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Comparison of post-lethality thermal treatment conditions on the reduction of *Listeria monocytogenes* and sensory properties of vacuum packed ricotta salata cheese

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Abstract

Ricotta salata is a whey protein cheese produced in Sardinia that in the last decades has been linked to several recalls and in 2012 to a severe human listeriosis outbreak. Contamination of ricotta salata with *L. monocytogenes* mainly occurs during post-process handling and generally origins from the processing environment. The application of water bath heat treatment in vacuum packed ricotta salata is a possible strategy to control *L. monocytogenes* superficial contamination. The objective of the present study was to select a heat treatment able to inactivate *L. monocytogenes* count of at least 5 log. Nine temperature time combinations, 75 °C, 85 °C and 90 °C applied for 15 min, 25 min and 40 min each were tested in ricotta wheels artificially contaminated with a mixture of 5 *L. monocytogenes* strains. Inactivation was assessed respectively one day and 30 days after heat treatment. The efficacy of treatments was evaluated based on the reduction in *L. monocytogenes* counts, on the impact on sensory properties and on the cost of the treatment. Two out of nine treatment combinations, i.e. 85 °C for 40 min and 90 °C for 40 min, were effective in reducing *L. monocytogenes* contamination level of 5 log. No significant difference was observed in sensory

properties after the heat treatments. Therefore both combinations are eligible to conduct a successive study aimed to extend the shelf-life of ricotta salata up to several months.

Keywords: *Listeria monocytogenes*, whey cheese, post-lethality treatment, sensory properties.

1. Introduction

Ricotta salata is a traditional whey protein cheese obtained in Sardinia (Italy) through the heat coagulation of the whey remaining after the production of sheep's milk cheeses. The main phases of production technology of ricotta salata are described as follows. Traditionally is manufactured using the whey remaining after the production of hard sheep's milk cheese, usually Pecorino Romano PDO (protected denomination of origin), which is stored in a silo at 45 °C until use. The whey is filtered and preheated at 60-70 °C using a plate heat exchanger. The whey is then transferred in large open kettles with approximately 1,200-1,500 liters capacity, added with 1% by weight of sodium chloride and heated to temperature above 80 °C for 30 minutes. As a result of heating, curd start floating on the top of liquid, this is collected using perforated scoops and transferred into plastic molds. The so called ricotta "*Toscanella*" is formed into cylindrical shapes and pressed to enhance drainage. The curd is salted either by dry-salting (5% w/v) or by brine-salting and dried for about 10 days in cold rooms at 10-12 °C. The manufacturing process result in cheese wheels weighing approximately 3 kg with a pH of 6.1-6.9, a_w of 0.940-0.970, moisture of 50-60% (< 50% if intended for grating), fat of 28-33% and protein of 14-23% (Spanu, Scarano, Spanu, Penna, Virdis, & De Santis, 2012; Spanu, Spanu, Pala, Virdis, Scarano, & De Santis, 2013). The final product is individually packed in vacuum bags and stored at refrigeration temperature with a set shelf-life which differs from three weeks up to several months, depending on the food business operators. Packaging of ricotta salata depends on the final use of the product, being ricotta salata wheels vacuum packed as a whole in shrinking bags if intended to be consumed grated, for mixing with other cheeses or as an ingredient, or cut into wedges before packaging if consumed plain. No preservatives are used for shelf life extension. In recent years contamination of ricotta salata with *Listeria monocytogenes* leaded voluntary recalls by international companies importing the product from Sardinia. In 2008 the European Commission documented a case of *L. monocytogenes* infection associated with the consumption of ricotta salata cheese (RASFF, 2008). The most recent and severe episode occurred

in the USA where a multistate outbreak of listeriosis linked to ricotta salata imported from Italy caused 20 hospitalization and 4 deaths (CDC, 2012). Heat treatments, such as thermization and pasteurization, applied to milk during cheese making and to whey during ricotta production inactivate *Listeria* cells to levels of approximately 3 to 6 $\log_{10}$ cfu (Buazzi, Johnson, & Marth, 1992; Casadei, Esteves de Matos, Harrison, & Gaze, 1998; ICMSF, 1996; Villani, Pepe, Mauriello, Moschetti, Sannino, & Coppola, 1996). Contamination of whey cheeses with *L. monocytogenes* origins from the processing environment and is localized almost exclusively on the rind, with a reported prevalence in ricotta salata of approximately 20% (Pintado & Malcata, 2000; Lioliou, Litopoulou-Tzanetaki, Tzanetakis, & Robinson, 2001; Ibba, Cossu, Spanu, Virdis, Spanu, Scarano, & De Santis, 2013; Spanu, Scarano, Ibba, Spanu & De Santis, 2015). The intrinsic properties of ricotta salata support the growth of *L. monocytogenes*, once onto the product, to level as high as 7.0 $\log_{10}$ cfu g^{-1} of rind, potentially harmful to human health (Spanu, Scarano, Spanu, Penna, Virdis, & De Santis, 2012). Ricotta salata produced in Sardinia is mainly exported in North America and in other European countries. However, international health authorities accept different health risk for *L. monocytogenes*, leading to an absence in 25 g recommended by FDA and 10^2 cfu g^{-1} criteria at the time of consumption set by European Commission (EC) Regulation No. 2073/2005. Even with a strict application of good hygienic practices during production, superficial contamination of ricotta salata could not be totally avoided, but only reduced (Tompkin, Scott, Bernard, Sveum, & Gombas, 1999). Therefore, the application of alternative control strategies should be applied if the product is exposed to environmental contamination after the lethality treatment (e.g., cooking) and before packaging (FSIS, 2014). In order to reduce *L. monocytogenes* contamination in ready to eat food, a number of post-package decontamination methods have been proposed, such as thermal pasteurization, irradiation and high-pressure. The efficacy of these decontamination technologies in different ready to eat products have been reviewed (Zhu, Du, Cordray, & Ahn, 2005). The final choice of the treatment to apply in ready to eat food stays on the food business operator based on scientific evidences on the efficacy, but is certainly cost-oriented. Heat post-lethality treatments (i.e. hot water bath and steam pasteurization) are widely used in the food industry due to their effectiveness in reducing the load of pathogenic microorganisms (Arnoldi, 2002; Orta-Ramirez & Smith, 2002). The effectiveness of a thermal treatment is influenced by several factors such as temperature-time

ratio, food composition, size and weight of the product and microorganism characteristics (Doyle, Mazzotta, Wang, Wiseman, & Scott, 2001; Ray, 2004; Sofos, 2002; Yen, Sofos, & Schmidt, 1991). Hence, the validation of a post-lethality treatment should be designed around the product, taking into account the formulation, packaging and the expected storage and use conditions. Therefore, results obtained on a specific product cannot be extended on another product, even if similar. Previous research demonstrated the efficacy of the immersion of vacuum packed ricotta salata wheels in water bath at 85 °C for 90 min in reducing *L. monocytogenes* counts of 6 $\log_{10}$ cfu g^{-1} of rind (Spanu, Spanu, Pala, Virdis, Scarano, & De Santis, 2013). However, the effect on sensory characteristics of such treatment was not investigated. Any technological interventions that negatively affect the sensory quality of a product become useless for a commercial purpose. For this reason sensory evaluation play a non negligible role in this type of investigation.

The objective of the present study was to compare 9 different temperature-time conditions for the superficial treatment of whole ricotta salata wheels. The efficacy will be evaluated taking into account the extent of reduction and survival of artificially inoculated *L. monocytogenes* and the impact on sensory properties. The results will be used to select the temperature-time ratio to perform a further study aimed to extend the shelf-life of ricotta salata up to 180 days.

2. Materials and methods

2.1. Ricotta salata samples

A total of 465 vacuum packed ricotta salata wheels were provided by a local cheese-making plant using sheep milk. Samples were randomly selected from 3 different batches (155 ricotta wheels for each batch) and stored in a cold room at 4±2 °C until the experiment was performed. Immediately after their arrival samples were labeled according to their use for the experiment. Experimental Units (EUs) were defined ricotta salata wheels artificially contaminated with *L. monocytogenes* and successively submitted to heat treatment. Positive Controls (PCs) were defined ricotta salata wheels artificially contaminated with *L. monocytogenes*. Blank Samples (BLs) were defined the units not inoculated and used to evaluate the level of natural contamination of ricotta salata with *L. monocytogenes*. Sensory Units (SEs) were defined the not inoculated

samples used to evaluate sensory properties either after heat treatment (SEt) or as control with no treatment (SEc). Composition Units (CUs) were defined not inoculated samples used for the determination of intrinsic properties (pH and a_W) and composition (moisture, fat and proteins) after heat treatments.

2.2. Artificial inoculation

The Technical Guidance document prepared by the EU Community Reference Laboratory (CRL) for *L. monocytogenes* (Beaufort, Cornu, Bergis, Lardeux, & Lombard, 2014) was used for the experiment designing. A mixture of 5 *L. monocytogenes* strains was used to artificially contaminate EUs and PCs ricotta salata wheels. Of the strains that composed the inoculum one was the reference strain ATTC 19111 (serovar 1/2a) obtained from American Type Culture Collection (Manassas, VA, USA), while the other four were 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. The wild-type strains were selected in order to be representative of the main serotypes associated with foodborne listeriosis. All the strains were stored at -80 °C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with glycerol (15% v/v). The inoculum level was aimed to demonstrate a reduction in *L. monocytogenes* level, or Performance Criterion (PC), of 5 $\log_{10}$ cfu g^{-1} of rind, considered to suffice to attain a Food Safety Objective (FSO) of 10^2 cfu g^{-1} throughout the entire storage period under refrigeration. Previous experiments were conducted to standardize the preparation of inoculum according to the indications contained in the Guidelines for conducting *Listeria monocytogenes* challenge testing of foods (Scott, Swanson, Frier, Pruett jr., Sveum, Hall, Smoot, & Brown, 2005). In order to prepare cells in the same physiological state (late exponential or early stationary phase) each strain was separately inoculated into tubes containing BHI broth and cultured overnight at 30 °C in a shaking water bath (100 rev min^{-1}). To adapt cultures at refrigeration temperatures, cells were then subcultured into 10 mL of BHI and incubated at 4±2 °C for approximately 15 days. A “mixed working culture” was obtained by transferring equal volumes of each individual culture into a sterile flask. The concentration was adjusted to ca. 10^7 cfu mL^{-1} using sterile saline solution (0.85% NaCl). Plate count on Trypticase Soy Agar (TSA, Biolife, Milan, Italy) was used to confirm concentrations. The whole surface of ricotta salata wheels was evenly sprayed with 2 mL of *L. monocytogenes* mixed culture using an atomizer. A

136 holding period of 15 min at room temperature was allowed to inoculated samples in order to let the
137 suspension attach, after which ricotta salata were individually vacuum packed in shrink bags (Criovac Cook-
138 In HT-3000, Sealedair Ltd., St Neots, UK) and stored at refrigeration temperature until further use.
139
140 *2.3. Heat treatment and experimental design*
141 The experiment was conducted in three independent trials, one for each batch, conducted one month apart.
142 Heat treatment was performed by immersion of vacuum packed ricotta salata wheels in hot water bath. Nine
143 different temperature- time conditions were tested: 75 °C, 85 °C and 90 °C applied for 15 min, 25 min and
144 40 min each. The number and the types of ricotta salata samples used for each treatment condition are
145 reported in table 1. Immediately after the heat treatment ricotta salata wheels were immersed in a tank
146 containing iced water for approximately 2 hours and then stored at 4±2 °C until analysis. The analysis points
147 or testing times (T) were: the day of inoculum and heat treatment, defined as T_0; 24 hours after heat
148 treatment, defined as T_1 and 30 days after heat treatment defined as T_{30}. T_1 was performed the day
149 subsequent the heat treatment to avoid false negative caused by the presence of sub-lethally injured *L.*
150 *monocytogenes* cells that may survive the heat treatment but are not immediately culturable. The PCs were
151 analysed at T_0, 6 hours after inoculation to assess if the level of contamination was effectively 10^5 $\log_{10}$ cfu g^{-1}.
152 The BLs were also examined at T_0, to account for eventual natural contamination of ricotta salata with *L.*
153 *monocytogenes*. The EUs and CUs were submitted to heat treatment and analysed at T_1 and T_{30}. Part of SE
154 units were treated (SEt) and part, used as negative controls (SEc), were no treated (Table 1).
155 The sampling plan with sample units, testing times and related analysis is summarized in table 2. The
156 effective temperature obtained on ricotta salata surface during each heat treatment was monitored using an
157 additional ricotta salata wheel where a data logger (KT 20T, Kimo, Montpon Ménestérol, France) was placed
158 1.5 cm below the surface and the temperature recorder during the treatment.
159
160 *2.4. Microbiological analysis*
161 Detection and enumeration of *L. monocytogenes* (ISO 11290-1:1996/Amd 1:2004; ISO 11290-2:1998/Amd
162 1:2004) and enumeration of aerobic mesophilic bacteria (ISO 4833:2003) were conducted on 25 g of ricotta

163 salata aseptically collected cutting the rind up to 2 cm. In order to detect the presence of sublethally injured
164 cells that may survive in the product but may not be cultured on selective media, on heat treated samples the
165 enumeration of *L. monocytogenes* was also conducted using the Thin Agar Layer (TAL) method. The TAL
166 method consists in the overlay of a nonselective agar medium onto agar plates containing a selective medium
167 that combines the ability to enumerate and to differentiate heat injured cells (Kang and Fung, 1999; Wu and
168 Fung, 2001). From each positive sample, 5 suspected colonies of *L. monocytogenes* were submitted to
169 phenotypic identification. Multiplex PCR was carried out to confirm identification and to separate the major
170 serovars (1/2a, 1/2b, 1/2c and 4b) into distinct serogroups (Doumith, Buchrieser, Glaser, Jacquet, & Martin,
171 2004). The *prs* gene, specific for *Listeria* spp. was used as internal amplification control. A selection of the
172 strains recovered from EUs at T_{30} was submitted to pulsed-field gel electrophoresis (PFGE) to confirm that
173 the strains recovered were the same that were inoculated. From each of the 3 replicate were selected up to
174 five strains for each temperature-time combination. In order to capture as much variability as possible a
175 preliminary screening of isolates to submit to PFGE was conducted based on the serogroups. PFGE was
176 carried out using the protocol proposed by Graves & Swaminathan (2001). The obtained restriction profiles
177 were analysed by visual examination to distinguish inoculated strains among each other and from strains
178 originating by natural contamination.
179
180 *2.5. Physico-chemical properties and composition*
181 Intrinsic properties and chemical composition of ricotta salata were determined to account for possible
182 interaction with *L. monocytogenes* survival and growth. PH and a_w were measured using pH meter GLP22
183 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab 4TE (Decagon, Pullman, WA,
184 USA), respectively. Near infrared transmittance (NIT) compositional analyzer (FOSS, Eden Prairie, MN,
185 USA) was used for the analysis of fat, moisture, protein and total solids. Differences in intrinsic properties
186 and composition ($\bar{x} \pm$SD) of ricotta salata cheese between the different temperature-time combinations used
187 for the heat treatment and over time (T_1 and T_{30}) were compared using Fisher's least significant difference
188 (LSD) test. Statistical analysis was performed with Statgraphics Centurion XVI software (StatPoint
189 Technologies, Warrenton, VA, USA).

190

191 *2.6. Sensory analysis*

192 The "Difference from control test" was applied to highlight sensory differences between heat treated samples
193 (SEt) and the negative control (SEc). This test is very helpful to determine difference between one or more
194 samples against the control and, if the difference is significant, to measure its size (Meilgaard, Civille, &
195 Carr, 1999). On the other hand this test can cause a sensory fatigue when many samples have to be taken into
196 account because, during each session, the control sample as reference and as blind sample must be served. In
197 order to avoid the sensory fatigue only five out nine temperature-time combination were evaluated: 75 °C
198 and 90 °C treated for 15 min and 40 min, and 85 °C treated for 25 min. Thirty judges (14 females and 16
199 males, aged 25-50 years) specialized in dairy products, previously selected for their sensitivity and after
200 attending a course of 60 hours in sensory analysis (ISO 8586-1: 1993), evaluated the samples against an
201 untreated control on a numerical category scale (0 = no difference and 9 = very large difference). Ricotta
202 salata samples were kept at 4-6 °C until sensory assessments. Before analysis the ricotta samples were
203 portioned extracting two opposing slices. The slices were further portioned into parallelepiped pieces (5 x 1.5
204 x 1.5 cm) and served, at room temperature, in odorless plastic containers marked with a random three-digit
205 number (Meilgaard, Civille, & Carr, 1999). Judges were also provided by a tray containing an unsalted
206 cracker and a glass of water. The evaluation was carried out in a randomized and balanced order (Macfie,
207 Bratchell, Greenhoff, & Vallis, 1989). Statistical analysis was performed with Statgraphics Centurion XVI
208 software (StatPoint Technologies) by the one-way ANOVA (factor: samples) and the Fisher's LSD.

209

210 *2.7. Validation of heat treatment*

211 The experiment was conducted in three independent trials for each of the 9 temperature-time combinations.
212 Samples used in each trial belonged to three different production batches (batch A, B and C). Analyses were
213 conducted at two different sampling times (T_1 and T_{30}). For each heat treatment combination and sampling
214 time were analyzed three samples. To account for a margin of safety, the effectiveness of heat treatment, i.e.
215 performance standard (Δ) was considered in the worst conditions, i.e. the minimum level of $\log_{10}$ cfu g^{-1}
216 reduction in *L. monocytogenes* counts. Reduction obtained as consequence of heat treatments was calculated

independently for each batch, by computing the minimum difference between the concentration (log_{10} cfu g^{-1}) before the treatment (T_0) and after the treatment (T_1 and T_{30}) observed in the triplicate samples.

2.8. Statistical analysis

Mean mesophilic bacteria counts (log_{10} cfu g^{-1}), intrinsic properties ($\bar{x}$ ±SD) and composition (%±SD) between the different temperature-time combinations at T_1 and T_{30} were compared using Fisher's least significant difference (LSD) test. All statistical analyses were performed with Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA, USA).

3. Results

3.1. L. monocytogenes contamination and background microflora

Natural contamination of ricotta salata rind with *L. monocytogenes* occurred in 6 out of 18 BLs (30.0%) all originating from the first batch. Enumeration of *L. monocytogenes* was possible in five BLs, showing a level of contamination of 2.68±0.51 log_{10} cfu g^{-1} ($\bar{x}$ ±SD). The mean level of artificial contamination expressed as log_{10} cfu g^{-1} ($\bar{x}$ ±SD) obtained on the rind of PCs units at T_0 was 4.82±0.43, 5.5±0.04 and 5.36±0.09 in the first, second and third replicate, respectively. Enumeration of aerobic mesophilic bacteria was conducted on 18 BLs, 9 PCs and 162 EUs. In BLs aerobic mesophilic population ($\bar{x}$ ±SD log_{10} cfu g^{-1}) was 7.41±0.47, 7.45±1.04, 7.83± 1.00 while in PCs was 7.21±0.31, 7.78±0.68 and 8.83±0.11 in the first, second and third batch respectively. The mean log_{10} cfu g^{-1} reduction in total bacterial counts observed at T_1 ranged between ca.1.0 and 3.0 for ricotta treated at 75 °C, 4.0 and 5.5 for treatment at 85 °C and from 5 to 6 for 90 °C. After 30 days of refrigerated storage the microbial population increased of less than 1.0 log_{10} cfu g^{-1} in ricotta salata treated at 75 °C and of ca. 1.0 and 2.0 log_{10} cfu g^{-1} in samples treated respectively at 85 °C and 90 °C. Pair-wise comparison of aerobic mesophilic bacteria counts between ricotta salata samples submitted to the 9 treatment combinations and between samples analysed at T_1 and T_{30} are reported in table 3.

3.2. Inactivation and survival of L. monocytogenes

The lethal effect was evaluated on 9 EUs (3 for each replicate) for each temperature-time combination at T_1 and T_{30}. The minimum differences in *L. monocytogenes* counts between T_1 and T_0 (Δ_1) and T_{30} and T_0 (Δ_{30}) for each of the 9 treatments are reported in table 4. At T_1 three out of nine combinations, i.e. 85 °C for 40 min, 90 °C for 25 min and 90 °C for 40 min, were effective either with the enumeration and detection methods. However, the 90 °C for 25 min combination showed the survival of *L. monocytogenes* after storage at 4 °C for 30 days. The complete description of the effect of each treatment at T_1 and T_{30} is reported in table 5 and figure 1.

Overall, 334 strains were confirmed as *L. monocytogenes* by molecular identification. Twenty-seven strains (90.0%) isolated from BLs were serogroup 1/2a, while 3 (10.0%) were serogroup 1/2c. Of the 49 strains isolated from PCs, 17 (34.7%) were serogroup 1/2a, 15 (30.6%) serogroup 1/2b, 11 (22.4%) serogroup 1/2c and 6 (12.2%) serogroup 4b. From EUs at T_1 were isolated 95 strains which were grouped as follows: 32 (33.7%) serogroup 1/2a, 12 (12.6%) serogroup 1/2b, 16 (16.8%) serogroup 1/2c and 35 (36.8%) serogroup 4b. From EUs at T_{30} were isolated 160 strains which were grouped as follows: 52 (32.5%) serogroup 1/2a, 23 (14.4%) serogroup 1/2b, 41 (25.6%) serogroup 1/2c and 44 (27.5%) serogroup 4b. Of *L. monocytogenes* recovered from EUs at T_{30} were submitted to PFGE 19, 13 and 30 strains from batch A, B and C respectively. Strains recovered showed the same PFGE profile of the inoculated strains and belonged exclusively to the wild type: 18 (29.0%) were 1/2a, 12 (19.4%) were 1/2b, 13 (21.0%) were 1/2c and 19 (30.6%) were 4b.

3.3. Temperature monitoring

The initial temperature of ricotta salata rind before the immersion in hot water was 6.0 ±0.8 °C. Figure 2 shows the temperature profile recorded on the rind of ricotta salata during water bath heat treatments for the three temperatures.

3.4. Sensory features

The results of sensory analysis (average values ±SD) are shown in table 7. The blind control allowed estimating the *placebo* effect, produced by asking to find a difference when in fact no differences exist.

Statistic inferences are estimates by comparing the samples and the blind control. All the heat treated samples are significantly different from the untreated one (blind control) with the exception of that treated at lowest temperature-time combination (75 °C for 15 min). However the difference size goes from 1.3 to 1.8 points that converted in the verbal scale correspond to "slight/moderate difference". The heat treated samples were not different between each other, meaning that heating from 75 to 90 °C for a time ranging between 15 and 40 min do not significantly change the sensory characteristics of ricotta salata.

3.5. Ricotta salata composition

Physico-chemical characteristics were determined on untreated samples (18 BLs) and heat treated samples (162 CUs). Intrinsic properties values ($\bar{x}$ ±SD) for untreated samples were 6.42±0.09 for pH and 0.963±0.01 for a_w. Composition values (%±SD) were respectively of 55.35±2.09 for moisture, 21.75±2.42 for fat, 14.55±1.37 for proteins and 4.56±1.38 for salt. Differences in intrinsic properties and composition between the 9 treatment combinations at T_1 and T_{30} are reported in table 6. PH and a_W were always within limits for *L. monocytogenes* growth and no significant difference was observed between values of ricotta salata submitted to heat treatment with different temperature-time conditions (P >0.05).

4. Discussion

In the last decades ricotta salata has been associated with several recalls due to *L. monocytogenes* contamination and more recently even with foodborne listeriosis outbreaks. Contamination of ricotta salata with *L. monocytogenes* mainly origins from food processing environment and is localized almost exclusively on the rind (Pilo, Marongiu, Corgiolu, Virdis, Scarano, & De Santis, 2007). Whole ricotta salata wheels are generally intended to be consumed grated including the rind. Post-process control strategies are needed in order to comply with international health authorities limits. Hot water bath treatment in vacuum packed ricotta salata has been previously evaluated, demonstrating to be an effective and economic method to inactivate surface contamination of ricotta salata cheese (Spanu, Spanu, Pala, Virdis, Scarano, & De Santis, 2013). However, optimization of the process was needed in order to account for the level of reduction in *L. monocytogenes* counts ($\log_{10}$ cfu g^{-1}), changes in sensory properties and cost of the treatment. In the present study was compared the listericidal effect of nine temperature-time combinations for the treatment of

297 artificially contaminated whole ricotta salata wheels. The recovery of *L. monocytogenes* from not inoculated
298 blank samples, confirms that natural contamination of ricotta salata is not a rare finding. The prevalence of
299 contamination of ricotta salata produced in Sardinia is estimated around 20% (Ibba, Cossu, Spanu, Virdis,
300 Spanu, Scarano, & De Santis, 2013; Spanu, Scarano, Ibba, Spanu & De Santis, 2015). This level can result,
301 when ricotta is stored at refrigeration temperatures for up to two months, in concentration of the pathogens of
302 approximately 10^7 $\log_{10}$ cfu g^{-1}, potentially harmful to human health (Spanu, Scarano, Spanu, Penna, Virdis,
303 & De Santis, 2012). The present study was aimed to validate the temperature-time combinations able to
304 reduce *L. monocytogenes* concentration of 5 log cfu g^{-1}, considered sufficient to comply with the food safety
305 objective of <100 cfu g^{-1} for the products placed on the market during their shelf-life (EC, 2005).
306 Enumeration of *L. monocytogenes* in positive control units confirmed that the desired level of contamination
307 of 10^5 cfu g^{-1} was obtained. Out of nine temperature-time combinations only two, 85 °C for 40 min and 90
308 °C for 40 min, showed to be effective in reducing *L. monocytogenes* to undetectable levels either at T_0 and
309 T_{30}. These combinations allowed to reach respectively 56.3±1.5 °C and 57.7± 1.4 °C on ricotta surface,
310 effective in killing *L. monocytogenes*. On one hand, little or no efficacy was observed for treatment
311 conducted at 75 °C, regardless of the time of application, while on the other hand no efficacy was
312 demonstrated for treatment conducted for 15 min, regardless of the temperature used. Despite an initial
313 inactivation of up to 5.0 log at T_0 as consequence of treatments performed at 75 °C (i.e. 25 and 40 min), *L.*
314 *monocytogenes* was still culturable with the detection method. The survival and successive growth during
315 storage at refrigeration temperature for 30 days resulted in counts as high as ca. 7 log. Failure of heat
316 treatment at 75°C to inactivate *L. monocytogenes* could be explained with the difference between water
317 temperature and the maximum temperature obtained on ricotta rind (47.5 °C). Treatment conducted at 85 °C
318 for 25 min resulted in a temperature on ricotta rind of 49.8±1.5 °C which allowed *L. monocytogenes* survival
319 to concentrations of up to 1 log at day zero. As consequence the microorganism grew during the successive
320 storage to level as high as 5 log. Although *L. monocytogenes* was not countable with the enumeration method
321 after 30 days in samples treated at 90 °C for 25 minutes, it was still detectable with the qualitative method.
322 No guarantee can be provided that the pathogen will not growth to levels potentially dangerous to human
323 during ricotta salata shelf-life. The highest temperature detected on ricotta salata rind after 25 min was 52.5

°C for the treatment at 90 °C. This could explain the presence of heat injured cells, which recovered after the subsequent storage at refrigeration temperature for 30 days. Strains capable to survive and growth after heat treatment belonged to the wild type, suggesting that they are characterized by a greater resistance as compared with reference strains. As far as the gap between the temperature of water during treatments and the temperature recorded on the ricotta salata rind it should be noted that temperatures were detected 1.5 cm below the ricotta surface, which may underestimate the effective temperature reached on the interface between packaged ricotta and water. The heat transfer is a function of the thermal properties of foods, which depend, among other factors, by chemical composition and temperature. However, due to the complexity of heat transfer calculations, specific experiments should be conducted in order to define the specific thermal properties of ricotta salata. Changes in sensory properties of heat treated ricotta salata were observed with respect of untreated samples, but no differences were among treatments. This indicates the feasibility of using more protective treatments with no negative implication for ricotta salata sensory profile.

5. Conclusion

Contamination of Ricotta salata with *L. monocytogenes* can effectively be controlled by the application of water bath heat treatment applied after packaging of the product. Treatments performed at 85 °C for 40 min or 90 °C for 40 min can be effectively used to obtain a reduction of 5 log of the pathogen. No significant difference was observed in the sensory properties between the treatments. Although treatments at 85 °C for 40 min might gather food processors favors as compared to 90 °C for 40 min, the latter may provide a greater safety of the product when is stored for periods of time longer than 30 days. Treatments applied for 40 min, either at 85 °C and 90 °C, are eligible as combination of choice to be used in a further study to assess the efficacy as post-lethality treatment aimed to extend ricotta salata shelf-life.

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Table 1. Temperature-time combinations used for water bath heat treatment of ricotta salata

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	15	-		18	18	12	48
	25	-		18	18	3	39
	40	-		18	18	12	48
85 °C	15	-		18	18	3	39
	25	-		18	18	12	48
	40	-		18	18	3	39
90 °C	15	-		18	18	12	48
	25	-		18	18	3	39
	40	-		18	18	12	48
Total		18	9	162	162	114	465

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

Table 2. Number of ricotta salata wheels and analysis performed at each sampling time.

		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	-	-	-
	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

Superscript letters are referred to the time between inoculation and analysis: [a] = day of inoculum and heat treatment; [b] = 24 hours after heat treatment; [c] = 30 days after heat treatment. Superscript numbers are referred to test units: [1]BLs (Blank Samples): not inoculated units; [2]PCs (Positive Controls): samples inoculated with *L. monocytogenes*; [3]EUs (Experimental Units): samples inoculated with *L. monocytogenes* and successively heat treated; [4]CUs (Composition Units): heat treated units for physico-chemical analysis; [5]SEs (Sensory Units): samples used to assess the effect of heat treatment on sensory properties.

Table 3. Comparison of aerobic mesophilic bacteria counts (log_{10} cfu g^{-1}; $\bar{x}$ ± SD) of heat treated ricotta salata with 9 different temperature-time combinations analyzed 24 h after the treatment (T_1) and after 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	15	9/9	6.94± 0.62^{A}	9/9	7.06±0.80^{A}
	25	9/9	5.69± 0.55^{B}	9/9	6.20±1.36^{A}
	40	9/9	5.12± 0.42^{B}	7/9	6.50±1.28^{A}
85 °C	15	9/9	3.90± 0.85^{C}	9/9	4.88±0.88^{B}
	25	9/9	3.71± 0.94^{C}	5/9	4.58±1.14^{B}
	40	9/9	2.45± 1.97^{D}	4/9	4.14±1.78^{BC}
90 °C	15	9/9	2.02± 1.22^{D}	6/9	4.15±0.82^{BC}
	25	9/9	1.68± 1.29^{D}	3/9	2.90±0.78^{C}
	40	9/9	2.01± 1.12^{D}	3/9	3.67±1.62^{BC}

Means in the same column on the same testing time (T_1 or T_{30}) with different capital letter are significantly different (P<0.05).

Table 4. *Listeria monocytogenes* reduction (Δ)[a] on ricotta salata rind after water bath heat treatment

		15 min		25 min		40 min	
Temperature	Batch	ΔT_1	ΔT_{30}	ΔT_1	ΔT_{30}	ΔT_1	ΔT_{30}
75 °C	A	-0.23	4.18	-4.54	2.26	-3.24	2.57
	B	-2.59	0.48	-5.45	-5.45	-5.45	-5.45
	C	-1.78	-1.36	-5.27	-1.73	-5.27	-5.27
85 °C	A	-4.54	-4.54	-4.54	-4.54	-4.54	-4.54
	B	-5.45	-1.51	-5.45	-0.22	-5.45	-5.45
	C	-2.15	-2.67	-3.97	-5.27	-5.27	-5.27
90 °C	A	-4.54	-4.54	-4.54	-4.54	-4.54	-4.54
	B	-5.45	-5.45	-5.45	-5.45	-5.45	-5.45
	C	-3.97	-2.13	-5.27	-5.27	-5.27	-5.27

[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.

Table 5. Enumeration and detection of *L. monocytogenes* in ricotta salata artificially contaminated and heat treated with different temperature-time combinations and relative compliance with Regulation CE limits evaluated 24 h (T_1) and 30 days (T_{30}) after the heat treatment.

Treatment			*L.monocytogenes*					
			T_1			T_{30}		
			Enumeration		Detection in 25 g	Enumeration		Detection in 25 g
Temperature	Minutes	Batch	+ve/n	log_{10} cfu/g	+ve /n	+ve/n	log_{10} cfu/g	+ve /n
75 °C	15	A	2/3	3.67±0.89	3/3[1]	3/3	8.20±0.48[2]	3/3
		B	1/3	2.86±0.00	1/3[1]	1/3	5.93±0.00[2]	3/3
		C	1/3	3.49±0.00	3/3[1]	3/3	3.47± 0.48[2]	3/3
	25	A	0/3	0.00±0.00	1/3[1]	2/3	6.39±0.58[2]	3/3
		B	0/3	0.00±0.00	1/3[1]	0/3	0.00±0.00[2]	0/3
		C	0/3	0.00±0.00	1/3[1]	1/3	3.54±0.00[2]	2/3
	40	A	2/3	1.30±0.00	3/3[1]	3/3	6.87±0.34[2]	3/3
		B	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3
		C	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3
85 °C	15	A	0/3	0.00±0.00	0/3	0/3	0.00±0.00	1/3
		B	0/3	0.00±0.00	0/3	2/3	2.82±1.58[2]	2/3
		C	1/3	3.12± 0.00	2/3[1]	1/3	2.60 ± 0.00[2]	3/3
	25	A	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3
		B	0/3	0.00±0.00	0/3	1/3	5.23±0.00[2]	1/3
		C	1/3	1.30± 0.00	1/3[1]	0/3	0.00±0.00	1/3
	40	A	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3
		B	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3
		C	0/3	0.0±0.00	0/3	0/3	0.00±0.00	0/3
90 °C	15	A	0/3	0.00±0.00	0/3	0/3	0.00±0.00	2/3
		B	0/3	0.00±0.00	0/3	0/3	0.00±0.00	1/3
		C	1/3	1.30± 0.00	3/3[1]	2/3	2.92±0.22[2]	2/3
	25	A	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3
		B	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3
		C	0/3	0.00±0.00	0/3	0/3	0.00±0.00	2/3
	40	A	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3
		B	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3
		C	0/3	0.00±0.00	0/3	0/3	0.00±0.00	0/3

Compliance are intended as follows: [1]not compliant with the Regulation CE 2073/2005 detection limits before the food has left the immediate control of the food business operator (T_1); [2]not compliant with the Regulation CE 2073/2005 enumeration limits for the products placed on the market during their shelf-life (T_{30}).

Table 6. 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	15	6.41±0.12^{A}	6.32±0.06^{A}	0.959±0.01^{A}	0.952±0.01^{A}	54.96±1.44^{A}	54.59±1.67^{A}	20.97±1.88^{A}	20.87±2.29^{A}	15.19±3.38^{AB}	14.75±1.35^{AB}	5.02±0.84^{A}	5.50±1.33^{A}
	25	6.42±0.11^{A}	6.36±0.05^{A}	0.954±0.01^{A}	0.951±0.01^{A}	54.90±2.08^{A}	54.94±1.36^{A}	21.73±2.01^{AB}	22.70±3.01^{A}	15.56±2.63^{B}	14.05±1.09^{A}	5.04±0.72^{A}	5.57±0.79^{A}
	40	6.41±0.13^{A}	6.35±0.07^{A}	0.950±0.01^{A}	0.654±0.01^{A}	55.09±2.23^{A}	53.53±1.93^{A}	20.90±3.11^{A}	22.71±2.32^{A}	14.44±1.29^{ABC}	14.56±0.93^{AB}	5.18±0.30^{A}	5.20±0.74^{AB}
85 °C	15	6.41±0.12^{A}	6.36±0.09^{A}	0.952±0.01^{A}	0.953±0.01^{A}	54.53±2.04^{A}	54.17±2.16^{A}	21.31±2.26^{AB}	21.65±2.52^{A}	14.20±2.32^{ABC}	15.01±1.54^{AB}	5.26±1.12^{A}	4.98±0.95^{AB}
	25	6.41±0.12^{A}	6.33±0.08^{A}	0.956±0.01^{A}	0.956±0.01^{A}	55.40±2.03^{A}	54.07±2.74^{A}	21.23±1.73^{AB}	22.29±3.13^{A}	13.97±1.04^{ABC}	15.37±1.12^{AB}	5.24±0.84^{A}	4.63±0.59^{B}
	40	6.38±0.10^{A}	6.33±0.11^{A}	0.953±0.01^{A}	0.956±0.01^{A}	54.72±1.81^{A}	53.91±2.12^{A}	21.65±2.21^{AB}	22.48±2.09^{A}	14.95±1.78^{ABC}	14.94±0.97^{AB}	5.16±0.76^{A}	5.09±0.76^{AB}
90 °C	15	6.40±0.11^{A}	6.33±0.09^{A}	0.953±0.01^{A}	0.954±0.01^{A}	54.30±1.64^{A}	53.61±1.62^{A}	23.07±0.88^{B}	22.75±2.15^{A}	13.33±0.56^{C}	14.48±1.50^{AB}	5.36±0.66^{A}	5.18±0.44^{AB}
	25	6.38±0.12^{A}	6.35±0.15^{A}	0.955±0.01^{A}	0.954±0.01^{A}	54.78±1.66^{A}	54.24±2.24^{A}	21.74±2.01^{AB}	22.52±2.36^{A}	14.38±2.00^{ABC}	14.33±0.82^{AB}	5.12±0.90^{A}	5.32±0.79^{AB}
	40	6.35±0.11^{A}	6.35±0.12^{A}	0.954±0.01^{A}	0.955±0.01^{A}	54.58±1.37^{A}	54.22±2.02^{A}	23.03±1.08^{B}	21.80±2.26^{A}	13.70±0.54^{AC}	15.50±2.66^{B}	5.19±0.87^{A}	5.03±0.93^{AB}

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 capital letter are significantly different (P<0.05).

62 Table 7. Mean values and standard deviations of sensory differences among the control (SEc) and the

63 samples heat treated (SEt).

Temperature-time condition	Differences
Blind samples	2.3^{a}* ± 1.5
75 °C x 15 min	3.4^{ab} ± 2.3
75 °C x 40 min	3.8^{b} ± 2.0
85 °C x 25 min	4.1^{b} ± 2.4
90 °C x 15 min	3.6^{b} ± 2.3
90 °C x 40 min	3.9^{b} ± 2.5

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

Figure 1. Reduction in *L. monocytogenes* counts (log_{10} cfu g^{-1}) of artificially contaminated Ricotta salata wheels (T_0) analyzed 24 h (T_1) and 30 days (T_{30}) after waterbath heat treatment with 9 temperature-time combinations.

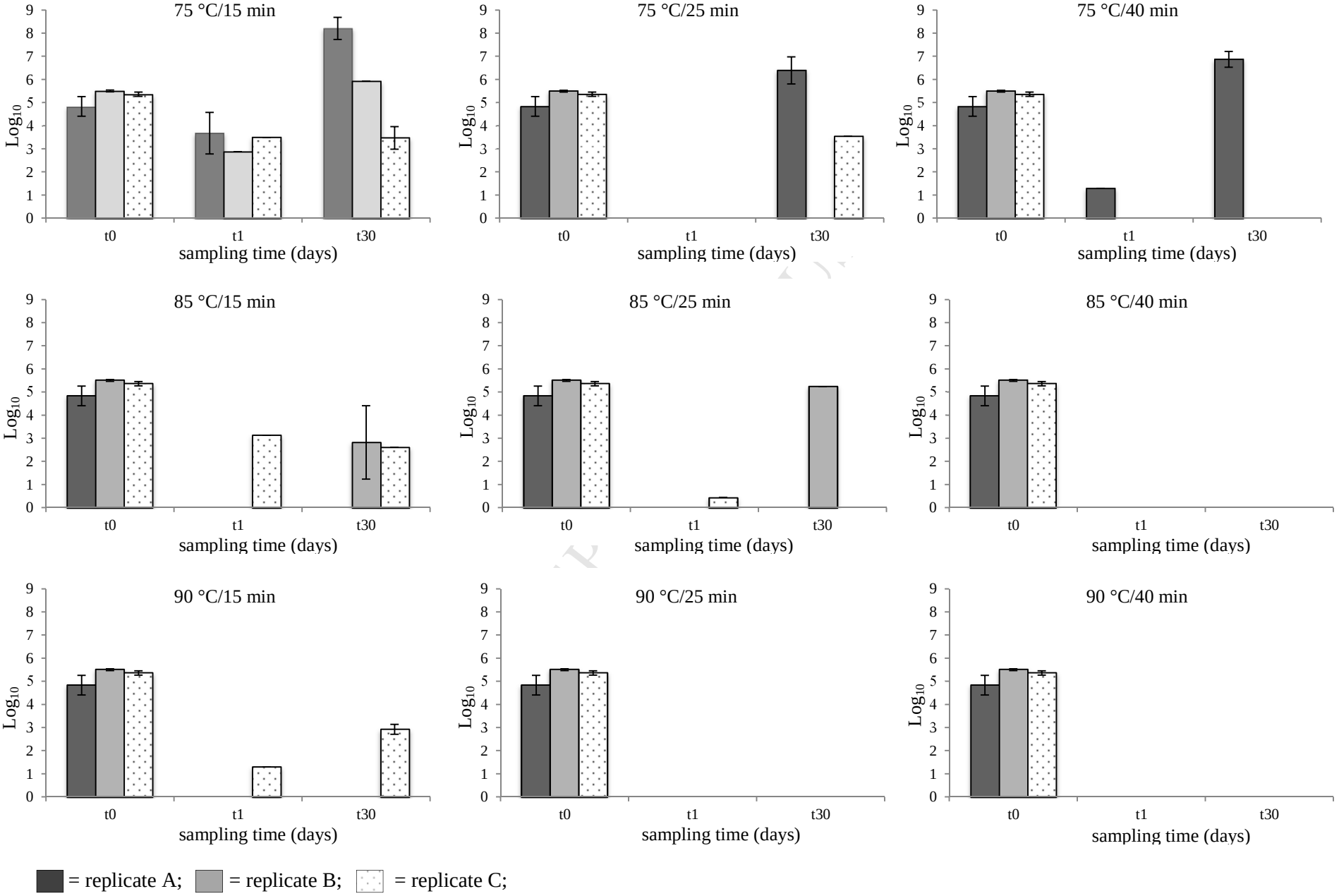
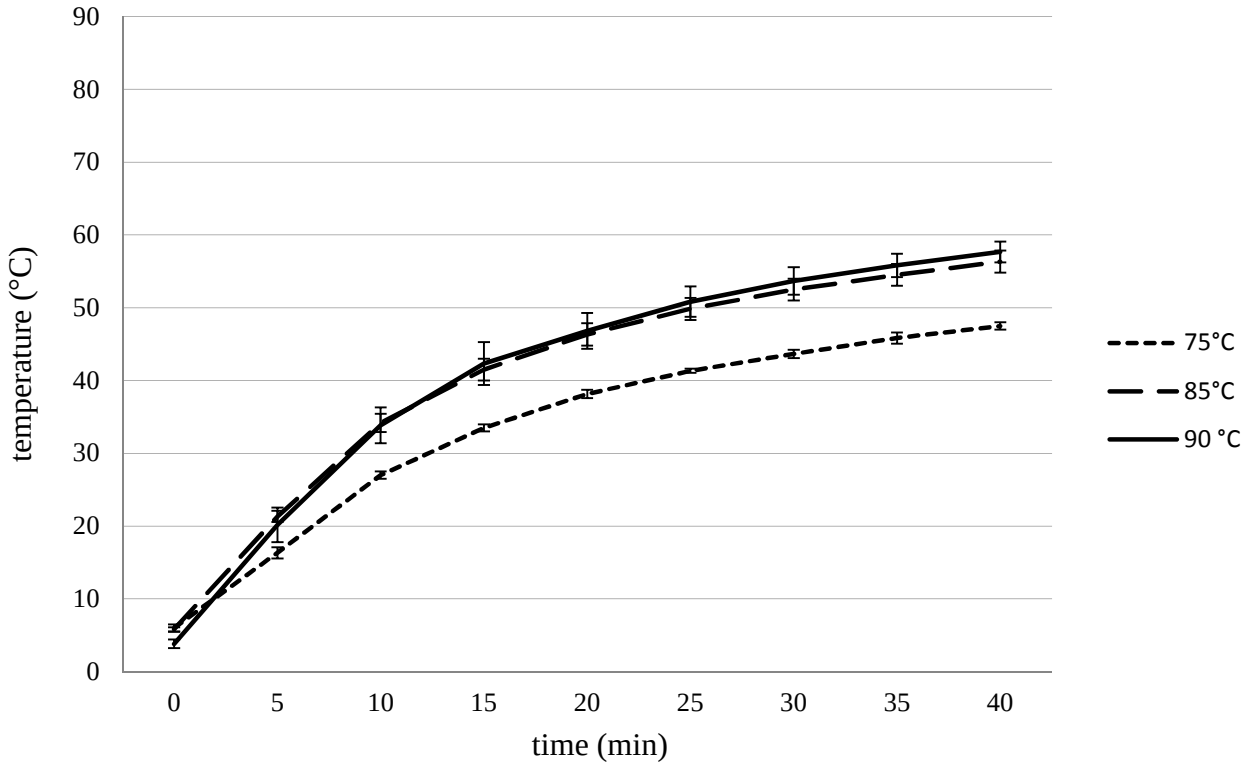


Figure 2. Temperatures recorded 1.5 cm below ricotta salata surface during water bath heat treatment



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

Highlights

1. Post-lethality treatment on *L. monocytogenes* was assessed in ricotta salata.
2. A reduction of 5 $\log_{10}$ cfu g^{-1} in *L. monocytogenes* count was validated.
3. No effect of treatments on sensory properties was observed.