harvest levels, with environment, irrigation water, handling of the product and food plant uncleanness among the main critical points (Dallaire et al., 2006). In particular, the ability of both food-borne pathogens to adhere to food and food-contact surfaces, thus forming or colonizing preexistent biofilm, associates the latter hypothesis to the higher risk for food safety, since potentially able to prejudice large quantities of products (da Silva and de Martinis, 2013; Srey et al., 2013). In the last years, several food surveys throughout the world reported on the detection of *L. monocytogenes* in raw RTE vegetable salads sold at market retails (Cordano and Jacquet, 2009; Ponniah et al., 2010; de Oliveira et al., 2010b; Jamali et al., 2013). From an industrial viewpoint, the prevalence of these events is a serious threat for fresh-cut producers because it involves economic losses due to the withdrawal of the food from the market and damage to the company image, impacting its intangible capital. The current European regulation for fresh-cut vegetables claims

that for *Listeria monocytogenes* a limit concentration of 100 CFU/g is tolerated at the end of the shelf life (European Commission Regulation 2073/2005). Since minimally processed vegetables are generally characterized by short shelf life (7-15 days), a rapid detection and quantification of human pathogens becomes a major challenge for producers so as for retail traders. Enumeration of *L. monocytogenes* in food is generally done by the Most Probable Number (MPN) method, which requires replicated dilution series of food in selective enrichment broth followed by plating on selective agar plates and subsequent biochemical assays for species identification (de Martinis et al., 2007). Although MPN method has the advantage of enabling detection of the target pathogen even when it is present in low numbers, it is laborious and requires several days for confirmation of results. Therefore, in the last years, a considerable number of detection methods using faster molecular tools have been proposed (de Oliveira et al., 2010b; Ponniah et al., 2010; Elizaquível et al., 2012). Currently, several genes have been suggested as target for the molecular detection of *L. monocytogenes* (Aznar and Alarcón, 2002; Rodríguez-Lazaro et al., 2004; O'Grady et al., 2008). The most recent advances in the field are focused on the reduction of the limits of detection and quantification (Elizaquível et al., 2011; Sánchez et al., 2012), on the discrimination between dead and live cells (Elizaquível et al., 2012) or the simultaneous detection of different foodborne pathogens (Elizaquível et al., 2011; Elizaquível et al., 2012; Sánchez et al., 2012; Garrido et al., 2013a). The performances of these methods were, in some cases, successfully compared with the official protocol or with diagnostic commercial kits (O'Grady et al., 2008; Sánchez et al., 2012; Kotzekidou 2013).

In the present work we integrated conventional MPN technique with qPCR, in order to keep the advantages of both methods, sensitivity of MPN and reliability and quickness of qPCR. Moreover, we developed a qPCR enrichment-based method for a fast and reliable detection of *L. monocytogenes* in minimally processed vegetables. In order to validate the proposed technique, the results were compared with conventional culture-dependent methods.

6.3. MATERIALS AND METHODS

Bacterial strains and growth conditions

The type strain *Listeria monocytogenes* CECT 4031 from rabbit origin was purchased from the Spanish Type Culture Collection (CECT, Valencia, Spain).

Five strains of *L. monocytogenes* (namely A.9.1, A.9.3, A.9.4, R.9.1 and R.9.3) isolated from strawberries were provided from the Athens University (Athens, Greece). The non-*monocytogenes Listeria* spp. strains, *Listeria ivanovii* IZPS B45 and *Listeria innocua* IZPS B48 were kindly provided by the Istituto Zooprofilattico Sperimentale di Puglia e Basilicata (IZPS, Foggia, Italy).

All strains were routinely cultured at 37 °C in TSB broth (Oxoid, Hampshire, UK) until reaching mid exponential phase.

Lactobacillus plantarum WCFS1 was used as further negative control and grown at 30 °C on MRS broth (Oxoid).

Food samples and artificial contamination

Minimally processed fresh-cut mix salads (lettuce, radicchio, endive) were randomly purchased at local markets in Foggia (Italy) and stored at 4 °C for a maximum of 24 h prior to analyses. All samples were investigated for the presence of *Listeria* spp. as recommended by the ISO protocol 11290-1 from International Organization for Standardization (Anonymous, 1996). All samples resulted negative for the presence of the pathogen and were used for subsequent artificial contamination experiments. For artificial inoculation, *L. monocytogenes* CECT 4031 grown at middle exponential phase were added to the corresponding enrichment selective media used for the MPN assays (see below) in order to obtain a contamination level ranging from 0 to 3 Log CFU g^{-1} of sample. For the qPCR detection without selective enrichment, salads samples were also inoculated at a level of 4 and 5 Log CFU g^{-1}.

MPN enumeration of *Listeria monocytogenes*

Salad samples (25 g) were added to 225.0 mL of Fraser broth (Oxoid) supplemented with Fraser Selective Supplement SRO156E (Oxoid) and

homogenized in a stomacher (BagFilter®, Interscience, FR) for 2 min. Triplicate series of tubes containing decimal serial dilution from 10 to 10^{-5} grams of homogenate were incubated for 48 hours at 37 °C in the same media. After incubation, aliquots of enrichment broth were taken from dark tubes (containing presumptive *Listeria* spp.) and streaked onto Oxford agar plates (Oxoid) and PALCAM agar (Oxoid). Plates were incubated at 37 °C for 48 h and five typical colonies were picked for purification on TSA + 0.6% yeast extract plates (Oxoid). Then, plates were incubated for 24 h at 37 °C and gram positive, catalase positive colonies were streaked on Blood agar (37 °C, 24 h). Hemolytic colonies were identified as *L. monocytogenes* using API Listeria strips (Biomerieux, Marcy l'Etoile, FR). The MPN value and 95% confidence intervals were determined by the number of positive tubes obtained in serial dilutions as reported by the USDA guidelines (USDA-FSIS, 2008).

DNA extraction

Genomic DNA was obtained from artificially inoculated salads samples, after homogenization in the enrichment broth, after 0, 2, 4, 6 and 24 hours, respectively. Briefly, 10 mL of the homogenate were centrifuged for 10 min at 5.000 x g. Supernatant was then discarded and DNA extracted by comparing the three methods reported:

i) *DNeasy Blood and Tissue kit* (Qiagen, Milano, IT), the extraction was performed following the manufacturer's protocol for Gram-positive. Slight modification were: adjustment to 40 minutes for lysozyme treatment and proteinase K digestion, double elution of DNA with 100.0 and 50.0 µL aliquots of elution buffer;

ii) *Power soil DNA isolation kit* (Mo-Bio, Carlsbad, CA, USA). Extraction was carried out following manufacturer's instruction. Mechanical lysis was performed using a Mini bead beater (Biospec products, Bartlesville OK, USA) for 30 sec at half power.

iii) *Phenol–chloroform extraction.* The organic extraction was performed according to the standard method reported by Sambrook and Russel (2001).

In addition, DNA was also extracted from dilution tubes considered for MPN enumeration by comparing two methods:

i) Microbial DNA isolation kit (MoBio) according to the manufacturer's instructions;

ii) the boiling method modified by de Oliveira et al. (2010b).

The amount and purity of DNA were assessed by measuring absorbance at 260 nm (concentration) and the ratio between absorbance at 260 and 280 nm (purity) using a BioTek Eon spectrophotometer (BioTek, VT, USA). DNA purity was considered satisfactory when the A260/A280 ratio was >1.50. The degradation level of the extracted DNA was evaluated by electrophoresis in agarose gel (1% w/v). All DNA samples were stored at -20 °C before analyses.

Real Time PCR conditions

Target genes, primers and probes employed are listed in Table 1. Four different assays were tested to amplify fragment of the 16S rRNA, *hlyA*, and *iap* genes. All reactions were carried out using iTaq SYBR green Supermix with ROX (BIO-RAD) or iTaq Universal probes Supermix (BIO-RAD) depending on the employed chemistry. Primers and probes were synthesized by PRIMM Biotech (Milano, IT). Reagent concentration and amplification cycles are reported in the reference papers listed in Table 1. *L. monocytogenes* specific primers and hybridization probe tagged with FAM fluorescent dye as designed by Rodríguez-Lázaro et al. (2004) were used to amplify a 64-base pair fragment of the listeriolysin O gene (*hlyA*). QPCR assays were performed on an AB 7300 Real Time PCR System (Life Technologies, Monza, IT). Amplification was carried out in a final volume of 20 µL including 3 µL of template DNA, 10 µL iTaq Universal probes Supermix (Bio-Rad, Milano, IT), 0.1 µM of each primer and 200 nM of probe. Amplification conditions were as follows: initial denaturation at 95 °C for 10 min followed by 45 cycles of 15 s at 95 °C and 1 min at 63 °C.

Table 1. QPCR assays, primers and probes. *SYBR green, +TaqMan.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'FAM-TAMRA3')	Size (bp)	Reference
16S rRNA*	CACGTGCTAC AATGGATAG	AGAATAGTTTTA TGGGATTAG	none	70	de Oliveira et al., (2010b)
*hlyA**	TGCAAGTCCT AAGACGCCA	CACTGCATCTCC GTGGTATACTAA	none	113	Nogva et al., (2000)
hlyA+	CATGGCACCA CCAGCATCT	ATCCGCGTGTTT CTTTTCGA	CGCCTGCAAGTC CTAAGACGCCA	64	Rodriguez et al., (2004)
iap+	CTAAAGCGGG AATCTCCCTT	CCATTGTCTTGC GCGTTAAT	CTTCTGGCGCACA ATACGCTAGCACT	174	Hein et al., (2001)

The specificity of the assay was tested by using DNA templates from pure cultures of *L. monocytogenes*, non-*monocytogenes Listeria* spp., and *L. plantarum*. The absence of PCR inhibitors was evaluated by adding 1 ng of purified DNA extracted from *L. monocytogenes* CECT 4031 to an aliquot of 5 µL of the extracted DNA and amplified using conditions described above. Quantification was performed by interpolating values from unknown samples in a standard regression curve generated from ten-fold dilutions of triplicate samples at known DNA concentration of the respective microorganism. Real-time PCR assays were performed in duplicate and included a negative control and a no template control in each run. Data were expressed as the mean of three independent experiments.

Application of the qPCR method to RTE food production chain

Minimally processed fresh-cut samples representative of 2 production batches of rocket, mixed salad and *piel de sapo* melons, respectively were provided by two different fresh-cut fruit and vegetables companies. For each food, samples were analyzed by MPN-qPCR and culture-dependent methods at three different stages: raw, after processing and at three days of shelf life at 4-5 °C. All samples were used for demonstration activity only and not purchased by the companies involved.

6.4. RESULTS

Optimizing bacterial DNA extraction from vegetable matrices

The first part of the present study was focused on the individuation of a suitable protocol for bacterial DNA extraction from fresh-cut vegetables. This preliminary step was necessary to individuate a procedure which could guarantee a good DNA yield combined with adequate purity and, especially, the absence of PCR inhibitors such as complex polysaccharides and chlorophyll. Furthermore, we needed a simple, time efficient, possibly low cost procedure. Finally, possible manipulation of toxics had to be taken into account. Therefore, four different DNA extraction protocols including two commercial DNA extraction kits were evaluated, in order to select the most suitable one, on the basis of cost, time, yield, quality and personnel skills required.

DNA extraction was performed on artificially inoculated samples using *Listeria monocytogenes* CECT 4031 as the test microorganism. Purity and concentration of DNA was assayed spectrophotometrically and its integrity checked on agarose gel electrophoresis.

As summarized in Table 2, all the extraction methods guaranteed adequate yield and purity. The phenol-chloroform method was inexpensive and allow to extract the higher yield of bacterial DNA. However, this method was discarded due to major drawbacks such as laboriousness, and necessity to store and manipulate significant amounts of toxic compounds, that make it inadequate for implementation in a laboratory as a routine analysis. When both commercial kits were used, no significant differences were observed (Fig. 1). Nonetheless, Qiagen Blood and Tissue kit appears to be more suitable for our purposes because either assuring more than acceptable purity and yield of DNA, or less expensive and time consuming than Mo-Bio Power Soil Kit. For this reason, and according to previous study (Elizaquível et al., 2008; Elizaquível et al., 2011) Qiagen kit was selected to extract the bacterial DNA from vegetable samples.

MPN enumeration is based on serial dilution of the sample, thus resulting in the dilution of the substances that could interfere with the PCR amplification reaction.

In addition, after 48 h of growth in a selective enrichment broth a high bacterial concentration is achieved in the positive tubes. Therefore two different DNA extraction methods have been compared because faster and less expensive: a commercial microbial DNA isolation kit and an approach based on a physical disruption of the cells subjected to heat exposure (Table 2). The boiling method (de Oliveira et al., 2010b) was the one providing the lower yield, but the least expensive and faster. Since, in previous assay we obtained the same results with both approaches (data not shown). Therefore, the protocol was adopted to extract the microbial DNA from cultures used to determine the MPN in order to confirm by qPCR the presence/absence of *L. monocytogenes* in MPN positive/negative tubes.

Table 2. Comparison between DNA extraction methods used in this work.

Extraction method	**Cost per reaction (€)**	**Time (20 sample-run)**	**Yeald (ng/µl)**	**Purity A260/A280**	**Manipulation of toxic compunds**	**DNA application**
MoBio - PowerSoil DNA isolation kit	7,00	2,2 h	75.13±8.46	> 1,9	-	Enrichment-qPCR
Qiagen - DNeasy Blood & Tissue kit	5,00	1,3 h	74.85±4.89	> 1,9	-	Enrichment-qPCR
Phen/chlor	0,50	4 h	87.17±6.74	> 1,9	Significant	Enrichment-qPCR
MoBio - Microbial DNA isolation kit	3,80	2 h	80.27±3.51	> 1,9	-	MPN-qPCR
Thermal shock	0,20	20 min	60.26±8.58	> 1,6	-	MPN-qPCR

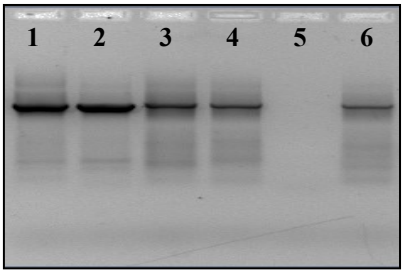


Figure 1. DNA extracted from fresh-cut salads artificially inoculated with *L. monocytogenes* CECT 4031 by using PowerSoil DNA isolation kit (Mo-Bio) (lines 1 and 2) or DNeasy Blood and Tissue kit (Qiagen) (lines 3 and 4). Lines 5: negative control. Lines 6: positive control.

Optimizing *Listeria monocytogenes* qPCR assay conditions

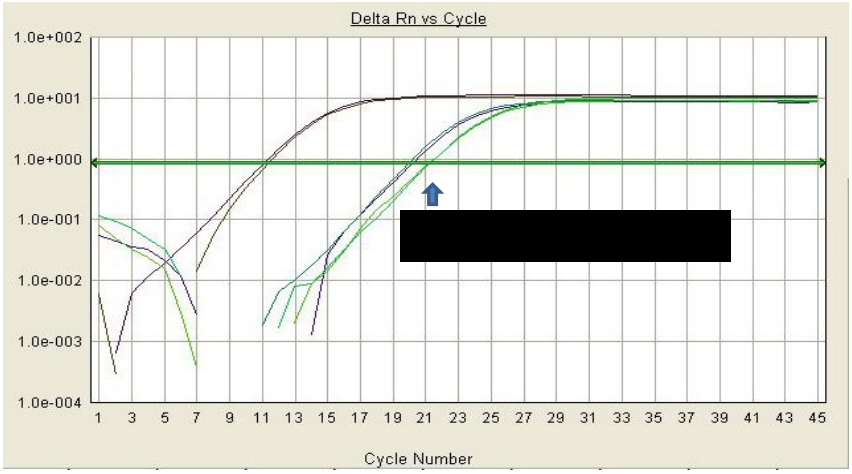
Q-PCR assay targeting *L. monocytogenes* 16S rRNA gene was initially chosen as it was assumed that presence of a multi-copy target sequence would enhance assay sensitivity, according to de Oliveira et al., (2010b). The assay was initially based on the use of SYBR green rather than fluorescent probes in order to reduce the cost per reaction. Assay specificity was tested against DNA extracted from the type strains *L. monocytogenes* CECT 4031*, L. innocua*, *L. ivanovii* and *L. plantarum* (used as negative control strain). The assay showed the expected signal in the case of *L. monocytogenes* and no signal in the case of the *Lactobacillus plantarum* strain. However, it lacked of specificity as a signal was also observed in the case of *L. innocua* and *L. ivanovii* (Fig. 2).

Due to this evidence, the assay was discarded and a SYBR green qPCR assay targeting *hlyA* gene was tested as reported by Nogva and coauthors (2000). This test was expected to improve specificity as it was directed to detect a specific virulence related gene. However, even in this case, similar results were observed, with positive signal generated also with non-*monocytogenes* DNA (data not shown). Therefore, this assay was also discarded.

Due to the unsatisfactory results obtained, we decided to perform further tests employing a probe–based assays in order to identify an adequate specificity.

Primers and probes targeting *iap* gene designed by Hein et al. (2001) were chosen for this assay. The assay was tested against the strains previously analyzed using the same protocols. In this case, the assay proved adequately specific, and only positive signal for *L. monocytogenes* strains were detected. Nonetheless, the assay was discarded as it was unsuitable for quantification purposes because of high serovar-related variability of results (data not shown).

A



B

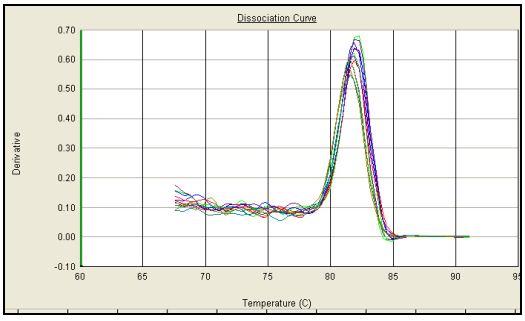


Figure 2. Amplification of a 70 bp of 16S gene from cultures of *L. monocytogenes* CECT 4031, *L. innocua* ATCC 33090 and *L. ivanovii* ATCC 19119 by using SYBR green as the fluorophore (A) and the corresponding dissociation curve (B).

Therefore, in the present study, the qPCR method developed by Rodríguez-Lázaro et al. (2004) was adapted to our purposes and employed in order to detect and quantify *L. monocytogenes* in artificially contaminated fresh-cut vegetables. Specificity of the assay was confirmed in all of preliminary assays performed using 1 ng of genomic DNA of *Listeria monocytogenes* from animal and vegetable origine and non-*monocytogenes* strains as template. Detection and quantification limits of the qPCR assay were investigated by using DNA extracted from overnight cultures of *Listeria monocytogenes* CECT 4031 strain. Serial dilutions of DNA were subjected to qPCR and a standard curve was constructed by using *hlyA* as target gene. Amplification profile of serial dilutions and standard curve are shown in Figure 3.

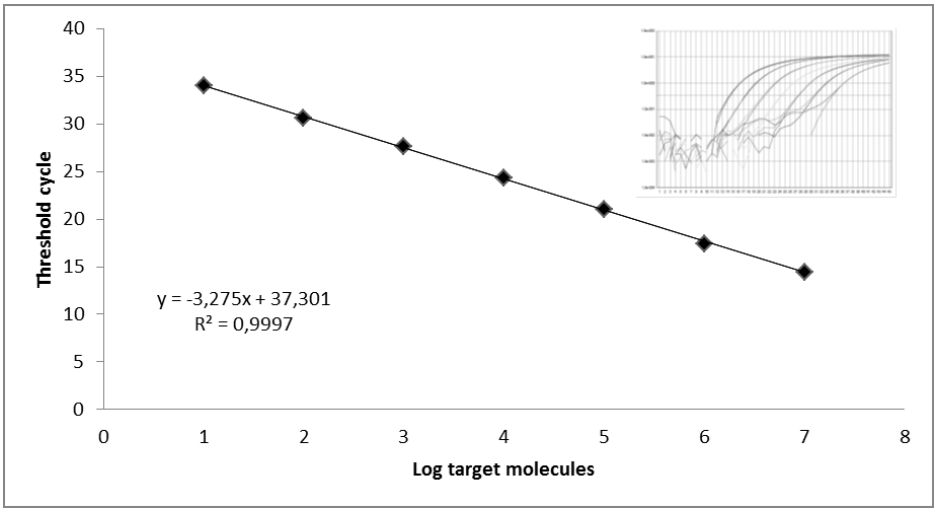


Figure 3. Standard curve and amplification plot of the 64 bp *hlyA* gene fragment generated by qPCR amplification of serially diluted purified DNA of *Listeria monocytogenes* represented as log of genome equivalents/reaction. Trend line equation and the corresponding square regression coefficient (R^2) are shown.

Amplification reactions were carried out with a range of DNA concentrations approximately corresponding to 1×10^7 – 1×10^0 target molecules. Being the target sequence part of a single copy gene, number of target molecules was estimated as

total DNA/mass of a single *L. monocytogenes* genome ≈ 2.94×10^{-15} g. The standard curve showed a linear relationship, spanning 7 logs, between log input DNA and threshold cycle. The slope of the curve was -3.27, close to the theoretical optimum (-3.32), and corresponding to 102% amplification efficiency, calculated by the formula (Efficiency $=10^{-1/slope}$-1). Square regression coefficient was R^2 = 0.999 indicating that the qPCR assay is highly linear in the considered range (Figure 3). There was no overlapping of confidence intervals based on standard deviation of C_T values down to 1×10^1 target molecules indicating that reliable quantification is possible to this limit. The LOD was found to be lower, corresponding to 5 target molecules (mean C_T value 37.8, st. dev. 3.7). This data led us to consider this qPCR assay suitable for development of MPN- qPCR and enrichment free detection protocols.

L. monocytogenes **enumeration by MPN-qPCR**

In our experiments we used a MPN protocol to quantify *L. monocytogenes* and qPCR to confirm positive and negative tubes by qPCR. In order to evaluate the reliability of our method on fresh-cut vegetables we applied our protocols on artificially inoculated salads (0 to 3 Log CFU g^{-1}) with both pathogens. Salad samples not inoculated and previously assayed for both the target pathogens were used as negative control. Enumeration of *Listeria monocytogenes* by the MPN method from artificially contaminated foods was performed after 48 h of incubation of triplicate serial dilutions in selective Fraser Broth. Aesculin hydrolysis in the positive tubes resulting in turning of culture medium to black (Fig. 4) allowed to define the three consecutive dilutions to determine the initial contamination by referring to the corresponding MPN tables (USDA-FSIS, 2008). Presumptive *L. monocytogenes* positive tubes were phenotypically and biochemically confirmed in all cases. Moreover, no typical *L. monocytogenes* colonies were observed on selective media when negative tubes from the same dilutions were assayed. Black tubes from negative controls were never observed by MPN. This result was also confirmed by conventional techniques (Table 1).

A **B**

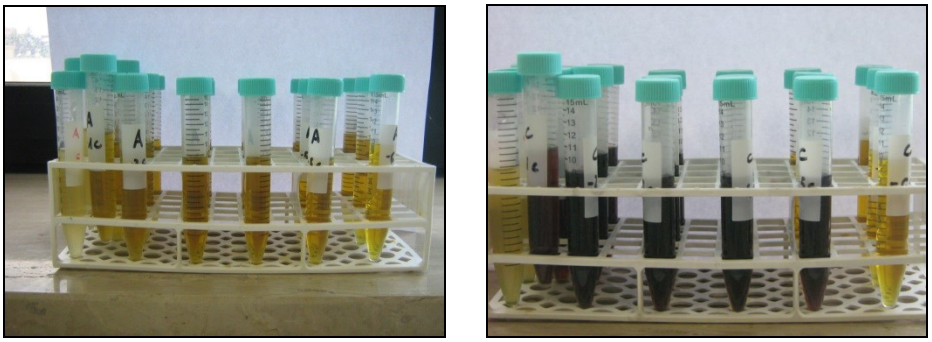


Figure 4. MPN enumeration of fresh-cut salads not inoculated (A) or inoculated with 10^2 cfu g^{-1} (B) of *L. monocytogenes* CECT 4031 after 48 h of incubation in Fraser Broth.

Table 1. Quantification of *L. monocytogenes* in artificially inoculated fresh-cut salads by MPN and confirmation of positive tubes by conventional methods and qPCR.

Theoretical inoculum (CFU g^{-1})	3 Tube Dilution[1] (mL homogenized)	Positive tubes (MPN)	True Positive[2]	MPN (g^{-1}) [3] (95% c.i.)	Positive (qPCR)
Control	10/1/0.1	0/0/0	n.d.	n.d.	n.d.
1	10/1/0.1	3/2/0	3/2/0	0.93 (0.23-3.80)	3/2/0
10	1/0.1/0.01	3/1/0	3/1/0	4.3 (0.90-18)	3/1/0
100	0.1/0.01/0.001	3/0/2	3/0/2	64 (17-180)	3/0/2
1000	0.01/0.001/0.001	3/1/1	3/1/1	750 (170-2000)	3/1/1

Detection of *L. monocytogenes* by qPCR

For the evaluation of LOQ and LOD of the qPCR methods without MPN step, artificially contaminated fresh-cut vegetables used for MPN enumeration, were in parallel incubated at 37 °C and samples analyzed by qPCR after 0, 2, 4, 6, 24 h of enrichment, respectively. As previously reported for vegetables matrices (Elizaquível and Aznar, 2008), DNA was extracted from 10 mL of homogenate by using the DNeasy Blood and Tissue kit (Qiagen). *L. monocytogenes* was detectable at contamination levels as low as 10 CFU g^{-1} with two hours of enrichment (mean CT value was 36.73, st. dev. 1.12). Limit of quantitation (LOQ) was 10^4 CFU g^{-1} without selective enrichment (data not shown).

Screening of RTE samples

Samples of ready to eat vegetables and fruits provided by two different companies were investigated for the presence of *Listeria* spp. by using both MPN-qPCR and conventional culture-dependent methods. Black tubes were not observed by MPN enumeration of all the samples, suggesting the absence of presumptive *Listeria* spp. According, *L. monocytogenes* was never detected by qPCR nor conventional methods (data not shown).

6.5. DISCUSSION

Fresh-cut packaged fruit and vegetables sold in the market are generally considered a product of high quality and freshness. However, they may represent an underestimated public health risk due to the potential presence of pathogenic bacteria, like *Listeria monocytogenes* (Francis et al., 2012). The existing regulations in Europe provides that the identification of these foodborne pathogens is carried out by culture-dependent methods. Nonetheless, an inexpensive analysis and a fast preliminary result based on a molecular approach could be remarkable for fresh-cut producers to integrate the conventional methods and thus allowing:

- a promptly intervention on presumptive contaminated product;
- a more drastic sanitization of the plant;
- a better internal quality control assessment;

- to ensure greater safety to the consumer.

In the last years, PCR-based techniques have been the subject of considerable focus and ISO guidelines have been established for the detection of food-borne pathogens (ISO 22174:2005, ISO/TS 20836:2005, ISO 20837:2006, ISO 20838:2006). Particularly, real-time quantitative PCR is considered as a method of choice for the detection and quantification of microorganisms (Postollec et al., 2011).

However, a methodology can be considered attractive to implement routine checks of commercial products only if sufficiently simple, fast, efficient and relatively inexpensive. In order to reduce the time for the analysis and the corresponding cost, some recent works addressed on the simultaneous detection by qPCR of pathogenic bacteria including *L. monocytogenes* (Elizaquível and Aznar, 2008; Elizaquível et al., 2011; Elizaquível et al., 2012; Sánchez et al., 2012; Garrido et al., 2013a). Nonetheless, the main efforts have been made to improve critical points such as the sensitivity and specificity in order to ensure a fast and reliable method.

Although, several works reported on a rapid detection of *L. monocytogenes*, only few study are focused on its quantification from vegetables matrices by coupling MPN with PCR (Ponniah et al., 2010) or qPCR (de Martinis et al., 2007; de Oliveira et al., 2010b) after an enrichment of 48 h. Therefore, in this study we aimed at the foodborne pathogens quantification by integrating MPN with qPCR in order to considerably reduce the time required for confirmation by conventional biochemical assays. The first goal was the comparison of the results obtained by MPN-qPCR with those one from culture dependent methods. In the present work, we were able to detect and -indirectly- quantify *L. monocytogenes* in artificially inoculated salads after 24 hours of incubation in the corresponding selective media. However, in real conditions the pathogen cells on vegetable surfaces are submitted to several stress such as washing with chlorinated water, low temperatures and microbial competition thus resulting in a slower recovery and growth rate. For this reason, an increase of the incubation time to 48 h should be anyway recommended for MPN enumeration when real samples are analyzed. With the aim of developing a faster method for the detection of *L. monocytogenes*, in parallel to the enumeration by the MPN-qPCR method, we assayed a qPCR approach on DNA

extracted after 0, 2, 4 and 6 h of enrichment in the corresponding selective media. We observed that after 2 h it was possible to detect *L. monocytogenes* with a limit of 10 CFU g^{-1}. With the same approach we could not achieve quantitative results for samples artificially inoculated at a level lower than 10^4 CFU g^{-1} without the enrichment step. According to our results, the detection of pathogenic bacteria by qPCR is generally limited at concentration above 10^2 CFU g^{-1} in artificially inoculated fresh, minimally processed vegetables without enrichment (Elizaquível and Aznar, 2008, Elizaquível et al., 2011). More recently, a method based on the recovery and concentration allowed the simultaneous detection and quantification of 10^2 CFU g^{-1} for *L. monocytogenes* in parsley and salad (Sánchez et al., 2012). However, detection values below 10 CFU mL^{-1} were only achieved after selective enrichment step which required approximately 30 hours (Oravcova et al., 2007; O'Grady et al., 2008; de Oliveira et al., 2010b; Garrido et al., 2013a). Conventional methods requires five days for determination of a negative result for *L. monocytogenes* contamination. If a positive test result occurs, additional days are required for biochemical tests to identify the species (Churchill et al., 2006).

The robustness of the results obtained by qPCR approach is closely related to the efficient recovery of bacterial DNA. Furthermore, DNA quality is critical because the efficiency of PCR amplification can be reduced by inhibitors from the matrix. In the last years, several works compared different microbial DNA extraction techniques in order to optimize yield, time and cost of the sample preparation process depending from the food (Amagliani et al., 2007; Di Pinto et al., 2007; Elizaquível and Aznar, 2008; Jara et al., 2008). The commercial DNeasy Blood and Tissue kit has been reported as an efficient DNA purification method from vegetable matrices (Elizaquível and Aznar, 2008; Elizaquível et al., 2011). Accordingly, in this study we used the same protocol to extract the DNA during the enrichment-qPCR assays. Agreeing, we were always able to detect the presence/absence of both pathogens when DNA obtained from a commercial kit for microbial DNA extraction or from the boiling method previously described (de Oliveira et al., 2010b; Ponniah et al., 2010) were used as template for qPCR amplification.

Several authors reported on the detection of *L. monocytogenes* sold at retail markets (Althaus et al., 2012; Sant'Ana et al., 2012b), but at our knowledge no study was addressed to improve companies internal quality control procedures. With this aim we applied the developed methods to assess the occurrence of *L. monocytogenes* in three different fresh cut foods including vegetables and fruits from the receipt of the raw materials to the early stages of shelf life.

Furthermore, in *in vivo* assays, qPCR always matched the outcomes of the conventional methods supporting that it is a reliable approach to discriminate both positive and negative presumptive results from MPN enumeration.

This focus could be interesting for industrial purposes since enumeration of the pathogenic microorganisms can provide an estimation of the efficacy of sanitizers treatment and represent an alarm bell to reduce the risk of cross contaminations in the plant.

Therefore, we believe that this work may represent a further step to confirm the strength of molecular methods as a useful and powerful tool to complement conventional methods for a rapid detection of foodborne pathogens in fresh-cut vegetables suggesting their routine implementation in the food industry.

7. PROBIOTIC LACTIC ACID BACTERIA AS A PROMISING STRATEGY TO FIGHT *LISTERIA MONOCYTOGENES* IN FRESH-CUT PINEAPPLES

7.1. ABSTRACT

Due to the increasing interest for healthy foods, the feasibility of using fresh-cut fruits to vehicle probiotic microorganisms is arising scientific interest. With this aim, the survival of probiotic lactic acid bacteria, belonging to *Lactobacillus plantarum* and *Lactobacillus fermentum* species, was monitored on artificially inoculated pineapple pieces throughout storage. The main nutritional, physicochemical and sensorial parameters of minimally processed pineapples were monitored. Finally, probiotic *Lactobacillus* were further investigated for their antagonistic effect against *Listeria monocytogenes* on pineapple pieces. Our results show that at eight-days of storage, the concentration of *L. plantarum* and *L. fermentum* on pineapples pieces ranged between 7.3 and 6.3 log cfu g^{-1} respectively, without affecting the final quality of the fresh-cut pineapple. The antagonistic assays indicated that both *L. fermentum* and *L. plantarum* were able to inhibit the growth of *L. monocytogenes*, being *L. plantarum* more effective than *L. fermentum*. This study suggest that both *L. plantarum* and *L. fermentum* could be successfully applied during processing of fresh-cut pineapples, contributing at the same time to inducing a protective effect against a relevant foodborne pathogen.

Kew words: Lactic Acid Bacteria, pineapple, probiotic, fresh-cut, *Listeria monocytogenes*

7.2. INTRODUCTION

A fascinating challenge for the food industry over the coming years is trying to meet the increasing demand for foods that encompass several levels of quality attributes including safety, nutritional and health value. Fresh-cut fruits and vegetables respond well to these requirements and their acceptance tends to be higher among specific categories of consumers (Ragaert et al., 2004). In recent years, some attempts were carried to further improve the added value of minimally processed fruits and vegetables, proposing them as functional foods. Thus, juices matrices have been purposed as carrier for probiotic microorganism (Espírito-Santo et al., 2011; Martins et al., 2013), and few examples of minimally processed fruits such as papaya and apple slices were enriched with commercial probiotic bacteria (Tapia et al., 2007; Rößle et al., 2010a; Alegre et al., 2011). In fresh-cut fruit processing, typical operations such as peeling and cutting can promote the microbial adhesion to the tissue, increasing the surface contact and the release of cellular content rich in minerals, sugars, vitamins and other nutrients, ideal substrates for probiotic bacteria growth (de Oliveira et al., 2011). In addition, a rather short shelf life of these products should contribute to the maintenance of a high microbial load during the storage time, and the low temperature of storage should contribute to control the undesirable effect of the microbial metabolic activity on quality. Moreover fresh fruits and vegetables contain mostly cellulose, which is not digested by humans and that it was suggested playing a protective role for probiotic microorganisms in the gastrointestinal system (Alegre et al., 2011; Bove et al., 2013; Arena et al., 2014). Standing to these considerations, an increasing interest for fresh-cut fruits as potential matrices to vehicle beneficial microorganisms is arising, which can be also considered a promising alternative to probiotic dairy products (Soccol et al., 2010).

From a microbiological point of view, it is known that minimally processed fruit and vegetables can be a risk for the safety of the consumers (Francis et al., 2012). Foodborne illness are oft related to the consumption of fresh-cut products contaminated by *Listeria monocytogenes* (Harris et al., 2003; EFSA, 2011; CDC 2012). Although the high acidity would hinder the proliferation of pathogens on

fresh-cut products, growth of *L. monocytogenes* was reported on several minimally processed fruits such as apples (Alegre et al., 2010a), peaches (Alegre et al., 2010b), and oranges (Caggia et al., 2009). Non-acidic fruits as melon, watermelon, papaya and persimmon have also shown to be a good substrate for foodborne pathogens' growth (Penteado and Leitao, 2004; Uchima et al., 2008).

For this reason, several methods have been developed in the last years to fight the growth of pathogenic microorganisms including both chemicals and biological approaches (Goodburn and Wallace, 2013). Among the green strategies, the employment of antagonistic bacteria, particularly lactic acid bacteria (LAB), as biocontrol agents against human pathogens on fresh produce has been reported with encouraging results (Olaimat and Holley, 2012).

In this work we proposed fresh-cut pineapple as a new carrier to drive potential probiotic strains belonging to *L. plantarum* and *L. fermentum* species. The main nutritional, physicochemical and sensorial features of pineapple pieces were also monitored to determine if the probiotic LAB used in this study would affect the overall quality of the fresh-cut product throughout storage. The same microorganisms were also investigated for their antagonistic effect against *L. monocytogenes*.

7.3. MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus plantarum B2 (CECT 8328) and *Lactobacillus fermentum* PBCC11.5 (CECT 8448), previously isolated from sourdoughs (Arena et al., 2014; Russo et al., 2014) and deposited at the Spanish Type Culture Collection (Valencia, Spain), were routinely grown on MRS broth at 30 °C.

The type strain *Listeria monocytogenes* CECT 4031 used for the antagonistic assays were grown on TSB at 37 °C.

Preparation of the probiotic solution

The probiotic solution was obtained as reported by Rößle et al. (2010a). Briefly, microbial strains were inoculated from a cryopreserved stock (1:1000 v/v) in 4 L of MRS broth and incubated at 37 °C until the late-exponential phase (OD_{600} = 3.5) corresponding to approximately 8×10^9 CFU mL^{-1} according to previously generated standard curve. Then, cells were recovered by centrifugation (5,000 rpm x 5 min), washed twice with citric acid-sodium citrate buffer (pH 3.8) (Sigma-Aldrich, St. Louis, MO, USA) and resuspended in 2 L of the same buffer to obtain a final concentration of 1 x 10^{10} CFU ml^{-1}. Inoculum concentration was checked by plating appropriate dilutions onto MRS agar.

Inoculation of pineapple pieces with probiotics bacteria

Pineapple fruits (*Ananas comosus* L.), purchased at local markets (Foggia, Italy), were stored at 12 °C until the assays. Fruits were sorted to eliminate damaged or defective samples, and washed in tap water. Peel was manually removed with a ceramic knife, then the fruits were cored and the pulp cut into 1-cm-thick wedges. From each wedge, 8 pieces were obtained (Fig. 1A). Forty five pieces randomly selected for each treatment were dipped for 2 min in agitation in approximately 700 mL of buffer solution (citric acid-sodium buffer, pH 3.8) containing *L. plantarum* or *L. fermentum*, respectively. Control samples were plunged only in the buffer solution. After treatment, pineapple pieces were air-dried, packed in polypropylene

plastic film bags (10 x 10 cm, OTR of 1100 cm^3 m^2 24 h^{-1} bar^{-1}) each containing 15 pineapple pieces, and thermally sealed in passive-modified atmosphere packaging. Analysis were performed after 0, 3, 6 and 8 days of storage at 5 °C. All treatments were performed in triplicate.

Determination of the microbial load in artificially contaminated pineapple pieces

For microbiological enumeration, three pieces of each treatment were weighted, diluted (1:10) with saline solution (NaCl 8.6 g L^{-1}) and homogenized in a blender (Bag Mixer®, Interscience, St Nom la Bretêche, France) for 2 minutes. Then, samples were submitted to ten-fold serial dilution. *L. plantarum* and *L. fermentum* concentration was determined by plating on MRS agar after incubation at 30 °C for 48 h. Mesophilic microorganism and yeasts and moulds were enumerated by plate counting on PCA (Oxoid, Hampshire, UK) or PDA added with chloramphenicol (100 mg L^{-1}) and incubated at 25 and 30 °C for 48 h, respectively.

Antagonistic assay

Pineapples wedges were made as previously reported. From each wedge, pieces (1.5 cm x 1.5 cm) were obtained with a corel (Fig. 1B). Samples were stored at 5 °C until analysis.

Microorganisms at middle exponential phase (OD_{600} = 0.8), were collected after centrifugation (5,000 rpm x 5 min), washed twice, then resuspended in 10 mL of sterile saline solution. Viability of the microbial solution was monitored by plate counting analysis.

Each pineapple plug was spread with 15 µL of solution containing 2 x 10^7 and 2 x 10^8 CFU mL^{-1} of pathogenic and probiotic bacteria, respectively. Controls were represented by pineapples inoculated with the same concentration of single microorganism. The microbial load was evaluated at 0, 2, 5, 7 days on pineapples pieces stored at 5 °C. MRS plate agar were used to count *L. plantarum* and *L. fermentum* after appropriate serial dilutions. At the same time, ten-serial dilution of *L. monocytogenes* were enumerated on Palcam Agar, respectively.

A **B**

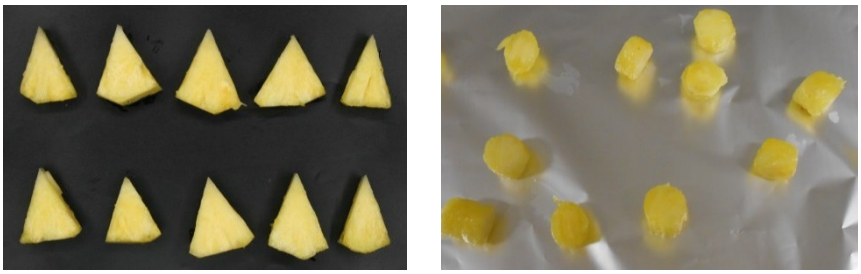


Figure 1. Pieces of pineapples used for the probiotic (A) and the antagonistic (B) assays

Physicochemical analysis

Color analysis

Color was measured elaborating the images acquired with a Spectral scanner (DV, Padova, Italia). The external surfaces of ten pineapple pieces for each replicate were scanned. The central region was manually selected. On these regions, color in CIE L*a* b* scale was measured. From the primary L*, a*, and b* values the following indexes were calculated:

- Hue angle ($h° = \arctan \frac{b^*}{a^*}$)
- Global color variation $\Delta E = \sqrt{(L_0^* - L_t^*)^2 + (a_0^* - a_t^*)^2 + (b_0^* - b_t^*)^2}$

Gas composition

Oxygen and carbon dioxide percentage inside the bags was measured in the headspace of each sample replicate using a handheld gas analyser (CheckPoint, Dansensor A/S, Denmark) during the storage time.

Firmness

Ten pieces for each replicate were cut into small cubes (10 mm side length) and compressed between two parallel plates using an Instron Universal Testing Machine (model 3340), with a crosshead speed of 30 mm x min^{-1}. Firmness of the fruit samples was defined as the rupture load of the force/deformation curve and expressed in Newton (N).

Total phenols and antioxidant capacity

The same fruit extract was used for total phenols and antioxidant activity. Total phenols were determined according to the method of Singleton and Rossi (1965). The content of total phenols was expressed as milligrams of gallic acid per 100 grams of fresh weight (mg GA 100 g^{-1}). Antioxidant assay was performed following the procedure described by Brand-Williams et al. (1995) with minor modifications. The diluted sample, 50 µL, was pipetted into 0.950 mL of DPPH solution to initiate the reaction. The absorbance was read at 515 nm after overnight incubation. Trolox was used as a standard and the antioxidant activity was reported in mg of Trolox equivalents per 100 g of fresh weight (mg TE 100 g^{-1}).

Simultaneous analysis of organic acids and sugars

Organic acids and sugars were extracted homogenizing 15 g of fresh pineapple tissue with 15 mL of ultrapure water for 1 min. The homogenate was centrifuged at 9,000 rpm for 10 minutes at 5 °C. The supernatant was filtered with a C_{18} Sep-Pak cartridge (Grace Pure ^{TM}, New York, USA) and then with a 0.2 µm filter (Incofar, Modena, Italy). Organic acids and sugars were identified using the method as described by Mena et al. (2011). The different organic acids and sugars were characterised and quantified by chromatographic comparison with analytical standards. Sugars and organic acids content were expressed as g per 100 g or mg per 100 g of fresh weight, respectively.

Total soluble solids, titratable acidity, and pH

Total soluble solids content (TSS) were measured with a digital hand refractometer (Atago, Japan). For pH and titratable acidity (TA), 5 g of juice were titrated with an automatic titrator (TitroMatic 1S, Crison, Spain). TA was expressed as per cent of citric acid (applying the acid milliequivalent factor 0.064 respectively) referred to the juice.

Vitamin C

Vitamin C content was assessed homogenising 5 g of pineapple tissue for 1 min with 5 mL of methanol/water (5:95), plus citric acid (21 g L^{-1}), EDTA (0.5 g L^{-1}) NaF (0.168 g L^{-1}). The homogenate was filtered and the pH adjusted to 2.2 – 2.4 by addition of 6 mol L^{-1} HCl. The homogenate was centrifuged at 10,000 rpm for 5 min and the supernatant was recovered, filtered through a C18 Sep-Pak cartridge

(Waters, Milford, MA, USA) and then through a 0.2 μm cellulose acetate filter. L-ascorbic acid (AA) and L-dehydroascorbic acid (DHAA) contents were determined as described by Zapata and Dufour (1992) with some modifications (Gil et al., 1999). AA and DHAA contents were expressed as mg of L-ascorbic or L-dehydroascorbic acid per 100 g of fresh weight.

Sensorial quality

A panel of six trained blind panelists carried out the sensory evaluations of fresh-cut pineapple at the processing day, and at each sampling time. Translucency, dehydration, browning, flavour, firmness, juiciness, sweetness, acidity, off-flavour, off-odors, color were evaluate using an hedonic scale from 1 to 5 where 1 = not present/very low/not typical and 5 = very pronounced/very typical of fresh fruits. For overall appearance a photographyc scale was used, which included 1 picture and a brief description for each point, with 1 = *really poor;* 2 = *browned flesh and translucent areas (limit of edibility);* 3 = *yellow flesh, slightly translucent areas (limit of marketability)*; 4 = *bright yellow flesh,* 5 = *excellent*. Every attribute was scored on a 1 to 5 scale, where 1= *absent*, 3= *moderate*; 5 = *full characteristic or fresh*.

Statistical analysis

The effect on quality parameters of treatment was tested by performing a one-way ANOVA using StatGraphics Centurion XVI.I (StatPoint Technologies, Inc., USA), and mean values within each sampling were separated applying Tukey test with significant difference when $P \leq 0.05$. Analysis of variance was performed separately for each sampling day.

7.4. RESULTS

Survival of probiotic strains in pineapple wedges

L. plantarum B2 and *L. fermentum* PBCC11.5 were tested for their ability to survive in pineapple pieces at refrigeration temperature during 8 days of storage. Strains were independently inoculated at a concentration of about 8.4 ± 0.42 log 10 cfu g^{-1}. A reduction in the survival of both inoculated strains was always observed. However, while *L. fermentum* achieved a final level of 6.3 ± 0.22 log 10 cfu g^{-1}, the survival of *L. plantarum* was higher (Figure 1). Plate count on MRS of uninoculated pineapple wedges revealed an initial contamination of about 3.5 ± 0.37 log 10 cfu g^{-1} (data not shown).

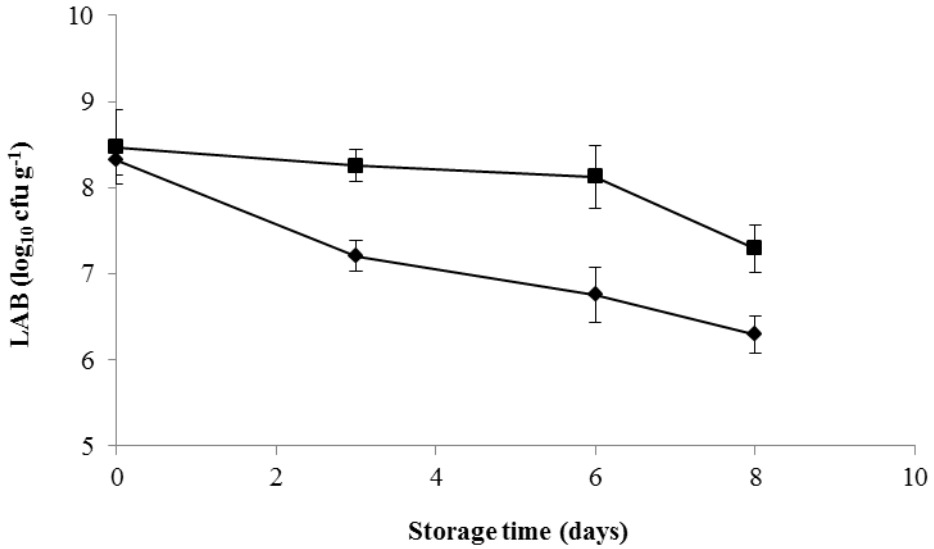
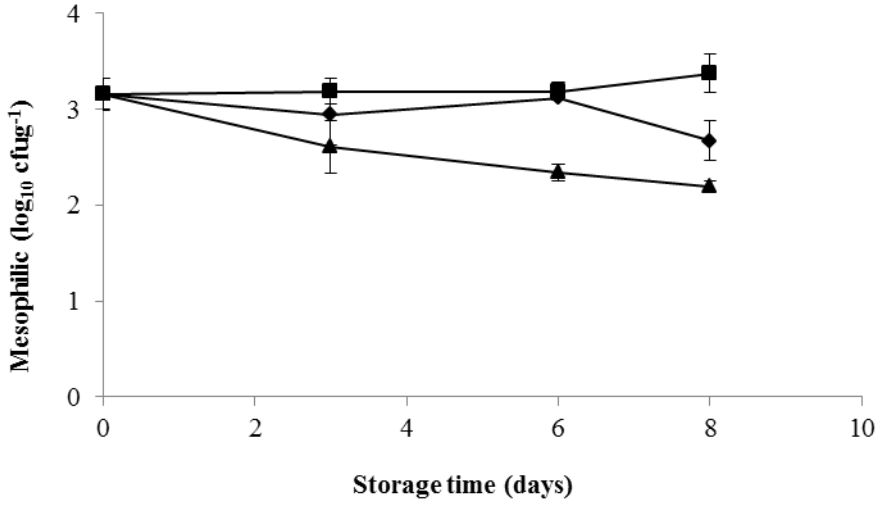


Figure 1. Population of *L. plantarum* B2 (square) and *L. fermentum* PBCC11.5 (diamond) on artificially inoculated pineapples and stored at 5 °C for 8 days. Experiments were performed in triplicate, and the standard deviations are indicated.

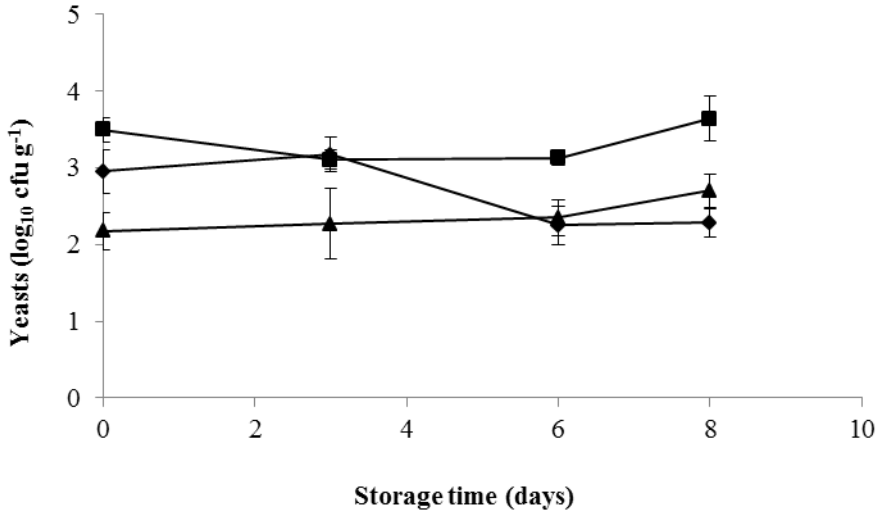
Initial mesophilic population of uninoculated pineapple wedges was 3.5 ± 0.16 log 10 cfu g^{-1}. This concentration remained almost stable during the 8 days of storage in control samples and when *L. plantarum* was added. In contrast a reduction of about

1 log was observed in samples inoculated with *L. fermentum* (Figure 2A). Similarly, yeast and moulds were found at an initial contamination level of 3.68 ± 0.42 log 10 cfu g^{-1} and no differences were found in their growth either during the storage time nor in the presence of probiotic bacteria (Figure 2B and 2C).

A



B



C

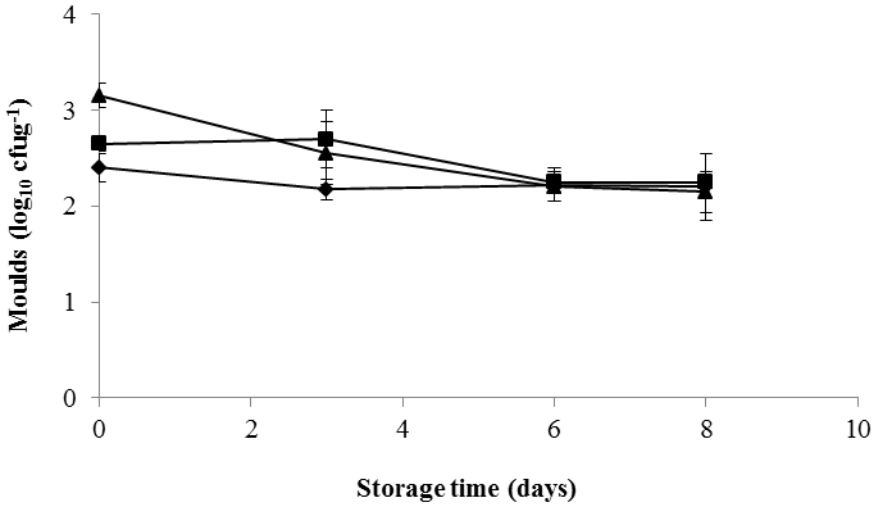


Figure 2. Population of mesophilic microorganisms (A), yeasts (B), and moulds (C) on pineapples not inoculated (diamond) or inoculated with *L. plantarum* B2 (square), *L. fermentum* PBCC11.5 (triangle) and stored at 5 °C for 8 days. Assays were performed in triplicate, and the standard deviations are indicated.

Quality evaluation

At the time of processing, pineapples had solid soluble content equal to 12 %, juice pH of 3.52 and titratable acidity of 0.68 %, expressed as citric acid.

Regarding the gas evolution inside the bags slight differences were found between the treated and control samples. Oxygen concentration dropped after two days of storage and then remained quite stable around values of 0.6-0.8 % up to the end of storage time. Carbon dioxide reached maximum values of about 18 % in all the bags with a slightly higher, but not significant, increase in samples inoculated with *L. plantarum* and *L. fermentum* (Figure 3).

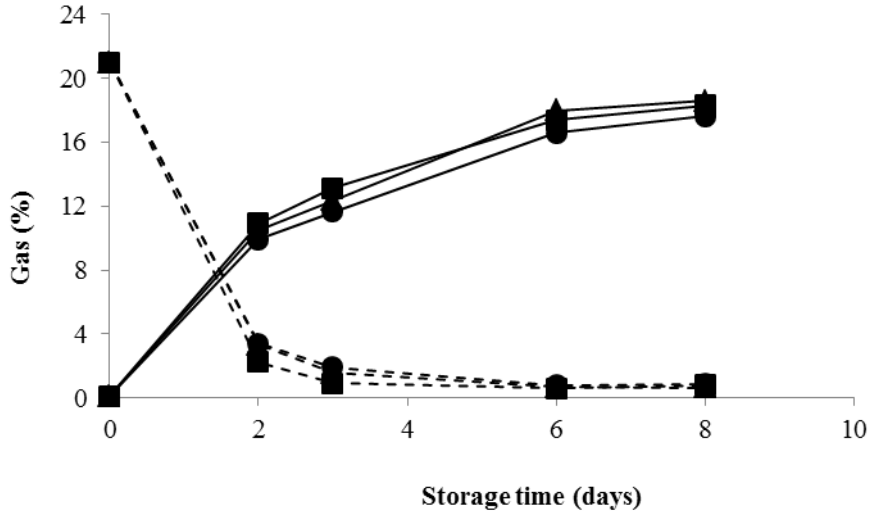
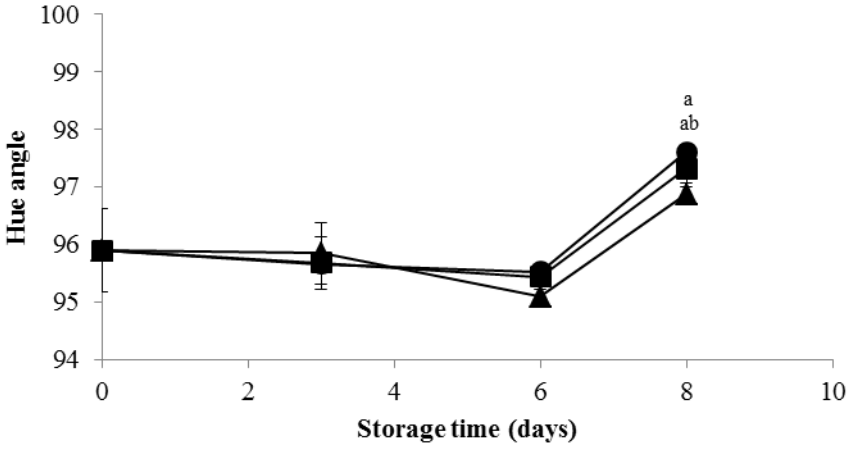


Figure 3. In-package atmosphere changes of O_2 (dashed lines) and CO_2 (continuous lines) of fresh-cut pineapples wedges untreated (circle) or inoculated with *L. plantarum* B2 (square), *L. fermentum* PBCC11.5 (triangle) and stored at 5 °C for 8 days. Data are means of three replicates for each sampling time.

Probiotic bacteria affected very little quality and composition of pineapple fruit pieces; some differences were observed in term of color, and overall appearance.
Color of the pieces showed significant differences in terms of a* and L* values (data not shown) and consequently on Hue Angle and ΔE variations. Particularly pineapple pieces inoculated with *L. fermentum* showed at the end of the storage less variation of a* values than control samples (Figure 4A), which in turn induced less color variation. Hue angle increased during storage for all treatment going from 96 to 98 °C, meaning a reduction of the yellow component, but with a minor extent for pieces inoculated with *L. fermentum* than for control pieces, while pieces inoculated with *L plantarum* showed intermediated results . This difference was not evident in terms of global color variation expressed as ΔE (Figure 4B), where the change in L* values are accounting for the major part of the variation. The samples treated with the probiotic strains, in fact, showed an higher reduction of L* values (data not shown) and as a consequence, higher value of ΔE compared to the untreated pineapples**.**

A



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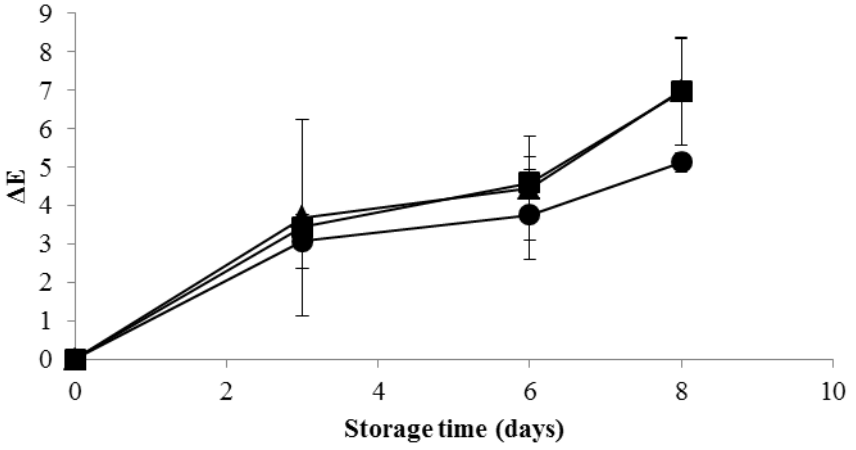


Figure 4. Color parameters evolution (Hue angle (A), ΔE (B)) of fresh-cut pineapple pieces untreated (circle) or inoculated with *L. plantarum* B2 (square), *L. fermentum* PBCC11.5 (diamond), and stored for 8 days at 5 °C. Reported values are means of ten pieces for each replicate for each sampling time. Means with different letters at the same time of storage are significantly different according to the Tukey test (P value ≤ 0.05).

From a sensorial point of view dipping in probiotic-enriched solution did not significantly affect neither the organoleptic characteristics of fresh-cut pineapples nor most of the external attributes (color, translucency and browning), despite some difference observed instrumentally for color. As reported in the radar graph (Figure5) the panelists did not observe any off-flavour or off-odor development in all the samples at the end of storage, as well as any sign of browning. Compared to initial values the judges observed a significant ($P \leq 0.05$) reduction of firmness and overall appearance after 8 days. Moreover, control and samples inoculated with *L. fermentum* still maintained a score higher than the limit of marketability (score 3), whereas *L. plantarum* inoculated pineapple pieces reached at the end of storage an average score of two. Also for pineapple firmness the panelist observed a significant reduction after 8 days of storage, if compared to the initial values, but without differences among the treatments. Also no difference among treatments was instrumentally observed on firmness.

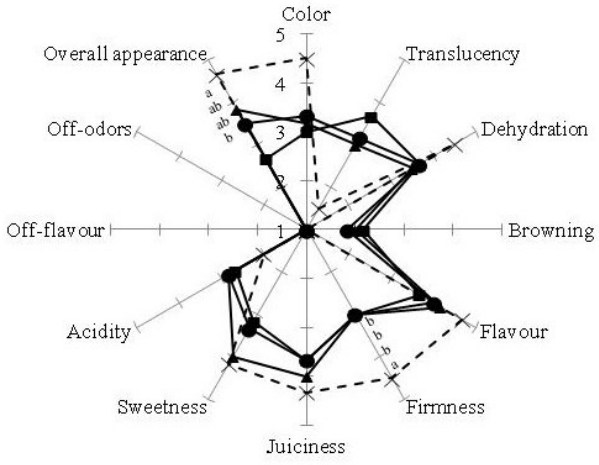


Figure 5. Sensory properties of fresh-cut pineapple pieces inoculated with *L. plantarum* B2 (square), *L. fermentum* PBCC11.5 (triangle), or not inoculated (circle) and stored for 8 days at 5 °C. Dashed line is referred to the initial values (day 0). Reported values are means of three replicates for each sampling time. Means with different letters are significantly different according to the Tukey test (p-value ≤ 0.05).

The evolution of antioxidant compounds and sugar and acids is reported in Table 1A and B, respectively. Total phenolics after an initial decrease, remained constant until the end of the trial and reached an average value of about 31 mg 100 g^{-1} of gallic acid. The treatment with *L. fermentum* had a higher level of antioxidant capacity of the after 6 days of cool storage, but not at the end when samples treated with *L. fermentum* showed the lowest antioxidant capacity. Concerning the sugars and organic acids content of fresh-cut pineapple any significant difference was observed, except for tartaric acid (Table 1B). For control samples and fruit pieces inoculated with *L. plantarum* sucrose concentration decreased during storage, from values of 2.9 to 2.35 and 1.9 g 100 g^{-1}, respectively, whereas fructose and glucose increased. In pineapples pieces inoculated with *L. fermentum*, sucrose content kept constant during storage at value of about 2.5 g 100 $g^{-1.}$. Regarding the organic acids, the content of tartaric acid at the third day of storage was lower in fruits inoculated with *L. fermentum* compared to *L. plantarum.* The trends of all the monitored acids were quite variable and not dependent on the type of treatment.

Antagonistic assays

In order to assess the antagonistic effect of *L. plantarum* B2 and *L. fermentum* PBCC11.5 on relevant pathogenic bacteria, the growth of *L. monocytognes* was monitored in pineapple pieces during a time of seven days when inoculated alone or in combination with each probiotic. *L. monocytogenes* CECT 4031 population was 7.16 ± 0.37 log 10 cfu g^{-1} and promptly decline about 1.5-log units and then rises at 6.61 ± 0.41 log 10 cfu g^{-1} if inoculated alone. When *L. monocytogenes* CECT 4031 was co-inoculated with the antagonistic *L. fermentum* strain, a fast reduction was observed after three days, followed by a further decrease at the all monitored steps until a final concentration of around 2-log units lower (4.67 ± 0.20 log 10 cfu g^{-1}). In contrast, the antagonistic effect of *L. plantarum* on *L. monocytogenes* CECT 4031 was barely more marked at three days of storage then increase to about 5.37 ± 0.12 log 10 cfu g^{-1} (Figure 6).

L. plantarum population remains almost constant after 7 days of storage when inoculated alone, while pineapple pieces inoculated with *L. fermentum* showed a

reduction of about 0.5-log in the final level. In contrast, the co-inoculum approach performed with *L. monocytogenes* CECT 4031 resulted in decrease of the *L. plantarum* and *L. fermentum* microbial population of around 1-log (Figure 7A and 7B).

Table 1. Chemical properties (antioxidant compound content (A), sugars and organic acids (B)) of fresh-cut pineapple pieces treated or inoculated with L. *plantarum* B2, *L. fermentum* PBCC11.5, and stored for 8 days at 5 °C. Reported values are means of three replicates for each sampling time. Means with different letters at the same time of storage are significantly different according to the Tukey test (P value ≤ 0.05).

A

		Antioxidant compound content				
	Day	**Ascorbic Acid** (mg/100g fw)	**Dehydroascorbic acid** (mg/100g fw)	**Vitamin C** (mg/100g fw)	**Total phenols** (gallic acid mg/100g fw)	**Antioxidant Capacity** (Trolox eq. mg/100g)
	0	20.68±3.59	3.21±1.25	23.89±4.72	41.49±5.19	42.39±3.39
Control	3	16.56±1.68	4.22±0.54	20.78±1.72	32.72±5.04	50.86±1.82
L. plantarum B2	3	12.65±4.72	5.84±1.53	18.49±5.94	30.65±4.14	51.12±2.21
L. fermentum PBCC11.5	3	16.82±3.14	5.59±0.74	22.41±3.16	32.26±3.94	51.03±3.69
Control	6	12.24±2.74	4.81±0.81	17.06±2.47	31.42±2.51	48.90±1.52^{b}
L. plantarum B2	6	11.90±3.43	4.84±1.31	16.74±4.74	31.40±4.64	48.59±2.31^{b}
L. fermentum PBCC11.5	6	11.30±1.18	3.25±0.29	14.55±0.97	26.90±2.70	55.43±2.09^{a}
Control	8	15.35±4.52	4.99±0.82	20.34±4.53	30.04±0.87	54.23±0.10^{a}
L. plantarum B2	8	14.48±4.08	4.63±0.76	19.11±4.84	33.06±2.21	49.80±2.49^{ab}
L. fermentum PBCC11.5	8	14.41±3.77	4.04±0.79	18.45±4.35	30.76±2.37	47.21±2.77^{b}

B

	Day	Sugars			Organic acids			
		Sucrose	Glucose	Fructose	Citric acid	Tartaric acid	Malic acid	Succinic acid
		g $100g^{-1}$	g $100g^{-1}$	g $100g^{-1}$	mg $100g^{-1}$	mg $100g^{-1}$	mg $100g^{-1}$	mg $100g^{-1}$
	0	2.80± 0.24	0.53± 0.05	0.09± 0.01	149.50± 7.53	10.02±0.47	161.50± 12.65	5.90±2.01
Control	3	2.91± 0.30	0.63± 0.09	0.10± 0.01	209.20± 32.68	11.86± 1.04[ab]	165.23± 20.17	10.97± 2.39
L. plantarum B2	3	2.87± 0.36	0.65± 0.06	0.11± 0.01	197.64± 30.87	12.30± 1.04[a]	165.46± 10.73	11.48± 2.91
L. fermentum PBCC11.5	3	2.50± 0.36	0.63± 0.07	0.09± 0.01	149.64± 20.64	9.68±0.46[b]	143.49± 13.05	7.02±0.18
Control	6	3.19± 0.57	0.73± 0.10	0.12± 0.02	217.74± 69.87	12.17±2.27	166.92± 29.63	11.16± 5.11
L. plantarum B2	6	2.70± 1.08	0.60± 0.16	0.10± 0.02	177.82± 29.03	11.33±4.01	149.95± 26.03	11.01± 1.97
L. fermentum PBCC11.5	6	2.53± 0.38	0.72± 0.09	0.12± 0.01	190.22± 31.95	11.13±0.47	163.92± 18.43	12.01± 2.82
Control	8	2.35± 0.59	0.76± 0.15	0.11± 0.03	156.63± 43.64	11.42±4.62	158.57± 39.72	7.69±5.09
L. plantarum B2	8	1.90± 0.04	0.65± 0.03	0.10± 0.01	175.76± 50.59	10.41±1.52	142.42± 26.01	8.22±4.39
L. fermentum PBCC11.5	8	2.48± 1.14	0.69± 0.15	0.10± 0.03	158.78± 19.06	11.60±3.63	140.58± 32.76	11.50±2.61

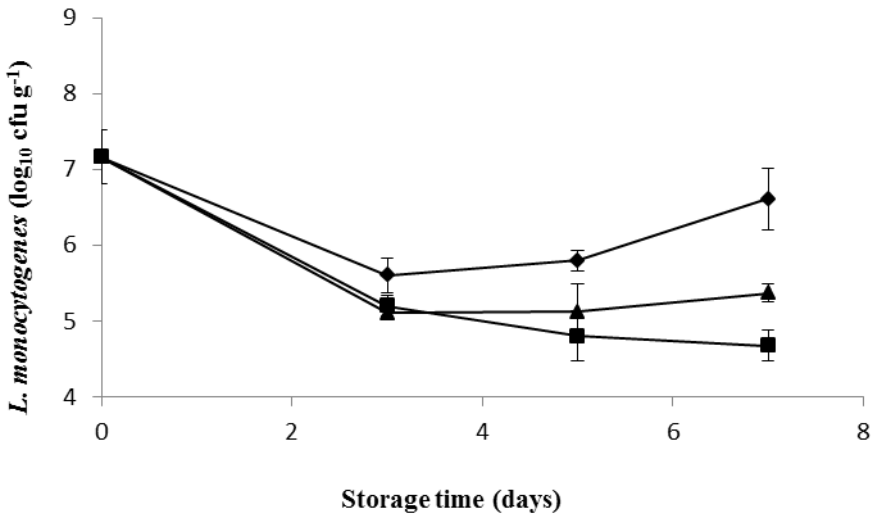
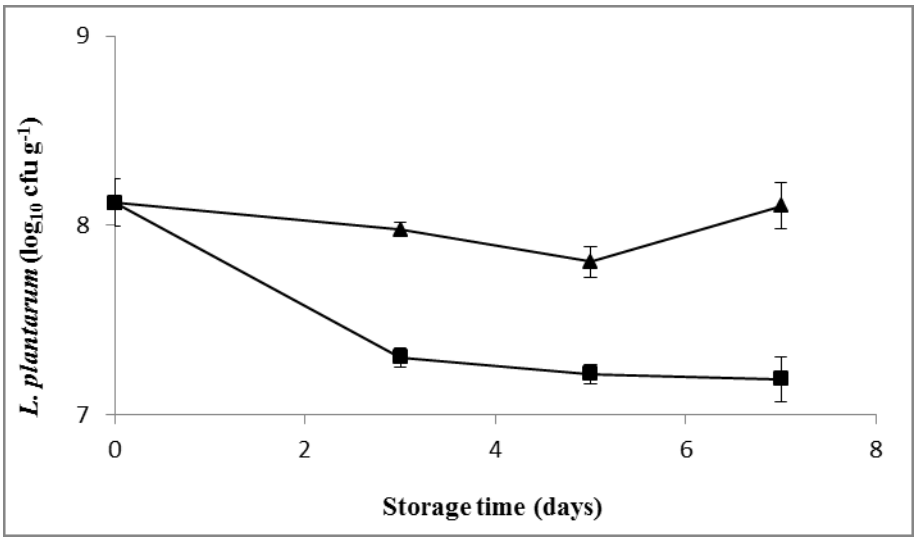


Figure 6. *E. coli* O157:H7 (A) and *L. monocytogenes* (B) population on pineapple pieces not inoculated (diamond) or co-inoculated with *L. plantarum* B2 (square), *L. fermentum* PBCC11.5 (triangle) and stored at 5 °C for 7 days. Experiments were performed in triplicate, and the standard deviations are indicated.

A



B

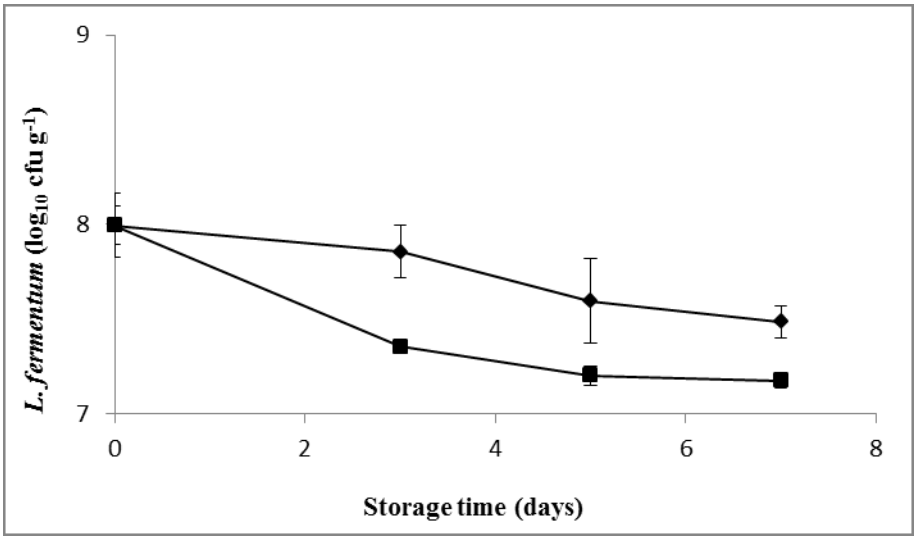


Figure 7. *L. plantarum* B2 (A) and *L. fermentum* PBCC11.5 (B) population on pineapple pieces not inoculated (diamond) or co-inoculated with *L. monocytogenes* (square), *E. coli* O157:H7 (triangle) and stored at 5 °C for 7 days. Experiments were performed in triplicate, and the standard deviations are indicated.

7.5. DISCUSSION

Pineapple is one of the most important tropical fruits in the world. However, the large size, the high percentage of the inedible parts and the difficulty of peeling operations, reduce the consumer acceptability, necessitating also large space for storage (James and Ngarmsak, 2010). Therefore, processing pineapple into ready-to-eat product is an attractive alternative for the market (Azarakhsh et al., 2014). At the same time the increasing interest for healthy food makes particularly charming the production of fresh cut fruits as a carrier for probiotic microorganisms. Recently, some attempts were carried to obtain functional foods adding to the natural freshness and nutritional value of minimally processed papaya and apples the beneficial effect of probiotic (Tapia et al., 2007; Rößle et al., 2010a; Alegre et al., 2011). Actually, the most common probiotic used and marketed in food worldwide belong to the genera *Lactobacillus* and *Bifidobacterium*. However, only few strains including the commercial *Bifidobacterium lactis* BB12 and *Lactobacillus rhamnosus* GG have been added to minimally processed fruit (Tapia et al., 2007; Rößle et al., 2010a; Alegre et al., 2011). In the present work we inoculated pineapples pieces with *L. plantarum* B2 and *L. fermentum* PBCC11.5 from vegetable origin and previously characterized for their probiotic potential (Arena et al., 2014). Probiotics can play their beneficial role if reach the gut lumen in a number enough to provide health gain to the host. It is considered that a food product should contain a concentration of viable cells not less than 10^6 cfu g^{-1} to be efficacious (Champagne et al., 2011). According to this condition, we observed that after 8 days at 5 °C, a possible expiration date for this kind of product, the survival of *L. plantarum* and *L. fermentum* on pineapples pieces ranged between 7.3 and 6.3 log cfu g^{-1}, respectively. Similarly, Rößle et al. (2010b) found that *L. rhamnosus* GG level in apple wedges was around 7.0 log 10 cfu g^{-1} after 10 days of storage at refrigeration temperature. This microbial concentration remained approximately constant even after 28 days of storage (Alegre et al., 2011). Comparable results are reported by Tapia and coauthors, (2007) using *Bifidobacterium lactis* BB12 as probiotic strain. As previously reported (Alegre et al., 2011; Rößle et al., 2010b) we inoculated microorganisms on pineapples pieces by immersion in a dipping solution

containing organic acid as browning inhibitor. Dipping is a processing step generally conducted after peeling and/or cutting to extend the fresh-cut fruit shelf life with the aim to add antimicrobial and antibrowning agents and texture preservatives (Martín-Belloso et al., 2012). Therefore, a dipping solution enriched with high concentration of lactic acid bacteria could be a practical and inexpensive way to obtain minimally processed probiotic fruits.

The main physical and chemical parameters were evaluated to determine if the addition of high amount of the probiotic LAB used in this study could affect the quality of pineapple fresh-cut without observing any particular problem due to this fruit manipulation. Beside some differences after 3 days on tartaric acid content and the antioxidant capacity, no other differences were observed on pineapple composition. Moreover tartaric acid was not increased by the bacterial activity, but decreased in *L. fermentum* treated pieces compared to control and *L. plantatum* treatments. Slight variation in color did not affected sensorial evaluation, except for overall appearance which at the end of the storage was higher in fruits inoculated with *L. fermentum* than *L. plantarum* but generally did not impacted clearly the main sensorial features of minimally processed pineapples, according to previously observed for apple wedges (Rößle et al., 2010a; Alegre et al., 2011). Overall, the panelists did not observe any off-flavour or off-odor development in all the samples at the end of storage, demonstrating that high concentrations of probiotic bacteria had no effect on the degradation rate of the sensory properties of the product, and this is in accord what reported by Rößle et al. (2010a) where panelists did not express a preference for pineapples containing probiotic bacteria over control pineapples.

It is known that the addition of probiotic LAB to ready to eat fruits could be a hurdle to the development of foodborne pathogens. In the last years, the employment of LAB has been proposed as an emerging bioprotective technology in fresh-cut fruit production. Trias et al., (2008b) reported that eight-teen LAB strains mainly belonging to *Leuconostoc* spp. and *Lactobacillus plantarum* were able to strongly inhibit the growth of foodborne human pathogens on Golden Delicious apples. Recently, Alegre et al., (2011) found that co-inoculation on apple wedges of

the probiotic *L. rhamnosus* GG reduced the growth of *L. monocytogenes* of about 1-log unit, while no effect was detected on Salmonella population. A similar antagonism against *L. monocytogenes* and *E. coli* O157:H7 was observed for the non-LAB strain *Pseudomonas graminis* CPA-7 (Alegre et al., 2013a). In a recent study, strains of *L. plantarum* and *L. fermentum* characterized for their probiotics properties exhibited antagonistic activity towards relevant foodborne pathogens including *E. coli* and *L. monocytogenes* (Ramos et al., 2013). Therefore, with the aim to simulate the antagonistic effect on the growth of *L. monocytogenes* in a probiotic food, we inoculated pineapple pieces with the same concentration of *L. plantarum* and *L. fermentum* previously tested to obtain the enriched with probiotic fruits. A level 1-log lower of pathogenic bacteria was used for co-inoculum. This is according to Alegre et al. (2013b) that found the more significant growth inhibition of *Listeria innocua* by *P. graminis* CPA-7 co-inoculating a level of 10^7 and 10^8 cfu mL^{-1} respectively, suggesting that CPA-7 should be present in at least the same amount as the pathogen to adequately reduce its level. Furthermore, in a semi-commercial trials the same authors reported an increase of the dynamic population of *L. monocytogenes* over 6.0 log cfu g^{-1} after 7 days of storage at 5 °C (Alegre et al., 2013a). However, if the storage temperature was 10 °C, *L. monocytogenes* reached a concentration of approximately 7.0 log cfu g^{-1} after a week (Alegre et al., 2013a), supporting the importance of a correct management of the cold chain during the shelf life of fresh-cut fruits. However, cold chain abruption with extended use after expiration date is a probable scenario and an incidence between 10 and 20 % of houses and commercial refrigerators working at a temperature >10 °C it was reported (Kovačević et al., 2013). In the present work, we observed that the viability of *L. plantarum* and *L. fermentum* inoculated on pineapple pieces remains almost constant over the time, further confirming their potential application to enrich fresh-cut fruits. The co-inoculum with *E. coli* O157:H7 not seems affect the dynamic population of the tested LAB. In contrast, *L. monocytogenes* reduced of about 1-log the level of both *L. plantarum* and *L. fermentum*. According, to this result *L. monocytogenes*, after an initial decline was able to growth during the storage time, suggesting a mechanism of competition with the probiotic strain.

However, we showed that *L. plantarum* was able to inhibit the growth of both pathogens, while *L. fermentum* was effective only against *L. monocytogenes*.
Therefore, our results suggest that *L. plantarum* and *L. fermentum* strains could be successfully employed for the elaboration of minimally processed pineapples within eight-day shelf life, contributing at the same time to carry a protective effect against relevant foodborne pathogens. Thus, this work fits in an attempt to expand the range of both food matrices and probiotic strains in order to obtain new functional foods.

SECTION 3.
CONCLUSION

8. CONCLUSION

8.1. Final conclusions

In the European Union (EU), listeriosis is a relatively rare but serious food-borne illness in humans, with high morbidity, hospitalization and mortality in vulnerable populations. The bacterial genus *Listeria* currently comprises several species, but human cases of listeriosis are almost exclusively caused by *Listeria monocytogenes*. The main route of transmission to humans is believed to be through consumption of contaminated food. The bacterium can be found in raw and processed foods that are contaminated during and/or after processing. The fact that *L. monocytogenes* is able to multiply in various foods at temperatures as low as 2 to 4 °C makes its occurrence in minimally processed foods of particular concern (Gandhi and Chikindas, 2007). In addition, the ability of *L. monocytogenes* to form biofilms pose a serious threat to the safety of fresh-cut products as they can persist for long periods of time in the food processing environment and thus represent a source of recurrent cross contamination at plant level (da Silva and De Martinis, 2013).

Fresh-cut packaged fruits and vegetables are generally considered a product of high quality and freshness. However they may represent an underestimated public health risk due to the possible occurrence of pathogenic *Listeria monocytogenes* because they are consumed raw and are susceptible to be contaminated by fecal material and soil on the farm (Francis et al., 2012). Minimally processed vegetables are often used as ingredients to prepare salads, which may have longer refrigerated shelf lifes, with risk of increasing of *L. monocytogenes* population (Sant'Ana et al., 2012a).

Currently, the EU microbiological criterion for *L. monocytogenes* is set as ≤100 cfu g^{-1} for ready to eat products on the market (Regulation (EC) No 2073/2005).

The official recommended methods to detect *L. monocytogenes* in foods are developed by the ISO 11290:1 (anonymus, 1996), the US Department of Agriculture–Food Safety and Inspection Service (USDA–FSIS), the Federal Drug Administration (FDA), or the Netherlands Government Food Inspection Service (NGFIS), which are carried out by culture-dependent methods. These procedures

can be coupled with the Most Probable Number (MPN) method in order to enumerate *L. monocytogenes* at low concentrations (up to 10^3 CFU g^{-1}) in foods. Thus, conventional methods requires, on average, five days for determination of a negative result for *L. monocytogenes* contamination. If a positive result occurs, additional days are required for biochemical tests to identify the species (Churchill et al., 2006). Therefore, the official methods may take 7-10 days to detect *L. monocytogenes*, a time corresponding approximately to the shelf life of the product itself. For this reason, a rapid detection and quantification of the pathogenic bacteria *L. monocytogenes* is a key issue to ensure the food safety to the consumers.

In the present work we developed a MPN quantitative real-time PCR (MPN-qPCR) method for a fast and reliable detection and quantification of *Listeria monocytogenes* in artificially contaminated minimally processed vegetables. With the proposed method we are able to quantify *L. monocytogenes* when inoculated a concentration of as low as 1 CFU g^{-1} after 48 hours with a gain of about 4-5 days compared to the official method. Furthermore, if qPCR was not associated to MPN enumeration, we can detect a limit of 10^1 CFU g^{-1} after only 2 hours of enrichment of *L. monocytogenes* in a selective media. A reduction of the time to detect *L. monocytogenes* in fresh-cut samples is of great interest, since ensure a major safety to the consumers, and at the same time it is a competitive advantage for the companies. From an industrial perspective the prevalence of *L. monocytogenes* on fresh-cut sold at markets is a serious threat because it determines a significant economic harm due i) to the loss of the products; ii) to its recall from the market; iii) to the damage of the company's image impacting its intangible capital. Furthermore, with the aim to propose a method for routine analysis, we optimized the protocol taking into account the most suitable method in terms of cost, efficiency, times for analysis, handling and production of toxic wastes.

As previously reported, fresh-cut contamination can occur at pre- and post-harvest levels (Dallaire et al., 2006), and several studies showed an increase of *L. monocytogenes* concentrations on fresh-cut vegetables stored under different conditions (Sant'Ana et al., 2012a; Likotrafiti et al., 2013;Vandamm et al., 2013). Therefore, the proposed methodology was adopted for the microbiological control

of *L. monocytogenes* in minimally processed vegetables and fruits, currently available on the market, at three different stages: raw, after processing and at three days of shelf life. Thus, in this study we monitor the prevalence of *L. monocytogenes* on rocket and mix salads due to their worldwide spread on the market. In addition, we analyzed a variety of fresh-cut melons, since contaminated cantaloupe were recently involved in the most important multistate listeriosis outbreak of the last years (CDC, 2012). This activity was carried out in collaboration with two companies from Italy and Portugal, with the aim of demonstrating the potential of this method for its implementation in the food industry.

This focus could be interesting for industrial purposes since enumeration of the pathogenic microorganisms can provide an estimation of the efficacy of sanitizers treatment and represent an alarm bell to reduce the risk of cross contaminations in the plant. A fast detection of *L. monocytogenes* could improve the internal quality control assessment allowing for example a promptly intervention on presumptive contaminated product and/or a more drastic sanitization of the plant.

Over the last years, there is clearly an urgent need to develop new and effective methods, which are regarded as safe and eco-friendly, to control the postharvest increase of foodborne pathogens. Among these, the occurrence of microorganisms with inhibitory properties could improve the shelf life and safety of vegetable products while reducing the need to use increasing levels of chemical additives (Schuenzel and Harrison, 2002). Growth of foodborne pathogens on fresh-cut vegetables and fruits has been prevented using epiphytic microorganisms including some species of lactic acid bacteria (Abadias et al., 2009; Trias et al., 2008a; Alegre et al., 2013b), that can play a protective role against pathogens in the product itself during storage by competing with pathogens for nutrients (vitamins, minerals, trace elements and peptides), producing organic acids and bacteriocins (Alegre et al., 2011).

In the present study we showed the ability of two *Lactobacillus* strains, belonging to the species *L. plantarum* and *L. fermentum*, to reduce the proliferation of *L. monocytogenes* on fresh-cut pineapples without affect the global quality of the

product along conservation. Interestingly, the LAB strains used in this study were previously characterized for their probiotic features (Arena et al., 2014), suggesting that fresh-cut pineapples could be used as a new carrier to vehicle probiotic microorganisms. With a similar approach, Alegre et al., (2011) proposed to enrich apple wedges with the probiotic *Lactobacillus rhamnosus* GG to contrast the growth of two foodborne pathogens (*L. monocytogenes* and *Salmonella*).

This is an interesting focus, since although a wide amount of new food formulations such as probiotic yogurt-like, soy- and cereal-based fermented products are emerging in addition to the traditional employment of probiotics to produce milk-based foods (Divya et al., 2012), only few products of vegetable origin have been proposed with this aim (Martins et al., 2013).

In addition, pineapple is one of the most important tropical fruits in the world, that it is often commercialized as minimally processed due to logistical reasons (large space for storage and transport) and an increasing acceptability of the consumer (difficulty of peeling operations). From a technological point of view, the addition of probiotic microorganisms to pineapples pieces could be achieved during dipping, a processing step generally conducted after peeling and/or cutting to extend the fresh-cut fruit shelf life with the aim to add antimicrobial and antibrowning agents and texture preservatives (Martín-Belloso et al., 2012).

Therefore, our results suggest that the addition of probiotic LAB could be successfully employed to protect fresh-cut pineapples from the risk of the *L. monocytogenes* growth, contributing at the same time to increase the healthy value of the product.

In conclusion, we believe that this thesis may represent a further step to confirm the strength of molecular methods as a useful and powerful tool to complement conventional methods for a rapid detection of foodborne pathogens in fresh-cut vegetables suggesting their routine implementation in the food industry. Finally, this work fits in an attempt to expand the range of both food matrices and probiotic strains in order to obtain new and more safety minimally processed foods.

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8.3 APPENDIX

Articles in journals with peer-review

1. **P. Russo**, G. Botticella, M. L. Amodio, G. Colelli, M. Cavaiuolo, A. Ferrante, S. Massa, G. Spano and L. Beneduce. Detection and Enumeration of *Listeria monocytogenes* in Fresh Cut Vegetables Using MPN-Real-Time PCR. *Acta Horticolturae* (in press).
2. **P. Russo**, G. Botticella, V. Capozzi, S. Massa, G. Spano and Luciano Beneduce. A fast, reliable and sensitive method for detection and quantification of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in ready-to-eat fresh cut products by MPN-qPCR. *BioMed Research International* (submitted).
3. **P. Russo**, M. L. V. de Chiara, A. Vernile, M. L. Amodio, M. P. Arena, G. Spano and V. Capozzi. Fresh-cut pineapple as a new carrier of probiotic lactic acid bacteria. *BioMed Research International* (submitted).

Book chapters

1. G. Botticella, **P. Russo**, V. Capozzi, M.L. Amodio, S. Massa, G. Spano and L. Beneduce. *Listeria monocytogenes*, biofilm formation and fresh-cut produce. In: A. Méndez Vilas ed. *Microbial pathogens and strategies for combating them: science, technology and education*. Badajoz, Formatex, 2013;114-123.

Proceedings of symposia

1. M. Cavaiuolo, A. Ferrante, **P. Russo**, L. Beneduce, G. Spano, S. Paramithiotis, A. Hadjilouka, P. Tzamalis and E. H. Drosinos. Validation of innovative methods for the detection of human pathogenic bacteria in fresh cut vegetables. 3rd International Conference on Effects of Pre- and Post-harvest Factors on Health Promoting Components and Quality of Horticultural Commodities. 24-25 March, 2014, Skierniewice (Poland).
2. **P. Russo**, A. Vernile, L. Beneduce, M.L. Amodio, G. Colelli, G. Lasalandra, G. Spano and S. Massa. Prevalence of *Listeria* spp., *Listeria monocytogenes*, *Esherichia coli* O157:H7 and *Esherichia coli* O104:H4 in mixed lettuce and

rocket leaves sold in apulian markets. III convegno nazionale Società Italiana di Microbiologia Agraria, Alimentare e Ambientale (SIMTREA). 26-28 June, 2012, Bari, Italy, pag. 144.

3. **P. Russo**, A. Vernile, M.L. Amodio, G. Colelli, S. Massa and G. Spano. MPN-QRTPCR method and quantification of *Listeria monocytogenes* in ready to eat vegetables. III convegno nazionale Società Italiana di Microbiologia Agraria, Alimentare e Ambientale (SIMTREA). 26-28 June, 2012, Bari, Italy, pag. 143.
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Oral communications

1. G. Botticella, **P. Russo**, M.L. Amodio, G. Colelli, M. Cavaiuolo, A. Ferrante, S. Massa, G. Spano, and L. Beneduce. Detection and enumeration of *Listeria monocytogenes* in fresh-cut vegetables using MPN - Real Time PCR and an enrichment free approach. XI International Controlled & Modified Atmosphere Research Conference. 3-7 June 2013, Trani, Italy, pag. 29.

Demonstration session

(in the framework of 7FP project QUAFETY-Comprehensive approach to enhance quality and safety of ready-to-eat fresh products)

1. **P. Russo**. Innovative diagnostic kit and procedures for bacteria detection in fresh cut vegetables. Workshop non-destructive methods for evaluation quality and safety of ready to eat fresh products. Escola Superior de Biotecnologia da Universidade Católica Portuguesa, Porto, 14 November 2013.

2. **P. Russo**. Innovative diagnostic kit and procedures for bacteria detection in fresh cut vegetables. Agronomia s.r.l. (San paolo d'Argon, Bergamo, 12 November 2013); NoviFruits s. l. (Lisbona, 13 November 2013).

International course

5° European short-course on "Quality and Safety of Fresh-cut Produce". Berlin, 6/8, February, 2012.