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# Growth of *Listeria monocytogenes* in ready to eat salads at different storage temperatures and valuation of virulence genes expression

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*Key words: Challenge test, food safety, Listeria monocytogenes, ready to eat salad, virulence genes*
*Parole chiave: Challenge test, sicurezza alimentare, Listeria monocytogenes, insalata pronta al consumo, geni di virulenza*

**Abstract**

***Background.*** *Vegetables are major components of a healthy and balanced diet. However, 25% of foodborne diseases are linked to the consumption of vegetables.*
***Study design.*** *The aim of this work was to assess the microbiological risks associated with consumption of ready to eat salads (RTE).*
***Methods.*** *Microbiological challenge tests were carried out for the evaluation of the L. monocytogenes growth potential in RTE salads stored at different temperatures.*
***Results.*** *The results indicate that L.monocytogenes was able to grow ($\delta \geq 0.5$) in all storage conditions considered at the end of shelf life. In order to evaluate the virulence role of L. monocytogenes, the temperature-dependent transcription of major virulence genes was also investigated by RT-PCR.*
***Conclusion.*** *The microbiological challenge test allowed us to confirm, as also demonstrated by other authors, that RTE salads are able to support the growth of L. monocytogenes strains ($\delta \geq 0.5$) stored under different temperatures.*

## Introduction

Processed ready-to-eat (RTE) vegetables with a prolonged shelf-life under refrigeration are good media for the growth of psychrophilic microorganisms (1). Surveillance data have indicated that vegetables were implicated in foodborne disease outbreaks caused by a variety of pathogenic microorganisms (2-4) such as *Listeria monocytogenes* (5-8). *L. monocytogenes* has been isolated from environmental sources, including food-processing environments (9-11). According to the European annual epidemiological report on sources of zoonoses, zoonotic agents and foodborne outbreaks, *L. monocytogenes* is one of the main causes of hospitalization and death in Europe (12). Most human Listeriosis cases appear to be caused by consumption of ready-to-eat foods (RTE) contaminated with high levels of *L. monocytogenes* (13-16). Current European legislation (17, 18) specifies microbiological criteria for *L. monocytogenes* in RTE. According to these regulations (17, 18), products with a shelf-life of less than five days, which are unable to support *L. monocytogenes* growth, are considered to be RTE. Considering the

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current wide distribution and consumption of RTE foods, producers should carry out challenge tests as defined by EC Reg. n. 2073/2005 (17), to establish the ability of individual foods to support the growth of *L. monocytogenes*. Previous studies have reported low prevalence and counts of *L. monocytogenes* in RTE vegetables (5, 6, 8) however, concentrations as high as $10^6$ CFU/g may be reached depending on storage conditions (19-21). *L. monocytogenes* infection is probably associated with the ingestion of high concentrations of this pathogen in healthy individuals (> 8 log CFU) or low concentrations in susceptible individuals (2-3 log CFU) (22-24). More information about the growth potential ($\delta$) of RTE vegetables could be very useful for identifying the critical storage conditions required to prevent pathogen growth to unacceptable levels in these products.

The infectious process is dependent on the production of several virulence factors. These include the hemolysin (hlyA, listeriolysin O) necessary for pore-forming, two distinct phospholipase (plcA, plcB) proteins involved in intracellular life, such as those responsible for escaping from phagosome. Moreover, they also include a protein responsible for motility (actA), and several internalin (inlA, inlB) proteins necessary for the adhesion and invasion of eukaryotic cells (14, 25, 26). Nevertheless, it is assumed that virulence is not a stable property and can be influenced by environmental conditions (temperature, water activity, pH, NaCl) (27). Many environmental factors may increase or decrease the virulence expression of *L. monocytogenes* (28, 29). Nearly all cases of human listeriosis have been associated with the consumption of contaminated food, and, therefore, the investigation of the virulence of *L. monocytogenes* after exposure to environmental conditions in food matrices is critical in order to understand and control its impact on public health (30, 31). The foodborne pathogen *L. monocytogenes* can grow in a wide range of temperatures, and several key virulence determinants of the organism are expressed at 37°C, but strongly repressed below 30°C (32). The aim of this work was to assess the microbiological risks associated with the consumption of RTE salads. Microbiological challenge tests were carried out for the evaluation of the *L. monocytogenes* growth potential in RTE salads stored at different temperatures. The virulence expression of major *L. monocytogenes* virulence genes (*inlA, inlB, hlyA, actA, plcA, plcB*) at different temperatures was also investigated.

## Methods

### *1. Salad composition*

The sample of ready-to-eat salads only contained fresh mixed vegetables (radicchio 25%, endive 50%, chicory 25%) packaged in plastic food containers.

### *2. Challenge tests*

The study was conducted on 180 pre-packaged mixed raw vegetables collected from local retail shops in Sardinia. Samples were randomly selected from three different batches (60 from each batch). The RTE vegetable samples were packed in flexible packaging and transported to the laboratory and stored at 4 ± 2 °C until the experiment was performed. Experimental samples (Es) were defined as the RTE vegetable samples artificially contaminated with *L. monocytogenes*.

Control samples (BS) were defined as the non-inoculated units and used to evaluate the natural presence of *L. monocytogenes* in RTE vegetable samples from the batches used in our experiment . During the work, the testing times (t) were defined as $t_0$, which was 6 h after inoculation, and $t_1$, $t_2$, $t_3$, $t_4$ which were, respectively, the analysis points every 2 d for a total of 8 d after inoculation.

*2.1 Artificial inoculation and experimental design*

The study was performed according to the Technical Guidance Document prepared by the EU Community Reference Laboratory (CRL) for *L. monocytogenes* (33). A mixture of three *L. monocytogenes* strains was used to challenge raw vegetable units. The inoculum was composed of *L. monocytogenes* reference strain ATCC 35152 obtained from the American Type Culture Collection and two wild type strains previously isolated from the ready-to-eat vegetable samples.

All the strains were stored at -80 °C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with glycerol (15%, v/v). Separate trials were conducted to determine the growth conditions necessary to standardize the level of inoculum to approximately 10-100 CFU/g. Cultures were then adapted at refrigeration temperature by storing at 4°C ± 2°C for ten days. Prior to starting the experiment, a bead of each strain was surface plated onto a Petri dish with Trypticase Soy Agar (TSA, Microbiol, Italy) and incubated at 37°C for 24 h. Then, a loopful of one isolated cell was transferred aseptically into 10 mL of BHI and incubated at 37°C overnight. To determine the initial concentration of each working cocktail, a suspension of 0.5 McFarland (McF), approximately $1.5x10^8$ CFU/mL, was prepared. Each working cocktail was diluted and mixed to obtain a "Challenge Working Culture" (CWC) of the three *L. monocytogenes* strains, approximately 2 log10 /mL at the stationary phase. Colony counts were confirmed by plate count on TSA. Samples of 10g raw vegetables were inoculated with 100 μL of CWC containing 2 log10 CFU/ml of *L. monocytogenes*, homogenized suspension with stomacher.

Subsequently, 180 inoculated samples were stored at four different temperatures, 4°C ,8°C, 25°C and 37°C. The challenge was carried out in independent trials for each batch (A, B and C) performed one week apart. *L. monocytogenes* enumeration was conducted according to International Standard methods UNI EN ISO 11290–2:2005. For the *L. monocytogenes* enumeration, the samples were subjected to a 1:10 dilution in Fraser Broth base (Microbiol Diagnostici, Uta, Cagliari, Italy) and maintained at 20 ± 2 °C for 1 h ± 5 min. A 1 mL aliquot from initial suspension was directly streaked on three ALOA (Agar Listeria Ottaviani&Agosti) and incubated at 37 °C for 24 and 48 h. After the incubation period, samples were taken using the typical colony count. For each time point, the results of the samples analyzed were aggregated and reported as the median concentration of microorganisms expressed in log10 CFU/g.

*2.2 Culture procedures*

Before carrying out the microbial challenge test, the absence of the microorganism target in the product was verified according to UNI EN ISO 11290-1:2005 standard methods.

Twenty-five grams of RTE salad samples were suspended in 225 mL of Half Fraser Broth (Microbiol Diagnostici, Uta, Cagliari, Italy), homogenized by Stomacher for 30 s and then incubated at 30 ± 1 °C for 24 ± 2 h (primary enrichment). After incubation, 0.1 mL of the primary enrichment was transferred into a 10 mL tube containing Fraser broth (Microbiol Diagnostici, Uta, Cagliari, Italy) and incubated at 37 ± 1 °C for 48 ± 2 h (secondary enrichment). After incubation, and, at the same time, the primary enrichment was streaked onto Agar Listeria Ottaviani Agosti (ALOA, Microbiol Diagnostici, Uta, Cagliari, Italy), and PALCAM agar (Polymyxin- Acriflavine-Lithium- Chloride- Ceftazidime- Aesculine Mannitol, Microbiol Diagnostici, Uta Cagliari, Italy) plates which were then incubated at 37 °C. After incubation, the secondary enrichment was streaked onto ALOA (Microbiol Diagnostici, Uta, Cagliari, Italy) and PALCAM agar (Microbiol

Diagnostici, Uta, Cagliari, Italy) plates and incubated, respectively, at 37 °C. Typical colonies, isolated from the ALOA and PALCAM agar, were cultured on Tryptone Soy Yeast Extract Agar (TSYEA, Microbiol Diagnostici, Uta, Cagliari, Italy) and incubated at 37 °C for 24 h. These were then subjected to the following tests for biochemical identification: Gram staining, determination of catalase activity (catalase test, Microbiol Diagnostici, Uta, Cagliari, Italy), hemolytic activity and Camp tests on sheep blood agar (Microbiol Diagnostici, Uta, Cagliari, Italy). The isolated strains were identified using the API Listeria system (bioMérieux, Marcy-l´Etoile, France). Reference strain *L. monocytogenes* ATCC 35152 was used in all biochemical reactions.

***3. Intrinsic properties***

For each sample, the pH and the water activity were determined using pHmetro Eutech Instruments pH 510 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and AquaLab4TE (Decagon, Pullman, WA, USA), respectively.

***4. Growth potential***

Growth potential (δ) was calculated as the difference between the median of log 10 CFU/g at $T_4$ and the median of log 10 CFU/g at $T_0$ (34). An independent growth potential was calculated for each trial, therefore, 5 data points were assessed for each trial.

***5. Molecular analysis***

Evaluation of the gene expression of *L. monocytogenes* strains used during the experimental challenge test was carried out by RT-PCR (StepOne Real-Time PCR System, Applied Biosystems Waltham, Massachussetts, USA). Total RNA was extracted from cultures of *L. monocytogenes* in stationary phase cells incubated at different temperatures (4°C, 8°C, 25°C, 37° C) using the PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) system, according to the manufacturer's instructions. The samples were centrifuged at 5000 g for 5 minutes at 4 ° C. Subsequently, the pellet was suspended in 100 µL of lysozyme solution (Tris HCl 10 mM pH 8.0, EDTA 0.1 mM, lisozyme) and 0.5 µL Sodium Dodecyl Sulfate (SDS 10%). After 5 minutes of incubation at room temperature 350 µL of lysis buffer with 2-mercaptoethanol were added. The samples were homogenized by centrifugation at 12000 g for two minutes at room temperature. To each sample 250 µL of 100% ethanol were added and, after centrifuging at 12000 g for 15 seconds treatment with DNase (TURBO DNase Applied Biosystems, Waltham, Massachussetts, USA) was carried out to eliminate genomic DNA from the samples. Subsequently, 700 µL of wash buffer (containing guanidine isothiocyanate) and 500 µL of a second wash buffer (added with ethanol 96-100%) were added. The reverse transcription of mRNA into cDNA was carried out using a SuperScript cDNA synthesis VILO kit (Invitrogen, Carlsbad, CA, USA): 4 µL of 5X VILO Reaction Mix, 2 µL of 10X SuperScript Enzyme Mix and 14 µL of RNA for a single reaction, in accordance with the manufacturer's instructions. The cDNA was quantified by spectrophotometric (Smart Spec Plus BioRad, Hercules, CA,USA) reading and purity was estimated using $A_{260}/A_{280}$. Each sample was then diluted in order to normalize the value to 50 ng/ µL. The following genes were taken into consideration: *hlya,* coding for listeriolysin; *actA,* coding for actin; *inlA* and i*nlB,* which code for two types of internalin; *plcA* and *plcB,* which encode for two phospholipases and *16S rRNA* was used as the housekeeping gene for its stability of expression (27). For the *hlyA, actA, inlA, inlB*, *plcA* and *plcB* genes, primers were designed using Primer Express 3.0 Applied Biosystem and produced by Invitrogen Live technologies (Table 1). They were

Table 1 - Details of primers and amplicon size used in this study.

| Gene | Forward primer 5'-3' | Reverse primer 5'-3' | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| 16S rRNA | TTAGCTAGTTGGTAGGGT | AATCCGGACAACGCTTGC | 550 | (27) |
| hlyA | TGCAAGTCCTAAGACGCCA | CACTGCATCTCCGTGGTATACTAA | 112 | This study (GenBank: DQ812517.1) |
| actA | TCGGACTTTCCACCACTACC | CGAATGAGCTCGGTTCTGAT | 110 | This study (GenBank: D88258.1) |
| inlA | GCCCAATTTCTAACCTGAAAA | TGGTTAAATTCGCAAGTGAGC | 153 | This study (GenBank: EF558944.1 |
| inlB | AAAGCACGATTTCATGGGAG | ACATAGCCTTGTTTGGTCGG | 145 | This study (GenBank:KR232386.1) |
| plcA | CCGCGGACATCTTTTAATGT | CGAGCAAAACAGCAACGATA | 129 | This study(GenBank: JF712529.1) |
| plcB | CAACCTATGCACGCCAATAA | TGCTACCATGTCTTCCGTTG | 111 | This study(GenBank: JF712529.1) |

subsequently amplified by Real Time-PCR, with SYBR Green being used for molecular intercalation. Amplification for each gene was carried out in a final volume of 20 µL including Power SYBR Green PCR Master Mix (Applied Biosystem life technologies), the template cDNA (≈ 350ng) and primers were used at a concentration of 25 µM. The thermal cycling conditions for the PCR were as follows: 1 cycle at 95 °C for 10 min, 40 cycles of amplification at 95 °C for 15 s and annealing at 60 °C for 1 min. A melting-curve analysis between 55 °C and 95 °C was also performed after each PCR to check the specificity of the amplification product. Gene expression at 37°C (RQ = 1) was used as a reference for the quantitative relative analysis (RQ). The experimental design was repeated three times.

### *6. Statistical analysis*

All tests to assess *L. monocytogenes* growth were run in triplicate and averaged Means (x), and standard deviations (SD) were calculated for tree replicates within the experiments. Analyses were done using Microsoft Excel XP 2010. The confidence interval of pH and $a_w$ is calculated as

$$\mu = \bar{x} \pm t_{0.05,n} SE(\bar{x}) = \bar{x} \pm t_{0.05,n} \sqrt{s^2/n}$$

where n= 3 is the number of replicates. Analysis of variance one-way ANOVA (using Microsoft Excel XP 2010) was used to evaluate the statistical significance of difference in gene expression. Differences of $P < 0.05$ were considered to be significant.

## Results and discussion

### *1. Challenge tests*

The microbiological challenge test was performed on RTE salads in order to assess the behavior of artificially inoculated *L. monocytogenes*. The experiments were

performed in such a way as to satisfy the ideal and extreme conditions that could realistically occur throughout the cold chain,using four different storage temperatures (4°C, 8°C, 25°C and 37°C). Our study shows, as also demonstrated by other authors (5, 6, 13, 20) that *L. monocytogenes* is able to survive and grow in all situations at all the storage temperatures considered. *L. monocytogenes* was not detected in the control samples (CS) during the challenge test. The *L. monocytogenes* concentration in Es samples stored at 4°C increased from 1.40 log10 CFU/g at $t_0$ to 2.04 log10 CFU/g at $t_4$ (2). On the 6th day, the product shows *L. monocytogenes* concentrations below 100 CFU/g, not exceeding the legal limit (18). On the contrary in Es samples stored at 8°C, the *L. monocytogenes* concentration increased from 1.40 log10 CFU/g at $t_0$ to 2.85 log10 CFU/g at $t_4$, and the product reaches this limit on the 2nd day of preservation. In Es samples stored at 8°C, 25° C and at 37°C the preservation limit is already exceeded after 24 hours. These results show that bacterial development was greater, as evidenced by $\delta$ = 5.94 log10 CFU/g at 25°C and $\delta$ = 6.32 log10 CFU/g at 37°C (Table 2). In all situations regardless the storage temperatures considered, the growth potential was always $\delta > 0.5$ log 10 CFU/g. The microbiological challenge test allowed us to confirm, as also demonstrated by other authors (1), that RTE salads are able to support the growth of *L. monocytogenes* strains ($\delta > 0.5$) stored under different temperatures (Figure 2). This is made possible by the pH and $a_w$ values measured in the food, which are favorable for growth (the pH was comprised within a range between 4.76 to 5.05, and the average value of $a_w$ was 0.995, with the presence of nutrients). In fact, apart from foods intended for infants and for special medical purposes, mixed salad is a food that is ready to support the growth of *L. monocytogenes* and regulated by Reg. (EC) 2073/2005 amending Reg. (EC) 1441/2007 (17, 18).

Table 2 - Results from the analyzed experimental samples ($E_s$): i) Evolution of the *L. monocytogenes* (L.m) concentration ii) intrinsic factors (pH and $a_w$) during storage and iii) growth potential ($\delta$) of *L. monocytogenes* when stored at 4°C, 8°C and 25°C. All values are expressed as median log10 CFU/g.

| Time | 4°C | | | 8°C | | | 25° | | | 37°C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *L.m* | pH | $a_w$ | *Lm* | pH | $a_w$ | *L.m* | pH | $a_w$ | *L.m* | pH | $a_w$ |
| $t_0$ | 1.40 | 6.35±0.057 | 0.996±0.003 | 1.40 | 6.23±0.025 | 0.992±0.002 | 1.40 | 6.19±0.080 | 0.991±0.002 | 1.40 | 6.15±0.076 | 0.998±0.004 |
| $t_1$ | 1.40 | 6.24±0.0248 | 0.993±0.0009 | 1.81 | 6.35±0.14 | 0.999±0.002 | 4.89 | 6.49±0.052 | 0.998±0.001 | 3.74 | 6.25±0.211 | 0.998±0.001 |
| $t_2$ | 1.30 | 6.27±0.266 | 0.993±0.0005 | 2.36 | 6.35±0.14 | 0.994±0.001 | 5.8 | 6.23±0.01 | 0.996±0.001 | 5.85 | 5.07±0.328 | 0.996±0.001 |
| $t_3$ | 1.85 | 6.38±0.014 | 0.996±0.002 | 2.88 | 6.52±0.065 | 0.994±0.002 | 6.48 | 6.12±0.01 | 0.997±0.002 | 6.6 | 5.40±0.103 | 0.992±0.002 |
| $t_4$ | 2.04 | 6.47±0.014 | 0.991±0.003 | 2.85 | 6.97±0.14 | 0.985±0.006 | 7.34 | 6.03±0.14 | 0.986±0.006 | 7.72 | 5.44±0.38 | 0.994±0.002 |
| $\delta (t_4 - t_0)$ | 0.044 | | | 1.45 | | | 0.274 | | | 6.32 | | |

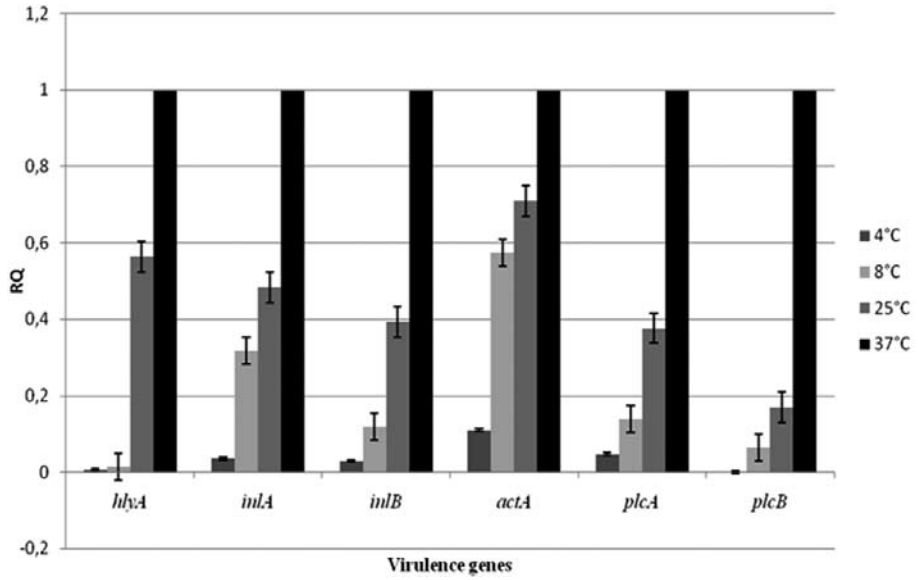


Figure 1 - Relative expression of the *L. monocytogenes* virulence genes hlyA, actA, inlA, inlB, plcA, plcB grown in RTE salads at different temperatures (4°C, 8°C, 25°C and 37°C) at the end of shelf life. The transcript levels were determined by RT-PCR and normalized to the housekeeping gene (16S rRNA). Values shown are the means from three independent RNA collections. Standard deviation is indicated as vertical bars. The experiment was repeated three times.

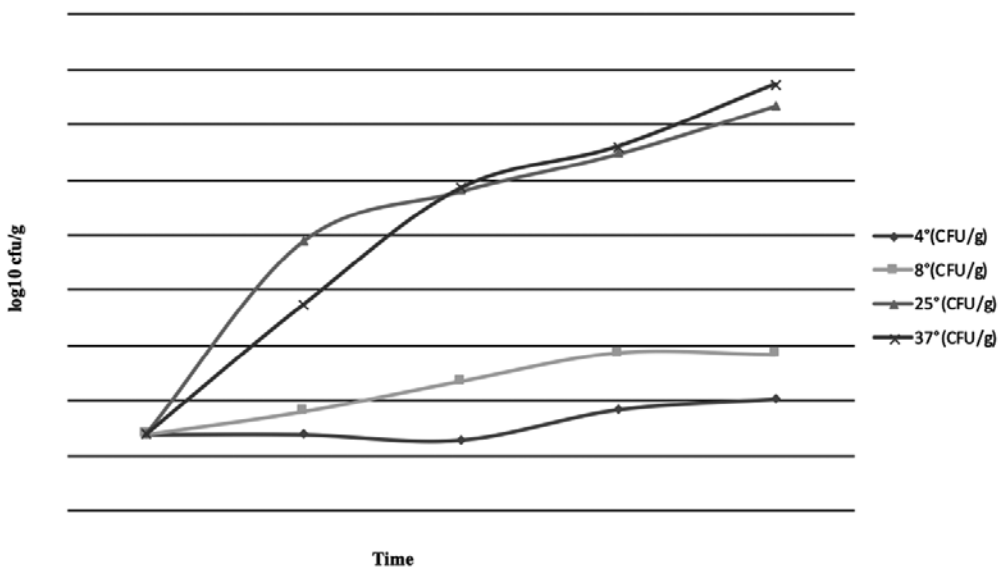


Figure 2 - *L. monocytogenes* (log10 CFU/g) challenge test. Evolution of *L. monocytogenes (L.m)* in Es stored at 4°C, 8°C, 25°C and 37°C.

As regards the risk of *L. monocytogenes* in these products during shelf life, with an artificial contamination level of 1.4 log10 CFU/g, their count did not exceed 2 log10 CFU/g at 4°C after 5 days, which confirms the importance of compliance with low temperatures for this type of food product. Instead, at 8°C, observations have shown that the limit is passed between day 2 and day 3 of conservation, while at 25° and 37°C this occurs after 24 hours (8).

***2. Intrinsic properties***

Among intrinsic properties our results showed that the initial $a_w$ values were 0.996 ± 0.003 for Es samples stored at 4 °C, 0.992 ± 0.002 for Es samples stored at 8 °C, 0.991 ± 0.002 for Es samples stored at 25 °C and 0.998 ± 0.004 for Es at 37 °C. These values change, in different times considered, until the end of the study (Table 2). pH values were similar between Es samples stored at 4 °C and at 8 °C and between Es samples stored at 25°C and at 37°C. In general the averaged values of pH and $a_w$ were typical of products able to support the growth for *L. monocytogenes*.

***3. Molecular analysis***

The relative quantification of transcript levels were determined by RT-PCR (35, 36) and normalised to the 16S rRNA housekeeping gene. Target gene transcript levels were quantified with StepOne v2.0 software using the maximum expression of the sample incubated at 37 ° C as a reference. Transcripts levels were determined in strains exposed to different (4°C, 8°C, 25°C) and optimal (37 °C) temperature conditions. The RQ of transcripts was calculated in relation to transcript levels at 37 °C. The results for expression levels of the target genes of *L. monocytogenes* after exposure to different temperatures are shown in Figure 1. The analysis of the results suggests a progressive reduction in the expression of all the virulence genes considered in this study (Table 3), which is directly proportional to the decrease in the incubation temperature to which they were subjected. However, a total shutdown of the expression of virulence genes only occurred for *pclB,* where the gene expression in the sample incubated at 4 ° C was below the detection limits of the method. In particular these results show that the most expressed gene at low temperatures (8 °C) is *actA* with an average RQ equal to 0.57±0.035, followed by *inlA* whose average RQ is equal to 0.32±0.035. Finally, *hlyA, inlB, plcA* and *plcB* result as being feebly expressed with an average RQ equal to 0.015±0.035, 0.09±0.03, 0.13±0.036 and 0.05±0.015 respectively. Lower transcript levels were observed for samples stored at 4°C.

The afore-mentioned preventive measure is crucial in light of the results obtained from molecular studies on the expression of *L. monocytogenes* virulence genes. In fact in conditions of thermal abuse (8°C, 25°C, 37°C), growth and also virulence gene expression increases, with a significant difference between storage temperatures ($P$<0.05).

Indeed, the analysis of the results suggests a progressive reduction in the expression of

Table 3 - Relative quantitation of virulence genes. Mean values of three experiments.

| Virulence genes | *hlyA* | *inlA* | *inlB* | *actA* | *plcA* | *plcB* |
|---|---|---|---|---|---|---|
| 4°C | 0.0063 | 0.0356 | 0.029 | 0,11 | 0.046 | 0 |
| 8°C | 0.015 | 0.3166 | 0.09 | 0.573 | 0.14 | 0.063 |
| 25°C | 0.5633 | 0.4833 | 0.393 | 0.71 | 0.376 | 0.17 |
| 37°C | 1 | 1 | 1 | 1 | 1 | 1 |

all the virulence genes considered in this study, which is directly proportional to the decrease in the incubation temperature to which the samples are subjected. This finding is in keeping with the results that other authors have evidenced for other kinds of stress adaptation (27, 37, 38). However, a total shutdown of virulence genes expression did not occur, except for *pclB,* where the gene expression in the sample incubated at 4 ° C was below the detection limits of the method. In fact, virulence is not stably associated with the strain, and as shown by other authors, is temperature dependent.

## Conclusion

The study of virulence genes expression conducted through a quantitative assessment associated with the maintenance of storage conditions at different temperatures could contribute to the advancement of knowledge of the mechanisms that lead from the state of infection to disease. While it is conceivable that for foodborne diseases, such as those caused by the intake of preformed toxin in the food (intoxication by *S. aureus* coagulase positive, *Cl. botulinum*), the possible applications of this methodology support the study of the factors that may inhibit the formation of toxins, for infections such as listeriosis, it may instead represent an approach to the study of the mechanisms underlying the attenuation of virulence. As well as the transition from the state of infection to disease, the virulence is certainly influenced by the concentration of the microorganism present in the food, and the total shutdown of the virulence gene expression of the microorganism may also underlie the pathogenesis or even be associated with incubation times shorter than the dilated time for listeriosis reported by previous studies in the scientific literature (39). The safety of food by the experimental inoculation of pathogenic micro-organisms in order to assess the risk of toxinfections could direct research on methods of decontamination or on the use of substances that may exert an inhibitory activity or compete for control of the pathogens in the salad (extracts, bacteriophages, bacteriocins) to allow action in view of prevention and health promotion.


### Riassunto

***Crescita di Listeria monocytogenes in insalate pronte al consumo a diverse temperature di conservazione e valutazione dell'espressione dei geni di virulenza***

**Introduzione.** Le verdure sono i componenti principali di una dieta sana ed equilibrata. Tuttavia, il 25% delle malattie di origine alimentare sono legate al consumo di verdure.

**Disegno.** Lo scopo di questo lavoro è stato quello di valutare i rischi microbiologici associati al consumo di insalate pronte al consumo (RTE).

**Metodi.** Sono stati effettuati challenge test microbiologici per la valutazione del potenziale di crescita di *L. monocytogenes* in insalate RTE conservate a temperature diverse.

**Risultati.** I risultati indicano che *L. monocytogenes* è stato in grado di crescere ($\delta \geq 0.5$) in tutte le condizioni di conservazione considerate alla fine della shelf life. Al fine di valutare il ruolo di virulenza di *L. monocytogenes*, è stata anche studiata la trascrizione dipendente dalla temperatura dei principali geni di virulenza mediante RT-PCR.

**Conclusioni.** Il challenge test microbiologico ci ha permesso di confermare, come dimostrato anche da altri autori, che le insalate RTE, conservate a temperature diverse, sono in grado di supportare la crescita di ceppi di *L. monocytogenes* ($\delta \geq 0.5$).


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