Detection of Virulence Genes and Growth Potential in *Listeria monocytogenes* Strains Isolated from Ricotta Salata Cheese

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Abstract: Ricotta Salata is a traditional ripened and salted whey cheese made in Sardinia (Italy) from sheep's milk. This product is catalogued as ready-to-eat food (RTE) since it is not submitted to any further treatment before consumption. Thus, foodborne pathogens, such as *Listeria monocytogenes,* can represent a health risk for consumers. In September 2012, the FDA ordered the recall of several batches of Ricotta Salata imported from Italy linked to 22 cases of Listeriosis in the United States. This study was aimed at evaluating the presence and virulence properties of *L. monocytogenes* in 87 samples of Ricotta Salata produced in Sardinia. The ability of this product to support its growth under foreseen packing and storing conditions was also evaluated in 252 samples. Of the 87 samples 17.2% were positive for the presence of *L. monocytogenes* with an average concentration of 2.2 $\log_{10}$ cfu/g. All virulence-associated genes (*prfA*, *rrn*, *hlyA*, *actA*, *inlA*, *inlB*, *iap*, *plcA*, and *plcB*) were detected in only one isolated strain. The Ricotta Salata samples were artificially inoculated and growth potential (δ) was assessed over a period of 3 mo. The value of the growth potential was always $>0.5 \log_{10}$ cfu/g under foreseen packing and storing conditions. This study indicates that Ricotta Salata supports the *L. monocytogenes* growth to levels that may present a serious risk to public health, even while stored at refrigeration temperatures.

Keywords: challenge test, food safety, *Listeria monocytogenes*, Ricotta Salata, virulence genes

Pratical Application: Ricotta Salata can be contaminated with different bacteria species, especially on the manufacturing process. Among the possible contaminants *L. monocytogenes* could be a main cause of foodborne illness in this product. A challenge test could be suitable for a study of risk evaluation during the shelf life of this product.

Introduction

Listeria monocytogenes is an important pathogen, responsible for many outbreaks related to the food products consumption, such as dairy products, meat, fish, and seafood (Barza 1985; Schlech 2000; Gaulin and others 2003; MacDonald and others 2005; Varma and others 2007; Jackson and others 2011). *L. monocytogenes* has been isolated from an environmental sources, including food-processing environments (Gandhi and Chikidas 2007; Swaminathan and Gerner-Smitdt 2007; Aissani and others 2012). According to the European annual epidemiological report on sources of zoonoses, zoonotic agents, and food-borne outbreaks, *L. monocytogenes* is the most responsible for cases of hospitalization and death in Europe (EFSA 2014). Most human Listeriosis cases appear to be caused by consumption ready-to-eat foods (RTE) that are contaminated with high levels of *L. monocytogenes* (Liu 2006; Lianou and Sofos 2007; Chan and Wiedmann 2009). Ricotta Salata is a typical cheese of the dairy tradition of Sardinia (Italy), produced from whey left over from production of cheeses made from sheep's milk (Spanu, and others 2013). The whey obtained after the cheese production is heated in a kettle to temperature of 85 °C for about 30 min. Subsequently floating curd is collected through the use of a perforated ladle and transferred into plastic moulds. The curd is then pressed in order to increase the drainage and dry salted (5% w/w) at a controlled temperature (10 to 12 °C) for about 10 d. The final product is individually packed in vacuum bags and stored at refrigeration temperature (Spanu and others 2013, 2015a). Large numbers of viable *Listeria* cells present in milk are killed by heat treatment applied during cheese-making and whey protein coagulation (Villani and others 1996; Spanu and others 2013). Previous studies have shown that the cheese-making plants are characterized by areas with conditions that favor the *L. monocytogenes* growth, such as refrigeration temperature, moisture and high salt concentration (Unnerrstad and others 1996; Tompkin 2002; Ibba and others 2013; Spanu and others 2015b). Several studies have found that *L. monocytogenes* contamination in Ricotta Salata is present in the surfaces of the product and has originated from the processing environment (Spanu and others 2013, 2015a).

In fact the Ricotta Salata production includes manual manipulation of the curd after floating and exposition to processing environment thus increasing the risk of *L. monocytogenes* contamination (Pintado and Malcata 2000; Lioliou and others 2001;Spanu and others 2013).

In September 2012, the FDA in the U.S. recalled several batches of Ricotta Salata imported from Italy contaminated with *L. monocytogenes* and involved in a multistate outbreak of Listeriosis (13 U.S. states) with 22 hospitalized cases and 4 deaths (FDA 2012). The current European legislation (EC Reg n.2073/2005 and n.1441/2007) specifies microbiological criteria for

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L. monocytogenes in RTE. According to these regulations, products with a shelf-life of less than five days, are considered to be RTE that are unable to support *L. monocytogenes* growth. Considering the current large distribution and consumption of RTE, the producer is recommended, amongst other measures, to perform laboratorial analyses, defined by EC Reg. n 2073/2005 as challenge tests, in order to document the ability of a food to support the growth of *L. monocytogenes*. Guidelines and procedures for performing challenge tests are available in a dedicated document produced by the Agence Française de Sécurité Sanitaire des Aliments (Beaufort and others 2008). The *L. monocytogenes* growth potential in food depends primarily on intrinsic properties of the food (for example, pH, NaCl content, a_w, food composition, associated microflora, antimicrobial constituents) and extrinsic properties (for example, temperature profile, gas atmosphere) (Beaufort and others 2008). Ricotta Salata is a RTE which constitutes a favorable ground for *L. monocytogenes* growth (Spanu and others 2013). Several studies have shown a prevalence of *L. monocytogenes* in Ricotta Salata by about 20% (Pintado and Malcata 2000; Lioliou and others 2001; Pilo and others 2008; Ibba and others 2013; Spanu and others 2015c).

Phylogenetic studies and molecular typing have also found that different *L. monocytogenes* strains are structured population, made up of divergent genetic lineages (Orsi and others 2011). The virulence varies significantly between strains and many isolated are ipovirulent or nonpathogenic to humans (Roche and others 2009; Renier and others 2011)

The aim of this work was to evaluate the presence and virulence factors of *L. monocytogenes* in Ricotta Salata produced in Sardinia. The ability of this product to support its growth under foreseen packing and storing conditions was also evaluated.

Material and Methods

Conventional microbiological analysis

The study was conducted on 87 Ricotta Salata samples collected from local cheese-making plants situated in different locations in Sardinia. The samples, transported under controlled temperature, were subjected to microbiological analysis at the Laboratory of Food Hygiene at the Univ. of Cagliari, which operates in conformity with European standard UNI CEI EN ISO/IEC 17025:2005.

Culture procedures

The Ricotta Salata samples were prepared according to the protocol of the International Standards Organization (UNI EN ISO 6887-5:2010).

The *L. monocytogenes* detection was conducted according to the standard methods UNI EN ISO 11290–1: 2005. Twenty-five grams of Ricotta Salata 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 10 mL tube containing Fraser broth (Microbiol Diagnostici, Uta, Cagliari, Italy) and incubated at 37 ± 1 °C for 48 ± 2 h (secondary enrichment). At the same time, after incubation, primary enrichment is streaked onto Agar Listeria Ottaiani Agosti (ALOA, Microbiol Diagnostici, Uta, Cagliari, Italy), and PALCAM agar (Microbiol Diagnostici, Uta, Cagliari, Italy) plates and incubated at 37 °C, respectively.

After incubation, secondary enrichment is 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 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.They were 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'Étoile, France). In all biochemical reactions the reference strain *L. monocytogenes* ATCC 35152 was used.

L. monocytogenes serotyping

The strains identified by biochemical tests as *L. monocytogenes* were characterized as a serotype using *Listeria* antisera kit (Denka Seiken, Tokyo, Japan) comply with the instructions provided from the manufacturer . Serotype is determined on the basis of reaction patterns obtained from combination of antigenic factors O and H according to the standard scheme proposed by Seeliger and Höhne (1979).

Molecular investigation and characterization

The *L. monocytogenes* detection was performed by real-time PCR (RTi-PCR) assay.

Twenty-five grams of Ricotta Salata samples were suspended in 225 mL of Half Fraser Broth (Microbiol Diagnostici), homogenized by Stomacher for 30 s and then incubated at 30 ± 1 °C for 24 ± 2 h. An aliquot of 1mL of each enrichment taken from the surface of the broth was centrifugated at 11000 × *g* for 3 min. DNA was extracted by DNeasy mericon food kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA concentration was determined by spectrophotometry using GeneQuant II (Applied Biotech, Plantation, Florida, U.S.A.).

The *L. monocytogenes* DNA was detected using Mericon *Listeria monocytogenes* kit (Qiagen, Hilden, Germany). The extracted DNA was stored at −20 °C until used.

Ten microliters of extracted DNA was added in 10 μL of reconstituted mericon assay in a final volume of 20 μL. Cycling protocol was as follows: 5 min at 95 °C, 40 cycles of 15 s at 95 °C, 23 s at 60 °C and 10 s at 72 °C. *L. monocytogenes* ATCC 35152 was used as PCR-positive controls in all amplifications. A negative control (molecular grade water) was included at each run. The RTi-PCR was performed with the Stratagene™ Mx3005P qPCR (Stratagene, La Jolla, Calif., U.S.A.).

The strains identified as *L. monocytogenes* were subjected to molecular characterization by PCR for the presence of the main virulence genes (*prf A*, *rrn*, *hlyA*, *actA*, *inlA*, *inlB*, *plcA*, *plcB*, and *iap*) which they were chosen for their importance in the *L. monocytogenes* pathogenesis.

Bacterial DNA was extracted following the protocol illustrated in the commercial kit "DNeasy Blood & Tissue Kit" (Qiagen, Hilden, Germany). DNA concentration was determined by spectrophotometry using GeneQuant II (Applied Biotech, Plantation, Florida, U.S.A.).

PCR detection of virulence genes

The oligonucleotide primers specific for the *L. monocytogenes* virulence genes (*prfA, rrn, hlyA, actA, inlA, inlB, plcA, plcB,* and *iap*) and the PCR protocols are described in Table 1. All primers were synthesized by Sigma Aldrich (Saint Louis, Missouri, U.S.A.).

Table 1–Target genes and PCR protocols used in this work for *L. monocytogenes* virulence associated gene detections in Ricotta Salata.

Target gene	*Name*	Primer sequence 5′——3′	Amplicon size (bp)	Primer (cycling conditions)
Prf A	*prf-A*	CTG TTG GAG CTC TTC TTG GTG AAG CAA TCG	274	Wernars and others 1992, Jofré and others 2005
	prf-B	AGC AAC CTC GGT ACC ATA TAC TAA CTC		
	lip1	GAT ACA GAA ACA TCG GTT GGC		
	lip2	GTG TAA CTT GAT GCC ATC AGG		
Rrn	*rrn-F*	CAG CAG CCG CGG TAA TAC	938	Conter and others 2007
	rrn-R	CTC CAT AAA GGT GAC CCT		
hlyA	*hlyA-F*	CCT AAG ACG CCA ATC GAA	702	
	hlyA-R	AAG CGC TTG CAA CTG CTC		
actA	*actA-F*	GAC GAA AAT CCC GAA GTG AA	268 or 385	
	actA-R	CTA GCG AAG GTG CTG TTT CC		
inlA	*inlA-F*	CCT AGC AGG TCT AAC CGC AC	255	
	inlA-R	TCG CTA ATT TGG TTA TGC CC		
inlB	*inlB-F*	AAA GCA CGA TTT CAT GGG AG	146	
	inlB-R	ACA TAG CCT TGT TTG GTC GG		
plcA	*plcA-R*	CGA GCA AAA CAG CAA CGA TA	192	
	plcA-F	CCG CGG ACA TCT TTT AAT GT		
plcB	*plcB-R*	GGG AAA TTT GAC ACA GCG TT	261	
	plcB-F	ATT TTC GGG TAG TCC GCT TT		
iap	*iap-F*	ACA AGC TGC ACC TGT TGC AG	131	Yadav and others 2010
	iap-R	TGA CAG CGT GTG TAG TAG CA		

For these genes detections, all primers were used at a concentration of 20 μM.

The *prfA* gene detection was performed with a nested PCR using the PCR protocols published by Wernas and others (1992) and Joffré and others (2005). In the first step 4 μL of extracted DNA was added in the Illustra PuReTaq Ready-To-Go PCR Beads reaction mix (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with 20 μL of water for molecular biology DNAse RNAse (Acros Organics, Geel, Belgium) and 0.5 μL each for forward and reverse primer *(prfA, prfB)* in a final volume of 25 μL. Reaction mix was subjected to the following programme : 5 min at 95 °C, 30 cycles of 15 s at 95 °C, 30 s at 60 °C and 90 s at 72 °C.

In the second step 2 μL of template DNA was added in the Illustra PuReTaq Ready-To-Go PCR Beads reaction mix (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with 22 μL of water for molecular biology DNAse RNAse (Acros Organics, Geel, Belgium) and 0.5 μL each for forward and reverse primer (*lip1* and *lip2*) in a final volume of 25 μL. The thermal cycles used were as follows: 5 min at 95 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 60 s at 74 °C and final extension of 5 min at 74 °C. For these gene detections, all primers were used at a concentration of 20 μM.

Three different multiplex PCR have been used for the detection of *rrn*, *hlya*, and *actA* genes, for *inlA*, *inlB*, and *iap* genes and for *plcA* and *plcB* genes.

For *rrn*, *hlya*, and *actA* genes detection 2 μL of DNA extracted, 0.5 μL each for forward and reverse primer (*rrn*, *hlya*, and *actA*) and 20 μL of water for molecular biology DNAse RNAse (Acros Organics, Geel, Belgium) were added in Illustra PuReTaq Ready-To-Go PCR Beads reaction mix (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in a final volume of 25 μL. The thermal cycles used were as follows: 5 min at 95 °C and 24 cycles of 80 s at 94 °C, 90 s at 55 °C, 2 min at 72 °C and a final extension of 10 min at 72 °C.

For *inlA*, *inlB*, and *iap* genes detection 2 μL of DNA extracted, 0.5 μL each for forward and reverse primer (*inlA*, *inlB*, and *iap*) and 20 μL of water for molecular biology DNAse RNAse (Acros Organics, Geel, Belgium) were added in Illustra PuReTaq Ready-To-Go PCR Beads reaction mix (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in a final volume of 25 μL. For *plclA* and *pcllB* genes detection 2μL of DNA extracted, 0.5 μL each for forward and reverse primer (*plclA* and *pcllB*) and 21 μL of water for molecular biology DNAse RNAse (Acros Organics, Geel, Belgium) were added in Illustra PuReTaq Ready-To-Go PCR Beads reaction mix (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in a final volume of 25 μL.

For both 2 multiplex PCR, the same thermal cycle was used: 5 min at 95 °C and 35 cycles of 1 min at 94 °C, 2 min at 55 °C, 1 min at 72 °C and a final extension of 5 min at 72 °C. For these gene detections, all primers were used at a concentration of 20 μM. *L. monocytogenes* ATCC 35152 was used as PCR-positive controls in all amplifications. A negative control (molecular grade water) was included at each run. The reactions were performed on a Mastercycler 5333 (Eppendorf, Hamburg, Germany). Finally, PCR products were separated by electrophoresis on 2% agarose/ethidium bromide gel (Roth Karlsruhe, Germany; Merk Darmstadt, Germany) in one TBE running buffer (Roth Karlsruhe, Germany), and were revealed by UV transillumination.

Challenge tests

The study was conducted on 252 Ricotta Salata samples produced by a local cheese-making plants. Samples were randomly selected from three different batches (84 from each batch). The Ricotta Salata samples were vacuum packed in flexible packaging and were transported to the laboratory and stored at 4 ± 2 °C until the experiment was performed. Experimental samples (E_s) were defined as Ricotta Salata samples artificially contaminated with *L. monocytogenes.*

Blank samples (B_S) were defined as the units not inoculated and used to evaluate *L. monocytogenes* natural presence in Ricotta Salata from 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, T_5, T_6 which were, respectively, the analysis points every 15 d for a total of 3 mo after inoculation.

Artificial inoculation and experimental design

The study was performed according to the Technical Guidance Document prepared by EU Community Reference Laboratory

(CRL) for *L. monocytogenes* (Beaufort and others 2008). A mixture of three *L. monocytogenes* strains was used to challenge Ricotta Salata units. The inoculum was composed of *L. monocytogenes* reference strain ATCC 35152 obtained from American Type Culture Collection and of two wild type strains previously isolated from the Ricotta Salata 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 to 100 cfu/g. Cultures were then adapted at refrigeration temperature by storing at 4 ± 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 Diagnostici, Uta, Cagliari, 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 McF, approximately 1.5×10^8 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 10^3 cfu/mL at stationary phase. Colony counts were confirmed by plate count on TSA.

Ricotta was aseptically removed from the commercial bags, cut in slices and transferred in sterile bags. Samples of 10g Ricotta slices were inoculated with 100 μL of CWC containing 10^3 cfu/g of *L. monocytogenes*.

Subsequently, n. 126 inoculated samples were vacuum packaged and stored at two different temperatures, 4 and 8 °C. These samples were defined as *Experimental samples vacuum packaged* (E_{sv}). The other inoculated samples (n.126) were not vacuum packed and were stored in sterile bags at the same temperature. These samples were defined as *Experimental samples not vacuum packaged* (E_{snv}).The challenge was carried out in independent trials for each batch (A, B, and C) performed one week apart.

L. monocytogenes detection and enumeration were conducted according to International Standard methods UNI EN ISO 11290–1:2005 and UNI EN ISO 11290–2:2005, respectively. 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. An aliquot 1 mL from initial suspension was directly streaked on three ALOA 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 Log_{10} cfu/g.

Intrinsic properties

Simultaneously with microbiological investigations intrinsic properties of Ricotta Salata, pH and water activity (a_w), were assessed. The pH and the a_w were determined using pHmetro Eutech Instruments pH 510 (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) and Rotronic Hygropalm with the AW-DIO (Rotronic ag, Bassersdorf, Switzerland), respectively.

Growth potential

Growth potential (δ) was calculated as the difference between the median of the $\log_{10}$ cfu/g at T_6 and the median of Log_{10} cfu/g at T_0 (Beaufort and others 2008). An independent growth potential was calculated for each trial.

Statistical analysis

All tests of the assessment of *L. monocytogenes* growth were run in triplicate and averaged. Means (x), Standard deviations (SD) and degrees of significance (using Student's *t*-test) were calculated replicates within the experiments, and analyses were done using Microsoft Excel XP 2010. Differences of $P < 0.05$ were considered to be significant.

Results and Discussion

Conventional microbiological analysis

The results of this study have provided information regarding the possible presence of virulent strains of *L. monocytogenes* in the Ricotta Salata samples analyzed. In the present work, natural contamination of Ricotta Salata samples analyzed was detected along the 3 mo observation period.

Of the Ricotta Salata samples, 17.2% (15/87) were contaminated by *L. monocytogenes*. Our result is consistent with those reported in literature; the incidence of *L. monocytogenes* in soft and semi-soft cheese in other investigations has been reported to range between 1.1% and 22%, whereas other *Listeria* species have been found in 0.5% to 24% of the cheese samples examined. Beckers and others (1987), Eppert and others (1995) Loncarevic and others (1995), and Pini and Gilbert (1988) found that 15% (10 out of 69), 26.1% (18 out of 69), 10% (18 out of 174), and 14% (12 out of 85) of French soft cheeses were positive for *L. monocytogenes.* Although cases of Ricotta Salata contaminated with *L. monocytogenes* have been reported (Pilo and others 2008; Ibba and others 2013; Spanu and others 2015c), it is usually limited to the rind when proper sanitation procedure is applied.

The average concentration in the samples analyzed was 2.2 $\log_{10}$ cfu/g. A previous study conducted on Ricotta Salata showed that contamination of the surface, even with a low level of *L. monocytogenes* (10^2 cfu/g), can result when stored at refrigeration temperature for 2 mo, in concentration of the pathogen potentially threatening to human health (Spanu and others 2013).

*L. monocyt*ogenes serotyping

From *15 L. monocytogenes* positive samples were recovered 75 strains which were characterized as a serotype. Among the isolates the most represented serotype was 1/2a (93%) followed by serotype 1/2b (7%). Our results are in compliance with those found in the literature (Spanu and others 2013, 2015a,b,c).

Molecular investigation and characterization

The Ricotta Salata *L. monocytogenes* positive samples obtained by the microbiological investigation was confirmed by molecular analysis.

Listeria monocytogenes is pathogenic at the species level, but various strains display varied virulence and pathogenic potential. There are different *L. monocytogenes* subtype which differ in their pathogenicity for humans and/or in their ability to transmit to human (Chen and Knabel 2007; Lomonaco and others 2012).

Regarding virulence genes Ricotta Salata-associated they were present in most isolated strains. The *prfA* and *actA* genes were predominant (100%) followed by *hlyA* (93%), *rrn* (80%) *inlA* (60%), *inlB* and *iap* (47%), *plcB* (40%), and *plcA* (20%).

Just one sample showed all nine virulence genes. Our results are in compliance, especially as regards the percentage of isolation of *inlA* and *InlB* genes, with previous investigations. In them, many lineage II isolates showed the presence of non sense mutations leading to premature stop codons ($PMSC_s$) in the *inlA* gene with

Table 2–Results from the analyzed experimental samples vacuum packaged (E_{sv}) stored at 4 °C and 8 °C at different testing times: (i) Evolution of the *L. monocytogenes* (L.m) concentration (ii) *L. monocytogenes* growth potential (δ) (median $\log_{10}$cfu/g), (iii) intrinsic factors (pH and a_w) ($x \pm$ SD).

	4 °C				8 °C			
Time	L.m		pH	a_w	L.m		pH	a_w
T_0	1.46		6.61 ± 0.1	0.962 ± 0.01	1.7		6.79 ± 0.1	0.954 ± 0.01
T_1	1.7		6.61 ± 0.17	0.959 ± 0.01	3.52		6.40 ± 0.1	0.946 ± 0.00
T_2	3.15		6.46 ± 0.14	0.949 ± 0.00	4.73		6.17 ± 0.1	0.954 ± 0.00
T_3	3.96	δ ($T_6 - T_0$)	6.26 ± 0.12	0.969 ± 0.01	6.17	δ ($T_6 - T_0$)	6.02 ± 0.13	0.958 ± 0.00
T_4	4.96	4.66	6.17 ± 0.16	0.944 ± 0.01	6.7	6	6.03 ± 0.15	0.928 ± 0.01
T_5	5.49		6.12 ± 0.1	0.957 ± 0.00	7.18		6.01 ± 0.12	0.939 ± 0.01
T_6	6.12		6.10 ± 0.1	0.954 ± 0.00	7.7		6.0 ± 0.17	0.935 ± 0.01

Table 3–Results from the analyzed experimental samples not vacuum packaged (E_{snv}) stored at 4 and 8 °C at different testing times: (i) Evolution of the *L. monocytogenes* (*L.m*) concentration (ii) *L. monocytogenes* growth potential (δ) (median $\log_{10}$cfu/g), (iii) intrinsic factors (pH and a_w) ($x \pm$ SD) Both experiments were interrupted at time T_3 due to the presence of molds and yeasts on Ricotta samples.

	4 °C				8 °C			
Time	L.m		pH	a_w	L.m		pH	a_w
T_0	1.73		6.65 ± 0.18	0.961 ± 0.01	1.56		6.86 ± 0.15	0.955 ± 0.00
T_1	1.78		6.63 ± 0.13	0.951 ± 0.01	3.72		6.76 ± 0.14	0.955 ± 0.00
T_2	3.5		6.20 ± 0.1	0.950 ± 0.00	5.4		6.16 ± 0.18	0.954 ± 0.01
T_3	4.04	δ ($T_3 - T_0$)	6.13 ± 0.14	0.969 ± 0.01	6.41	δ ($T_3 - T_0$)	5.85 ± 0.1	0.965 ± 0.01
ND	ND	2.31	ND	ND	ND	4.85	ND	ND
ND	ND		ND	ND	ND		ND	ND
ND	ND		ND	ND	ND		ND	ND

ND, not determined.

reduced invasion and attenuated virulence. Further studies have shown that reduced virulence are linked to PMSC$_s$ in atypical *inlB* and *inlA* genes (Jaquet and others 2004; Nightingale and others 2008; Van Stelten and others 2010). In general, a lower virulence cold result from point mutations in some virulence genes (Orsi and others 2011). *L. monocytogenes* strains may significantly vary in invasion efficiency and virulence phenotypes are not exclusively responsible for the association between epidemic strains and listeriosis outbreaks (Roberts and others 2009). There are few studies in Italy on *L. monocytogenes* virulent strains isolated in dairy products and literature focuses mainly on fish and RTE meat products and their processing environments (Conter and others 2007; Mazzette and others 2007; Lomonaco and others 2012). For this reason, in our study we considered it important to assess the presence of *L. monocytogenes* virulence genes isolated from Ricotta Salata to improve knowledge about the potential risks associated with the consumption of this product.

Challenge test

Microbiological challenge test was performed in Ricotta Salata cheese to assess the behavior of artificially inoculated *L. monocytogenes*. The experiments were performed in a manner to satisfy conditions (ideal and extreme) that could realistically occur all along the chain of cold, using two different storage temperatures (4 and 8 °C) and two types of packaging (vacuum packed and not vacuum packed). Our studiy show that *L. monocytogenes* is able to survive and grow in both situations regarding packaging and refrigeration.

L. monocytogenes was never detected in Blank samples BS during the challenge test. The *L. monocytogenes* concentration in E_{sv} samples stored at 4 °C increased from 1.46 $\log_{10}$ cfu/g at T_0 to 6.12 $\log_{10}$ cfu/g at T_6. The growth potential (δ) was always >0.5 $\log_{10}$ cfu/g (Table 2).

In E_{sv} samples stored at 8 °C the *L. monocytogenes* concentration increased from 1.70 $\log_{10}$ cfu/g at T_0 to 7.7 $\log_{10}$ cfu/g at T_6. These results show bacterial development was greater as evidenced by $\delta = 6$ $\log_{10}$ cfu/g (Table 2).

In E_{snv} samples and stored at 4 °C, significant differences ($P > 0.05$) were not observed compared to E_{sv} tested at the same temperature. (Table 3). In E_{snv} stored at 4 °C, an increase of the number of colonies from the time T_0 with a value of 1.73 $\log_{10}$ cfu/g to the time T_3 with a value of 4.04 $\log_{10}$ cfu/g was observed. In E_{snv} stored at 8 °C the *L. monocytogenes* concentration increased from 1.56 $\log_{10}$ cfu/g at T_0 to 6.41 $\log_{10}$ cfu/g at T_3. These results show a growth potential (δ) equal to 4.85 $\log_{10}$ cfu/g.

In both samples E_{snv} stored at 4 and 8 °C the experiments were interrupted at time T_3 due to the presence of molds and yeasts on Ricotta samples.

In all cases the ATCC 35152 strain showed the least growth potential in comparison with the wild type strains.

Our experimental data indicated that all Ricotta Salata analyzed was able to support the growth of *L. monocytogenes* because estimated growth potential (δ) was >0.5 $\log_{10}$ cfu/g. These results are in compliance with previous studies that have shown a listeria growth potential in samples of cottage cheese contaminated by about 5 $\log_{10}$/g (Spanu and others 2012). The greater mean increase of *L. monocytogenes* was observed between T_0 and T_6 in the ricotta sample that was vacuum packed and stored at a temperature of thermal abuse (8 °C). This data showed that low initial levels of contamination in the product could lead to concentration potentially harmful to human health during the first month of storing. In the frame of AFSSA document (Beaufort and others 2008) the food is classified into RTE able to support the growth of *L. monocytogenes* when the growth potential >0.5 $\log_{10}$ cfu/g. Concentration of *L. monocytogenes* should not lead to the level of 100 cfu/g at the end of shelf-life as indicated in Commission Regulation (EC) No. 2073/2005.

Intrinsic properties

Among intrinsic properties our results showed that the pH and a_w changed over time for both situations regarding packaging and refrigeration. Initial a_w values were 0.962 ± 0.01 for E_{sv} samples stored at 4 °C and 0.954 ± 0.01 for E_{sv} samples stored at 8 °C. These values change, in different times considered, until the end of the study (Table 2). Even samples E_{svn} have shown some variability in the values of a_w as shown in Table 3. pH was similar between E_{sv} samples stored at 4 °C and at 8 °C (Table 2). The E_{svn} samples stored at 8 °C showed a decrease in pH values from 6.86 to 5.85 (Table 3). For both the situations regarding packaging and refrigeration, samples stored at 8 °C showed the maximum (6.79 ± 0.1; 6.86 ± 0.15) and minimum (6 ± 0.17; 5.85 ± 0.1) pH values compared to samples stored at 4 °C.

In general the a_w and the pH values were always within the limits of growth for *L. monocytogenes*, although overall, intrinsic properties of Ricotta Salata were able to support *L. monocytogenes* growth throughout the observation period.

Conclusion

Our study showed that according to the physico-chemical characteristics of pH and a_w, Ricotta Salata could support *L. monocytogenes* survival and/or growth in certain storage and packaging conditions. Food business operators should therefore take action to provide adequate assurance that the microbiological criteria will be met through the product shelf-life. The control of environmental contamination is not sufficient to reduce the risks associated with *L. monocytogenes*, therefore post-lethality treatment (ie pasteurization) applied after the packaging of the product are required to limit the growth. The PCR screening protocols proposed in this paper can be used for surveillance and epidemiological investigation of *L. monocytogenes* in Ricotta Salata. Finally our results, are one of the first reports of the screening of potentially virulent strains of *L. monocytogenes* isolated from Ricotta Salata and may be useful in the risk assessment.

Author Contributions

V. Coroneo and C. Sanna : realized the study, maintained the isolates, the strains, prepared cultures and performed the experiments and interpreted the results. V. Carraro and A. Sanna: critical appraisal and editing of the manuscript. Aissani N: prepared cultures and performed the experiments. All authors have read and approved this manuscript.

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