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Inactivation of *Listeria monocytogenes* using water bath heat treatment in vacuum packed ricotta salata cheese wedges

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ABSTRACT: Ricotta salata cheese is frequently contaminated on the surface with *L. monocytogenes*. Water bath heat treatment in vacuum packed whole ricotta salata cheese wheels demonstrated to be effective in inactivating *L. monocytogenes*. However the risk of cross contamination in ricotta salata wedges is increased during cheese cutting. Therefore, the effectiveness of heat treatment in ricotta salata wedges has to be demonstrated conducting a new validation study. In the present study nine different time temperature combinations, 75°C, 85°C and 90°C applied for 10 min, 20 min and 30 min each, were tested on artificially contaminated ricotta salata cheese wedges. The extent of the lethal effect on *L. monocytogenes* was assessed one day and 30 days after the application of the hot water bath treatment. Five out of nine combinations, 75°C for 30 min, 85°C for 20 and 30 min and 90°C for 20 and 30 min, demonstrated to meet the process criteria of at least 5 log reduction. Sensory analyses were also conducted in order to account for the potential impact on sensory features of ricotta salata wedges which showed no significant differences between treatments.

Keywords: *Listeria monocytogenes*, Whey cheese, Post-lethality treatment, Sensory properties.

Practical Application:

The present study allowed to select water bath heat treatments of vacuum packed ricotta salata wedges effective to reduce *L. monocytogenes* contamination. Such treatments can be successfully applied by food business operator to meet compliance with microbiological criteria through the designated shelf-life.

Introduction

Ricotta salata is a whey protein cheese largely produced in Sardinia (Italy) using the whey remaining after the production of sheep's milk cheeses, mainly Pecorino Romano PDO (protected

denomination of origin). After filtration and pre-heating at temperature of 60-70°C, the whey is heated in open kettles under gentle agitation for about 30 min to temperature of 85-90°C. As consequence of whey protein coagulation, the curd starts floating on the surface and then is scooped into perforated plastic hoops lined with cheesecloth. In order to increase drainage, the curd is pressed for approximately 24 hours. Ricotta is salted by dry salting or by brine-salting and dried in storage rooms under controlled temperature (10-12°C) and humidity for 10-15 days. The final moisture of the product depends on its intended use, usually 53-55% if ~~intended~~ for grating and between 55-60% if is to be consumed as such. Traditionally ricotta salata is a cylindrical shaped wheel (ricotta "*Toscanella*") with an average weight of approximately 3 kg, pH of 6.1-6.9, a_W of 0.940-0.970, fat of 28-33%, protein of 14-23% and NaCl of 3-4% (Spanu and others 2012; Spanu and others 2013; Spanu and others 2015a). Ricotta salata wheels are individually packed in vacuum bags as a whole or after cutting into smaller wedges of ca. 200-300 g. Wedges are shaped as triangular prisms with height, width and length of ca. 3.5 cm, 8.5 cm and 12 cm, respectively. The shelf-life of the product differs from one business operator to another, but it usually ranges between few weeks up to several months at refrigeration temperature. Despite the application of good hygiene practices together with the application of procedures based on the HACCP principles during manufacturing, recalls of ricotta salata produced in Sardinia due to *Listeria monocytogenes* contamination have been reported in recent years (RASFF 2008; CDC 2012). Raw milk and whey can be excluded as the origin of the contamination in consideration of heat treatments usually applied during cheese making (i.e. thermization and pasteurization) and whey protein coagulation (Buazzi and others 1992; Casadei and others 1998; ICMSF 1996; Villani and others 1996). *L. monocytogenes* contamination in ricotta salata is generally limited to the product surfaces and origins from the processing environment. Previous studies reported a prevalence of *L. monocytogenes* in ricotta salata rind of ca. 20% (Pintado and Malcata, 2000; Lioliou and others 2001; Ibba and others 2013; Spanu and others 2015b). Contamination of ricotta salata is rarely

observed in the inner paste and is generally a consequence of flaws in the application of production hygiene procedures (Spanu and others 2015a). Cutting ricotta salata wheels into smaller portions and repacking for retail sale can expose the product to cross-contamination originating from utensils used to cut the cheese, thus increasing the risk of *L. monocytogenes* contamination. Previous investigation demonstrated a *L. monocytogenes* growth potential in contaminated ricotta salata wheels of ca. 5 $\log_{10}$ cfu g^{-1} on the rind during the first two months of storage at refrigeration temperature (Spanu and others 2012). This represents a serious concern in such product, since ricotta salata does not have an actual rind and it is generally assumed to be whole edible. There is evidence that food business operator cannot demonstrate compliance with microbiological food safety criteria of 10^2 cfu g^{-1} at the time of consumption set by European Commission (EC) Regulation No. 2073/2005 throughout the shelf-life. This issue is even more stringent considering that ricotta salata is largely exported in North America where the limit recommended by FDA is more restrictive, requiring the absence in 25 g.

Post-process decontamination procedures are widely used in ready to eat food industry to reduce the risk of *L. monocytogenes* contamination in packaged products (Zhu and others 2005). These include methods such as thermal pasteurization, irradiation and high-pressure referred to as post-lethality treatments since they are applied to the final product or sealed package of product in order to reduce or eliminate the level of pathogens resulting from contamination from post-lethality exposure (FSIS 2014; Spanu and others 2014). To describe the overall effect of a control measure on a specific hazard at a step it is used the term *performance criterion* (PC), which is defined as: the effect in frequency and/or concentration of a hazard in a food that must be achieved by the application of one or more control measures to provide or contributes to an food safety objectives or appropriate level of protection, as applicable (CAC, 2004).

Hot water bath treatments applied in whole ricotta salata cheese wheels have been previously proven to be effective in reducing *L. monocytogenes* contamination on the rind up to 5-6 $\log_{10}$ cfu

98 g^{-1} (Spanu and others 2015a). However, a thermal treatment should take into account several factors
99 such as temperature-time combination, microbiological profile, composition and product size and
100 weight (Doyle and others 2001; Ray 2004; Sofos 2002; Yen and others 1991).
101 Effectiveness of the immersion of portioned vacuum packed ricotta salata in hot water bath needs to
102 be validated with an ad hoc study.
103 The aim of the present study was to evaluate the efficacy of hot water bath treatment on the
104 reduction of *L. monocytogenes* in artificially contaminated ricotta salata wedges. Nine different
105 temperature-time combinations were compared in order to identify the process criteria required to
106 deliver a 5 log reduction of *L. monocytogenes* concentration. The impact of post-lethality treatments
107 on sensory properties of ricotta salata has been also evaluated. Inactivation of *L. monocytogenes*
108 was assessed 24 hours and 30 days after the treatment. The suitable treatment combination will be
109 selected to conduct a successive shelf -life study to assess compliance of treated ricotta salata
110 wedges for the designated durability.
111
112 **Materials and methods**
113
114 *Ricotta salata samples*
115 A local cheese-making plant supplied 465 vacuum packed ricotta salata wedges, obtained from the
116 whey remaining after the production of sheep milk cheeses. Ricotta salata samples belonged to 3
117 different production batches (155 from each batch). Ricotta salata wedges were transported and
118 stored at refrigeration temperature (4±2°C) until use for the experiment within 24 h. Part of ricotta
119 salata wedges (inoculated units) were artificially contaminated with *L. monocytogenes* while
120 another part (uninoculated units) was not contaminated. Inoculated units included ricotta salata
121 samples submitted to heat treatment, or experimental units (EUs), used to assess the efficacy of the
122 treatment and positive controls (PCs), used to check for the inoculum level. Uninoculated samples

included blank samples (BLs), used to evaluate the level of natural contamination of ricotta salata with *L. monocytogenes*; sensory units (SEs) used to evaluate sensory properties after heat treatment; composition units (CUs) samples used for the determination of intrinsic properties (pH and a_W) and composition (moisture, fat and proteins). Detailed description of the number of ricotta salata wedges included in each of the sample types is showed in table 1.

Artificial contamination

The artificial contamination of EUs and PCs ricotta salata wedges was conducted according to the Technical Guidance document prepared by the EU Community Reference Laboratory (CRL) for *L. monocytogenes* (Beaufort and others 2014). In the preparation of the inoculum was used a mixture of 5 *L. monocytogenes* strains composed by the reference strain ATTC 19111 (serovar 1/2a) obtained from American Type Culture Collection (Manassas, VA, USA) and by other four wild-type strains (respectively serotypes 1/2a, 1/2b, 1/2c and 4b), previously recovered from the cheese-making plant environments or from ricotta salata. Selection of strains and preparation of the inoculum have been previously described (Spanu and others 2015a). The target level of contamination was 5 $\log_{10}$ cfu g^{-1} of rind, which was obtained spraying ricotta salata wedges with 2 mL of inoculum at a concentration of 10^7 cfu mL^{-1}. The concentration of the inoculum was confirmed by plate count on Trypticase Soy Agar (TSA, Biolife, Milan, Italy). The whole surface of ricotta salata wedges was evenly sprayed using an atomizer. Before ricotta salata samples were individually vacuum packed in shrink bags (Criovac Cook-In HT-3000, Sealedair Ltd., St Neots, UK) a holding period of 15 min at room temperature was allowed to let the inoculum attach (Health Canada, 2012). Vacuum packed ricotta salata wedges were then stored in cold room at 4±2°C until further analyses. The inoculum was performed under a microbiological safety cabinet and the operator worn protection devices.

148 *Heat treatment and testing times*

149 Independent trials were conducted for each of the three production batches. The post-lethality
150 treatment was performed by immersion of vacuum packed ricotta salata wedges in a stainless steel
151 vat containing hot water. Nine different temperature-time conditions were tested: 75°C, 85°C and
152 90°C applied for 10 min, 20 min and 30 min each. Ricotta salata samples used for each treatment
153 condition are reported in table 1. Once treated, in order to firm up ricotta salata wedges, the samples
154 were immersed for approximately 2 hours in iced water and then stored at 4±2°C until analysis. The
155 testing times (T) were: T_0 which was the day of the artificial contamination and heat treatment; T_1
156 and T_{30} which were respectively 24 hours and 30 days after heat treatment. At T_0 were analyzed
157 PCs to assess if the level of contamination was effectively 10^5 $\log_{10}$ cfu g^{-1} and BLs to check for
158 eventual natural contamination of ricotta salata with *L. monocytogenes*. The EUs and CUs were
159 submitted to heat treatment and analysed at T_1 and T_{30}. The SE units were either submitted to heat
160 treatment (SEt) or used as negative controls (SEc). Types of ricotta salata samples, analysis
161 performed and sampling times are summarized in Table 2. Temperatures obtained during treatments
162 were monitored using datalogger (KT 20T, Kimo, Montpon Ménestérol, France) positioned on the
163 surface of an additional ricotta salata sample. Loggers were button shaped stainless steel recorder
164 with a diameter of ca. 17 mm and height < 6 mm which were inserted with their lower side 0.5 cm
165 deep in the paste and their upper side in the interface between ricotta rind and packaging bag.
166
167 *Microbiological analysis*

168 Ricotta salata rind was aseptically collected cutting the wedges surfaces up to 2 cm in depth.
169 Detection and enumeration of *L. monocytogenes* (ISO 11290-1:1996/Amd 1:2004; ISO 11290-
170 2:1998/Amd 1:2004) and enumeration of aerobic mesophilic bacteria (ISO 4833:2003) were
171 conducted in accordance with standard methods. The potential presence of sublethally injured cells
172 that may survive the heat treatment but may not be cultured on selective media was investigated

using the Thin Agar Layer (TAL) method (Kang & Fung, 1999; Wu & Fung, 2001). This method combines the ability of nonselective agar media to growth injured cells and of selective media to differentiate microorganism. Preliminary identification of *L. monocytogenes* was confirmed by picking 5 suspected colonies from each positive sample which were submitted to phenotypic and molecular identification (Doumith and others 2004; De Santis and others 2007). To confirm that strains recovered at T_{30} were the same as the ones used in the inoculum, a selection (up to 5 strains from each temperature time combination) was submitted to pulsed-field gel electrophoresis (PFGE), according to the protocol proposed by Graves and Swaminathan (2001). The PCR serogroups were used as preliminary screening criteria of isolates to be submitted to PFGE. Comparison of restriction profiles allowed to differentiate between inoculated strains and strains originating by natural contamination.

Centesimal composition and intrinsic factors

Ricotta salata centesimal composition (fat, moisture, protein, total solids and sodium chloride) and intrinsic factors (pH and a_W) were determined to account for possible interaction with *L. monocytogenes* survival and growth. Compositional data were obtained by near infrared transmittance (NIT) using a compositional analyzer (FOSS, Eden Prairie, MN, USA), while pH and a_w were measured using pH meter GLP22 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab 4TE (Decagon, Pullman, WA, USA), respectively. Comparison of centesimal composition and intrinsic factors ($\bar{x} \pm$SD) of ricotta salata wedges submitted to heat treatment with the nine different temperature-time combinations and over time (T_1 and T_{30}) was conducted using the Fisher's least significant difference (LSD) test, performed with Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA, USA).

Sensory analysis

198 The "Difference from control test" allowed to determine if a significant difference between the
199 treated (SEt) samples and the negative control (SEc) exist and to estimate the size of this difference
200 (Meilgaard and others 1999). In order to quantify the experimental noise (*placebo* effect), the
201 control sample (SEc) must be served during each session both as reference and as blind sample.
202 This cause a sensory fatigue especially if all of nine time-temperature combinations samples have to
203 be taken into account, for this reason the sensory analysis was restricted to only five heat treatments
204 corresponding to the lowest and highest time-temperature combinations (75°C and 90°C, 10 min
205 and 30 min) and the central point (85°C treated for 20 min). All of these heat treatments were
206 performed and evaluated in triplicate. Thirty assessors equally distributed between females and
207 males, aged from 25 to 50 years were selected after attending a course of 60 hours in sensory
208 analysis (ISO 8586-1: 1993) applied to dairy products. Assessors were asked to determine the
209 difference between the control sample (SEc) and the blind coded test sample (SEt) on a numerical
210 ten points scale (0 = no difference and 9 = very large difference). The Ricotta salata wedges were
211 portioned into parallelepiped pieces (5 x 1.5 x 1.5 cm) and served in a randomized and balanced
212 order (Macfie and others 1989), in odorless plastic containers marked with a random three-digit
213 number (Meilgaard and others 1999) at room temperature. Assessors were also provided with an
214 unsalted cracker and a glass of water in order to rinse their mouth after each evaluation. Raw data
215 (n=540) were analyzed by the two-way ANOVA ($P \leq 0.05$) using the Statgraphics Centurion XVI
216 software (StatPoint Technologies, Warrenton, VA, USA).
217
218 *Validation of heat treatment*
219 The effectiveness of each of the nine heat treatment combinations was evaluated computing the
220 minimum difference between *L. monocytogenes* concentration ($\log_{10}$ cfu g^{-1}) before the treatment
221 (T_0) and after the treatment (T_1 and T_{30}) and expressed as performance criterion or performance

222 standard (Δ.) The Δ was computed independently for each of the three production batches and for
223 each value was obtained from triplicate samples.
224
225 *Statistical analysis*
226 Comparison of Mean mesophilic bacteria counts (log_{10} cfu g^{-1}), intrinsic properties ($\bar{x}$±SD) and
227 centesimal composition (%±SD) between temperature-time combinations at T_1 and T_{30} was
228 conducted using Fisher's least significant difference (LSD) test. All statistical analyses were
229 performed with Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA,
230 USA).
231
232 **Results**
233 *L. monocytogenes contamination and background microflora*
234 In all BLs natural contamination with *L. monocytogenes* was never observed. In artificially
235 contaminated ricotta salata wedges (PCs) *L. monocytogenes* mean count expressed as log_{10} cfu g^{-1}
236 ($\bar{x}$±SD) was 5.24±0.11, 5.4±0.05 and 5.03±0.4 in the first, second and third batch, respectively.
237 Aerobic mesophilic bacteria enumeration ($\bar{x}$±SD log_{10} cfu g^{-1}) in BLs was 4.86±0.31, 6.44±0.46,
238 6.53± 0.41 while in PCs was 5.67±0.19, 7.12±0.26 and 6.35±0.62 in the first, second and third
239 batch respectively. At T_0 the reduction in mean log_{10} cfu g^{-1} total bacterial counts changed
240 according to temperature, ranging between ca.1.5 and 3.0 for ricotta treated at 75°C and up to 5.0
241 for treatment at 85°C and 90°C, while bacterial counts at T_{30} was a function of the time of heat
242 treatment, with an average increase of 2.5-3.0 observed only in ricotta samples treated for 10 min.
243 Pair-wise comparison of aerobic mesophilic bacteria counts between temperature-time
244 combinations at T_1 and T_{30} is reported in table 3.
245
246 *Post lethality treatment*

247 The fate of *L. monocytogenes* (inactivation, survival or growth) was assessed for each temperature-
248 time combination at T_1 and T_{30}. Despite treatment applied for 10 min showed a certain degree of
249 reduction of *L. monocytogenes* concentration at T_0 (between ca. 1 log at 75°C and 5 log at 90°C), in
250 the successive 30 days of refrigerated storage *L. monocytogenes* concentration increased to levels as
251 high as the initial contamination or even higher. After the water bath treatment applied for 30 min
252 with all three different temperatures, *L. monocytogenes* was not recovered with the detection
253 method, suggesting a full efficacy of treatments (figure 1). The treatments for 20 min were not
254 effective only for the lowest temperature tested. The performance standard or Δ of each of the nine
255 treatment combinations is reported in table 4.
256
257 *L. monocytogenes strains characterization*
258 Overall, 217 strains were confirmed as *L. monocytogenes* by molecular identification, 45 from PCs
259 and 172 from EUs, respectively. Of the strains recovered from PCs, 11 belonged to serogroup 1/2a,
260 12 to serogroup 1/2b, 3 to serogroup 1/2c and 19 to serogroup 4b. Of the strains recovered from
261 EUs 68 (39.5%) were serogroup 1/2a, 48 (27.9%) serogroup 1/2b, 32 (18.6%) serogroup 1/2c and
262 24 (13.9%) serogroup 4b. Comparison of PFGE profiles of the 48 *L. monocytogenes* strains selected
263 from EUs at T_{30} showed identical profiles with strains used to contaminate the ricotta salata wedges.
264 None of the strains showed similar profile with the reference strain ATCC 19111.
265
266 *Temperature monitoring*
267 The temperature recorded on ricotta salata rind before hot water bath treatment was 3.7±0.5°C. The
268 graph in figure 2 shows the evolution of the temperature on ricotta salata rind over time for each of
269 the three water temperature used during treatments.
270
271 *Ricotta salata composition*

Intrinsic properties values ($\bar{x}$ ±SD) determined on the 18 BLs were 6.40±0.16 for pH and 0.977±0.01 for a_w, while composition values (%±SD) were respectively of 53.92±3.29 for moisture, 21.50±3.75 for fat, 16.50±1.52 for proteins and 3.42±0.28 for NaCl. Composition analysis was also conducted on 162 treated samples (CUs) and differences in intrinsic properties and composition between the 9 treatment combinations at T_1 and T_{30} are reported in table 5. Ricotta salata wedges pH and a_w showed no significant difference with respect to the heat treatment (P>0.05) and were always above the lower limits for *L. monocytogenes* growth.

Sensory features

Difference from the control test performed by ANOVA showed no significant difference (P=0.45) (treatment effect) between the control sample (SEc) and the test sample (SEt) (table 6). Mean and standard deviation of the control, provided as blind samples (blind control), are statistically comparable with the means and the standards deviation of the heat treated samples, indicating that the heat treatment do not produce an effect greater than the experimental noise. Since all of the five heat treated samples, the lowest and highest time-temperature combinations (75°C and 90°C, 10 min and 30 min) and the central point (85°C treated for 20 min), are not significant different, it is reasonable to extend the same result on the four time-temperature combinations included in this field and not evaluated by the sensory analysis: (85°C for 10 min and 30 min, 75°C and 90°C for 20 min). Moreover the ANOVA shows that also the factor "replicate" is not significant (P=0.84) (table 6) validating both the heat treatments and the sensory analysis.

Discussion

Contamination of ricotta salata with *L. monocytogenes* is mainly a post process contamination originating from the processing environment and is generally limited to the rind (Pilo and others 2007). This represents a serious concern since ricotta salata wedges are consumed as such and the

297 rind is edible. Ricotta salata production includes pressing of the curd which could favour deepening
298 of the microorganism in the first centimeters of the paste. In addition the risk of cross contamination
299 in ricotta salata wedges is increased by transfer of the microorganism through blades during
300 mechanical cutting. Despite the contamination of ricotta salata with *L. monocytogenes* is not a rare
301 finding, the observed prevalence could range from 0.0% to as high as 20.0-30% (Spanu and others
302 2015b), in the present study was never detected from blank samples. The processing environment
303 represents the main contamination route. Therefore, the reason for this wide variability of
304 contamination between premises is largely due to difference in the implementation of good hygienic
305 and good manufacturing practices by the processor. A cheesemaking plant of proven history in
306 implementing and maintaining hygienic procedures provided ricotta salata samples used in the
307 present study. In addition, in order to avoid as much as possible the occurrence of natural
308 contamination, the production of ricotta salata batches intended to be used in the present study
309 where monitored, to guarantee that all the necessary measure where put in place to limit
310 contamination of the product. However, in many circumstances the prevention of environmental
311 contamination is almost impossible to avoid by the mere use of hygienic measures. Therefore, it is
312 essential for food business operator to implement strategies to control the contamination in order to
313 comply with microbiological limits for *L. monocytogenes* in RTE foods. A number of post-lethality
314 treatments have been proposed, among which hot water bath of vacuum packed ricotta salata
315 demonstrated to be effective (Spanu and others 2013; Spanu and others 2015a). The present study
316 evaluated the listericidal effect of nine different water bath temperature-time combinations in
317 vacuum packed ricotta salata wedges. The performance criterion of the treatment was to reduce *L.*
318 *monocytogenes* concentration of 5 log cfu g^{-1} from ricotta salata surface. In order to account for the
319 potential presence of sublethally injured cells, which during this period may recover their ability to
320 growth, the fate of *L. monocytogenes* was evaluated also after 30 days of refrigerated storage.
321 Efficacy of treatments was conditioned by exposition time rather than temperature. None of the

322 treatments applied for 10 min resulted effective, while all temperatures inactivated *L.*
323 *monocytogenes* when the treatment was prolonged up to 30 minutes. Intermediate situation was
324 observed for the 20 minutes treatments which were effective at 85°C and 90°C. Although *L.*
325 *monocytogenes* was inactivated to some extent (ca. 4 log) by treatments at 85°C and 90°C for 10
326 minutes, the cells surviving to the heath treatment increased in the subsequent storage to levels as
327 high as 5 log. Temperatures registered by dataloggers on ricotta salata surface explain the effect on
328 *L. monocytogenes* inactivation. Treatments conducted at 75°C only when applied for 30 min
329 allowed to obtain temperature on the product > 70°C which were maintained for 10 min, while after
330 the 10 min and 20 min treatments the maximum temperature registered were respectively 65.5°C
331 and 70°C. Although the treatment at 85°C for 10 min reached 72.6°C, it stayed above 70°C on
332 ricotta surface only for 2 min, justifying the survival of *L. monocytogenes*. Treatments at 85°C for
333 20 min and 30 min guaranteed on ricotta surface temperature above 70°C for 12 min and 23 min,
334 respectively. Similar behavior was observed for treatment at 90°C for 10 min which reached 76.3°C
335 on the product surface but temperature above 70°C were maintained only for 4 min. The 90°C for
336 20 min and 30 min hesitated in maximum temperature of 84.6°C and 88.1°C on ricotta rind which
337 were above 70°C for 14 min and 24 min respectively. Strains capable to survive and growth after
338 heat treatment belonged to the wild type, suggesting that they are characterized by a greater
339 resistance or a better adaptation to the substrate as compared with reference strains. However it
340 should be considered that the actual temperature recorded by dataloggers is the balance between hot
341 water bath temperature at the interface product-packaging and cold product temperature on the
342 lower face of the datalogger. Despite differences in treatment efficacy, the sensory analysis reveals
343 that the heat treated samples (SEt) are not significant different from the untreated control samples
344 (SEc), indicating that any heat treatments between 75°C for 10 min and 90°C for 30 min does not
345 affect the sensory properties of ricotta salata wedges.
346

Conclusion

Superficial contamination of ricotta salata rind with *L. monocytogenes* is difficult to avoid even with strict production hygiene procedures. Compliance with microbiological limits could be obtained with the application of control strategies aimed to reduce the contaminations originating in the post-processing environment. Post-lethality treatments such as hot water bath applied on packaged ricotta salata are effective and feasible treatment to kill *L. monocytogenes* from ricotta salata surface. Eligible treatments in ricotta salata wedges are 75°C for 30 min, 85°C for 20 and 30 min and 90°C for ~~10,~~ 20 and 30 min. These treatments should not be intended as an alternative measure to good manufacturing and good hygiene practices but rather an additional tool to obtain compliance with microbiological criteria.

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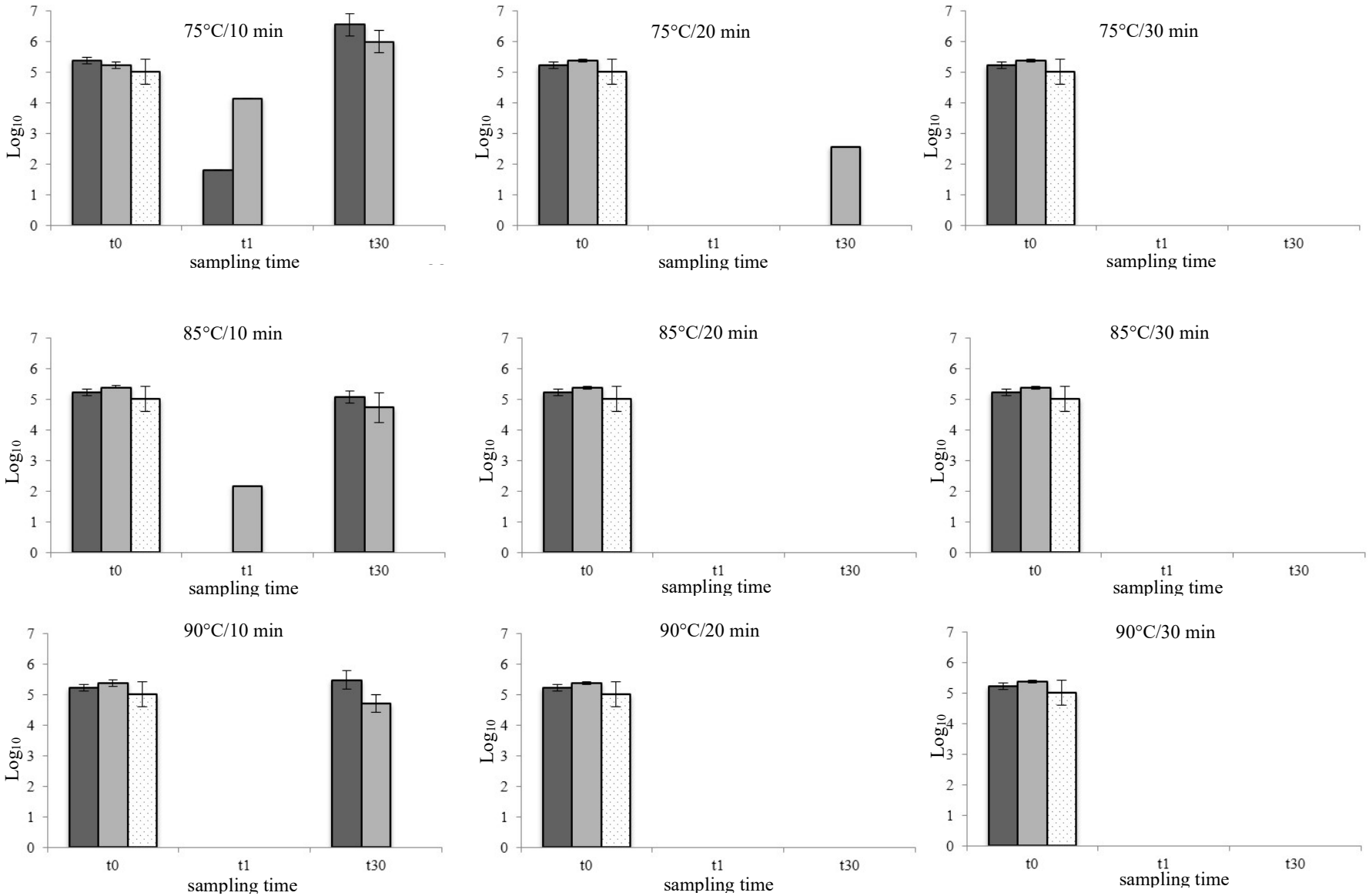
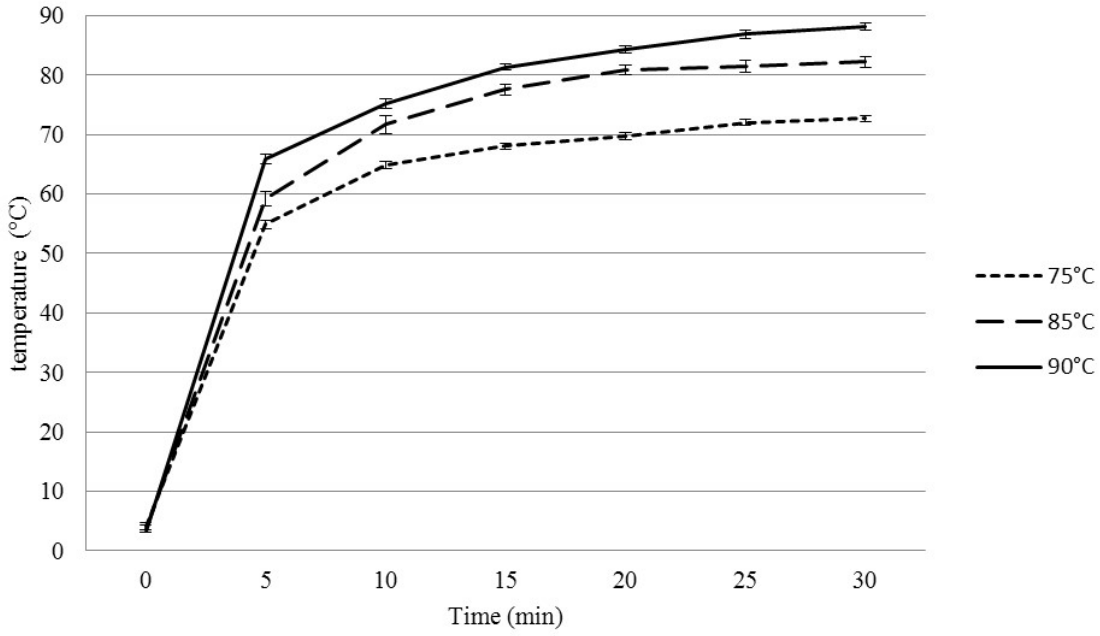
Figure 1. Inactivation of *L. monocytogenes* (log_{10} cfu g^{-1}) in artificially contaminated ricotta salata wedges analyzed 24 h (T_1) and 30 days (T_{30}) after water bath heat treatment with 9 temperature time combinations.



505 ■ = replicate a; ■ = replicate b; ▦ = replicate c; Sample size: t0 n = 3 for each replicate; t1and t30 n = 3 for each replicate and
506 temperature/time combination.

Figure 2. Temperatures recorded during water bath heat treatment on the interface ricotta salata rind packaging bag surface.



Each data point is the mean of temperatures recorder in the three replicates (batch A, B and C).

523 Table 1. Types of treatment and ricotta salata wedges samples used in the hot water bath validation study.

Temperature-time condition		Ricotta salata samples					
Temperature	Minutes	BL[1]	PC[2]	EUs[3]	CUs[4]	SEs[5]	Total
No treatment	-	18	9	-	-	42	69
75°C	10	-		18	18	12	48
	20	-		18	18	3	39
	30	-		18	18	12	48
85°C	10	-		18	18	3	39
	20	-		18	18	12	48
	30	-		18	18	3	39
90°C	10	-		18	18	12	48
	20	-		18	18	3	39
	30	-		18	18	12	48
Total		18	9	162	162	114	465

524 [1]BLs (Blank Samples): not inoculated units; [2]PCs (Positive Controls): samples inoculated with *L.*
525 *monocytogenes*; [3]EUs (Experimental Units): samples inoculated with *L. monocytogenes* and successively
526 heat treated; [4]CUs (Composition Units): heat treated units for physico-chemical analysis; [5]SEs (Sensory
527 Units): samples used to assess the effect of heat treatment on sensory properties.
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536 Table 2. Types of ricotta salata samples, analysis and testing times.

		Sampling time			
Analysis	Test units	T_0[a]	T_1[b]	T_{30}[c]	Total
Detection and enumeration of *L. monocytogenes* and aerobic mesophilic bacteria	BLs[1]	18	-	-	18
	PCs[2]	9	-	-	9
	EUs[3]	-	81	81	162
	CUs[4]	-	81	81	162
Intrinsic properties and composition	BLs[1]	18	-	-	-
	CUs[4]	-	81	81	162
Sensory analysis	SEs[5]				38

537 Superscript letters are referred to the time between inoculation and analysis: [a] = day of inoculum and heat
538 treatment; [b] = 24 hours after heat treatment; [c] = 30 days after heat treatment. Superscript numbers are referred
539 to test units: [1]BLs (Blank Samples): not inoculated units; [2]PCs (Positive Controls): samples inoculated with
540 *L. monocytogenes*; [3]EUs (Experimental Units): samples inoculated with *L. monocytogenes* and successively
541 heat treated; [4]CUs (Composition Units): heat treated units for physico-chemical analysis; [5]SEs (Sensory
542 Units): samples used to assess the effect of heat treatment on sensory properties.
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552 Table 3. Comparison of aerobic mesophilic bacteria counts (log_{10} cfu g^{-1}; $\bar{x}$ ± SD) of heat treated ricotta
553 salata wedges with 9 different temperature-time combinations analyzed 24 h after the treatment (T_1) and after
554 storage at refrigeration temperature for 30 days (T_{30}).

Treatment		Aerobic mesophilic bacteria			
Temperature	Minutes	+ve/n	T_1	+ve/n	T_{30}
75°C	10	8/9	3.89 ± 0.90^{A}	9/9	6.64 ± 0.27^{A}
	20	5/9	3.14 ± 1.08^{A}	7/9	3.01 ± 0.38^{C}
	30	2/9	3.41 ± 0.58^{AB}	1/9	1.48 ± 0.0^{C}
85°C	10	6/9	3.04 ± 1.14^{ABC}	9/9	5.51 ± 1.18^{B}
	20	3/9	1.10 ± 0.17^{D}	5/9	1.91 ± 0.75^{C}
	30	2/9	1.15 ± 0.21^{D}	2/9	1.67 ± 0.95^{C}
90°C	10	7/9	1.98 ± 0.93^{BCD}	8/9	5.08 ± 1.55^{C}
	20	1/9	1.00 ± 0.0^{CD}	1/9	1.48 ± 0.0^{C}
	30	1/9	1.00 ± 0.0^{CD}	1/9	1.00 ± 0.0^{C}

555 Means in the same column on the same testing time (T_1 or T_{30}) with different superscript letter are
556 significantly different ($P\leq 0.05$). +ve/n are the number of samples where aerobic mesophilic bacteria could
557 be enumerated.
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570 Table 4. Enumeration, detection and reduction (Δ)a of *Listeria monocytogenes* on the surface of ricotta salata wedges artificially contaminated and heat treated in

571 hot water bath with different temperature-time combinations.

Treatment			*L.monocytogenes*							
			T_1				T_{30}			
Temperature	Minutes	Batch	Enumeration		Δ	Detection in 25 g	Enumeration		Δ	Detection in 25 g
			+ve/n	log_{10} cfu/g	T_1-T_0	+ve /n	+ve/n	log_{10} cfu/g	T_{30}-T_0	+ve /n
75 °C	10	A	1/3	4.16±0.00	-0.99	3/3	3/3	6.01±0.37	1.28	3/3
		B	2/3	1.82±1.16	-2.73	3/3	3/3	6.56±0.47	1.73	3/3
		C	0/3	ND	-4.76	3/3	3/3	6.01±0.38	1.63	3/3
	20	A	0/3	ND	-5.15	0/3	3/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	1/3	2.57±0.00	2.79	1/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
	30	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
85 °C	10	A	0/3	ND	-5.15	2/3	2/3	5.09±0.20	0.08	2/3
		B	1/3	2.18±0.00	-3.18	3/3	3/3	4.74±0.49	-0.29	3/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-0.25	0/3
	20	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
	30	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
90 °C	10	A	0/3	ND	-5.15	3/3	3/3	5.48±0.30	0.67	3/3
		B	0/3	ND	-5.36	1/3	2/3	4.72±1.83	0.65	2/3
		C	0/3	ND	-4.76	2/3	3/3	5.58±0.07	0.88	3/3
	20	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
	30	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3

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[a]Values are the difference between concentration (log_{10} cfu g^{-1}) the day of artificial inoculation (T_0) and 24 hours (ΔT_1) and 30 days (ΔT_{30}) after treatment. For each batch and for each temperature-time combination values are the minimum difference between the initial contamination level and the maximum count after the treatment in the triplicate samples. +ve/n are the number of samples where *L. monocytogens* could be enumerated. ND are the samples where *L. monocytogenes* was below the detection limit.

Table 5. Intrinsic properties ($\bar{x}$ ±SD) and composition (%±SD) of ricotta salata submitted to 9 different heat treatment combinations and analyzed 24 h (T_1) and 30 days (T_{30}) after storage at refrigeration temperature.

Treatment		pH		a_W		Moisture %		Fat %		Proteins %		NaCl %	
Temperature	Min	T_1	T_{30}	T_1	T_{30}	T_1	T_{30}	T_1	T_{30}	T_1	T_{30}	T_1	T_{30}
75°C	10	6.36±0.13^{a}	6.30±0.12^{a}	0.975±0.001^{a}	0.976±0.000^{a}	52.94±2.58^{a}	52.69±3.33^{a}	20.49±2.20^{a}	22.88±4.16^{a}	18.00±1.74^{a}	16.30±1.56^{a}	3.30±0.52^{a}	3.36±0.26^{ab}
	20	6.33±0.10^{a}	6.27±0.14^{a}	0.976±0.000^{a}	0.977±0.001^{a}	53.50±2.91^{a}	54.85±3.15^{a}	21.45±4.08^{a}	20.67±3.58^{a}	17.16±1.35^{a}	16.75±1.62^{ab}	3.23±0.36^{a}	3.46±0.22^{abc}
	30	6.38±0.10^{a}	6.23±0.17^{a}	0.977±0.000^{ab}	0.977±0.000^{a}	53.04±2.84^{a}	54.05±2.64^{a}	20.96±2.28^{a}	22.23±3.16^{a}	18.13±1.50^{a}	16.20±1.28^{a}	3.25±0.15^{a}	3.34±0.26^{ab}
85°C	10	6.35±0.08^{a}	6.24±0.13^{a}	0.978±0.000^{ab}	0.976±0.000^{a}	53.21±3.57^{a}	54.41±2.86^{a}	20.92±2.65^{a}	20.62±2.23^{a}	17.85±1.72^{a}	16.62±1.12^{ab}	3.31±0.26^{a}	3.56±0.38^{bc}
	20	6.36±0.11^{a}	6.27±0.07^{a}	0.979±0.000^{ab}	0.975±0.000^{a}	54.67±2.07^{a}	53.43±4.10^{a}	20.15±2.08^{a}	20.61±2.11^{a}	17.29±1.50^{a}	18.19±2.25^{b}	3.31±0.23^{a}	3.52±0.22^{a}
	30	6.39±0.09^{a}	6.28±0.08^{a}	0.979±0.000^{ab}	0.980±0.001^{a}	54.13±3.65^{a}	55.31±3.63^{a}	20.42±2.05^{a}	20.41±1.96^{a}	18.04±2.17^{a}	17.15±2.19^{ab}	3.22±0.26^{a}	3.28±0.18^{a}
90°C	10	6.40±0.11^{a}	6.26±0.13^{a}	0.981±0.000^{b}	0.978±0.000^{a}	53.27±3.42^{a}	55.02±3.98^{a}	22.33±3.89^{a}	20.51±3.37^{a}	16.99±1.77^{a}	16.82±1.45^{ab}	3.24±0.27^{a}	3.43±0.28^{abc}
	20	6.39±0.11^{a}	6.25±0.11^{a}	0.981±0.000^{b}	0.977±0.000^{a}	53.65±4.02^{a}	54.02±2.80^{a}	21.00±3.00^{a}	21.28±2.38^{a}	17.91±1.97^{a}	17.44±1.71^{ab}	3.35±0.30^{a}	3.61±0.27^{c}
	30	6.34±0.09^{a}	6.24±0.08^{a}	0.981±0.000^{b}	0.978±0.000^{a}	54.92±2.84^{a}	54.73±4.02^{a}	20.84±2.93^{a}	21.53±3.74^{a}	16.88±0.97^{a}	16.97±1.61^{ab}	3.41±0.27^{a}	3.42±0.20^{abc}

Each data point is the mean of three samples. For each parameter means in the same column on the same testing time (T_1 or T_{30}) with different superscript letter are significantly different ($P \leq 0.05$).

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600 Table 6. Means and Standard deviation of sensory results

Treatment	Differences from the control		
Blind control	3.0 [a]	±	2.1
75°C x 10 min	2.9 [a]	±	2.0
75°C x 30 min	3.3 [a]	±	2.2
85°C x 20 min	3.5 [a]	±	2.3
90°C x 10 min	3.1 [a]	±	2.1
90°C x 30 min	3.1 [a]	±	1.9

601 Mean values with different superscript letters are significantly different among samples.* ($P \leq 0.05$).