Thermal inactivation and growth potential of *Listeria monocytogenes* in smoked tench

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Abstract

An experimental study for the evaluation of *Listeria monocytogenes* inactivation during a hot smoking process in tench was performed using *Listeria innocua* strains. Furthermore, the survival of *L. monocytogenes* in smoked tench was determined after post-processing in contaminated samples, evaluating the growth potential during storage. *L. innocua* was not detected after the smoking process. In the challenge test, the growth potential of *L. monocytogenes* was 5.68 log colony forming unit g^{-1}. The results showed that hot smoking at an inner temperature around 72°C is able to eliminate the microorganism. Nevertheless, the product is able to support the growth of the pathogen if post-process contamination occurs, as the food is suitable for *Listeria* multiplication. Product recontamination should be prevented by means of appropriate application of hygiene measures.

Introduction

Listeria monocytogenes is a foodborne pathogen that causes a potentially severe foodborne disease with a high mortality rate (CDC, 2013). *L. monocytogenes* has been observed in a large variety of foods, especially in those that are generally eaten with little or no prior heating (Bremer *et al.*, 2003; Burnett *et al*., 2005; Bouayad and Hamdi, 2012). Cold smoked seafood has been categorised as one of these high-risk, ready-to-eat (RTE) products because the heat applied during processing is not sufficient to inactivate the organism, and the products are consumed without further cooking (Rocourt *et al.*, 2003; Reij and Den Aantrekker, 2004).

Smoking is an ancient fish preservation method and it has economic importance for the seafood market (Ozogul *et al.,* 2010). As well as providing a specific aroma and colour to the fish, smoking extends the shelf-life of fish due to dehydration, and the antibacterial and antioxidant effects of the smoke components such as formaldehyde, carboxylic acids and phenols (Ozogul et *al.*, 2010; Tocmo *et al.*, 2014). In Umbria region (Italy), smoked tench is an artisanal fish product made by a small fishery consortium along lake Trasimeno and is known to be an economically important fish product which has started to be available in many markets; as a result, it is necessary to investigate the effect of different processing steps in the fish smoking process on the growth potential of *L. monocytogenes* and to determine whether the product supports the growth of *L. monocytogenes*, according to EC Regulation No. 2073/2005 (European Commission, 2005).

The aim of this study was to evaluate the inactivation of *L. monocytogenes* during the hot smoking process in tench, and its survival during storage after post-processing contamination.

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Materials and Methods

Production of smoked tench

The production of hot smoked tench includes: gutting fish; salting in brine containing 20% salt for 2 h at 10°C; washing to remove the brine; dripping for 16-24 h at 0±4°C; smoking for 6 h in a convection smoking oven (inner temperature around 72°C); and vacuum packaging. The shelf-life of the vacuum-packaged smoked tench was 30 days at 4°C, as established by the manufacturer.

Evaluation of *Listeria inactivation* during the hot smoking process

The study was performed directly in a fish processing plant. To avoid the use of the pathogen strain, *Listeria innocua* was used. Before fish contamination, the heat tolerance of a multi-strain mix of *L. innocua* (ATCC® 33090™, Li 1/2015 and Li 2/2015 from the IZS collection and derived from fish fillets) and three strains of *L. monocytogenes* (ATCC® 7644™, Lm 48189/13 and Lm50308/13 from the IZS collection and derived from fish fillets) were compared. This evaluation was carried out as reported by Lorentzen *et al.* (2010) in order to investigate the suitability of using *L. innocua* as a non-pathogenic indicator.

L. innocua was kept at -80°C in Cryoinstant (VWR Prolabo Chemicals, Leuven, Belgium) and was regenerated into Brain Heart Infusion (BHI) and incubated at 37°C for 24 h. Aliquots of each culture were transferred into tubes containing BHI and incubated at 12±1°C, a temperature close to that registered in the processing plant. The stationary phase was reached after 18-20 h. After centrifuging each strain at 2178 g at 10°C for 5 min, the supernatant was discarded and the pellet was resuspended in 10 mL of sterile physiological solution (H_2O with 0.9% NaCl). Counts were confirmed by serial decimal dilution and inoculation in Agar Listeria Ottaviani (ALOA Selective Supplement, ALOA Enrichment Supplement; Biolife, Vernon Hills, IL, USA); plates were incubated at 37°C for 24-48 h.

The inoculation of tench was performed after brining with a culture mix of *L. innocua*. Before inoculation, tench were screened for *Listeria* spp. (AFNOR BIO 12/11-03/04; AFNOR, 2010) and were found to be negative. The study was conducted in triplicate (three batches). Brined tench were contaminated with *L. innocua* on the external and internal surfaces before the smoking process with 1% v/wt of *L. innocua* to a final concentration of *ca*. 5 log colony forming unit (CFU) g^{-1}. The inoculum was distributed over the entire surface with a sterile L-shaped plastic cell spreader (Incofar, Modena, Italy). The level of contamination was analytically confirmed before the smoking process.

Afterwards, the tench were placed inside the

smoking chamber. The temperature during the smoking process was logged continuously using a temperature logger (Thermo Button-Plug; Proges Plus, Willems, France) placed inside five tench located in five different places in the smoking chamber (Figure 1). Temperature data logged for each batch during the smoking process are reported in Figure 2.

After processing, samples were aseptically collected in three different points (head, body and tail) and transferred to the laboratory within 15' for the enumeration of *L. innocua.* Briefly, 10 g of sample of each point was weighed into a sterile stomacher bag, then 90 mL of peptone water was added. Counts were confirmed by serial decimal dilution and inoculation in ALOA; plates were incubated at 37°C for 24-48 h. The reference method (ISO 7218:2007; ISO, 2007) was used for enumeration of bacteria. The typical colonies were tested for biochemical identification in micromethod (API Listeria; BioMérieux, Marcy-l'Étoile, France). The detection of *L. innocua* was also performed using the validated method AFNOR BIO 12/11-03/04 (AFNOR, 2010).

The approach used for calculating death kinetics only referred to thermal inactivation because evaluation of the compounds developed during smoking was not performed. The D and z values used were those reported for *L. monocytogenes* by van Asselt and Zwietering (2006). Data were then analysed by software of the Food Safety and Inspection Service of the United States Department of Agriculture (www.fsis.USDA.gov) to evaluate the worst-case scenario for the process. For this evaluation, the D70 value was set at 0.52 and the z value at 7°C.

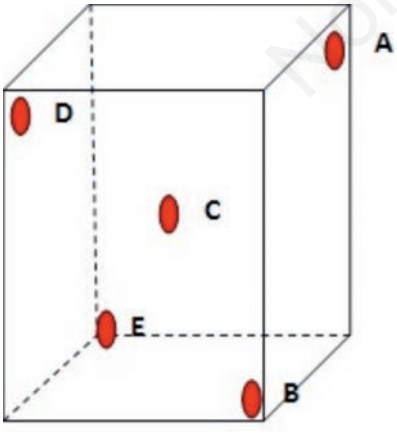

Figure 1. Data logger position inside the smoking chamber. Position of tench containing a data logger: A) in the top right of the chamber; B) in the bottom right; C) in the centre of the chamber; D) in the top left; E) in the bottom left.

Listeria monocytogenes contamination of ready-to-eat tench after smoking process, without further thermal treatment

For the experiment, three batches of vacuum-packaged smoked tench were produced, and each batch consisted of three experimental groups of 2 kg smoked tench. For the first group (CLm), 12 sample units were produced and contaminated by dipping the smoked tench in a suspension of the above-mentioned multi-strain mix of *L. monocytogenes*. These sample units were used to evaluate the growth potential of *L. monocytogenes* during the product's shelf-life. The cultures were treated as reported before for *L. innocua*. Each of the three strains was combined in an equal quantity at a concentration of about 7 log CFU g^{-1}. Dilutions of the mixed cultures were made to obtain a concentration in the foodstuff that was similar to the concentration occurring in it naturally (Ortenzi *et al.*, 2015). These second stationary-phase subcultures were used for contamination.

For the second group (CTRIZS), 12 units were treated with 1% v/wt of the sterile physiological solution (control samples) in order to compare the products submitted to challenge testing (CLm) with products that were routinely produced. For the first and second group, the smoked tench were kept for 3 h at 12°C after contamination to allow the bacteria to stick onto the fish. The smoked tench were then repacked under vacuum conditions into sterile polyethylene bags. For the third group (CTR), six units were produced following the normal process adopted by the manufacturer in order to compare the products of the second group with products routinely produced by the processor.

In total, 30 smoked tench were sampled during the testing period and submitted to analytical determination. After packaging, the vacu-

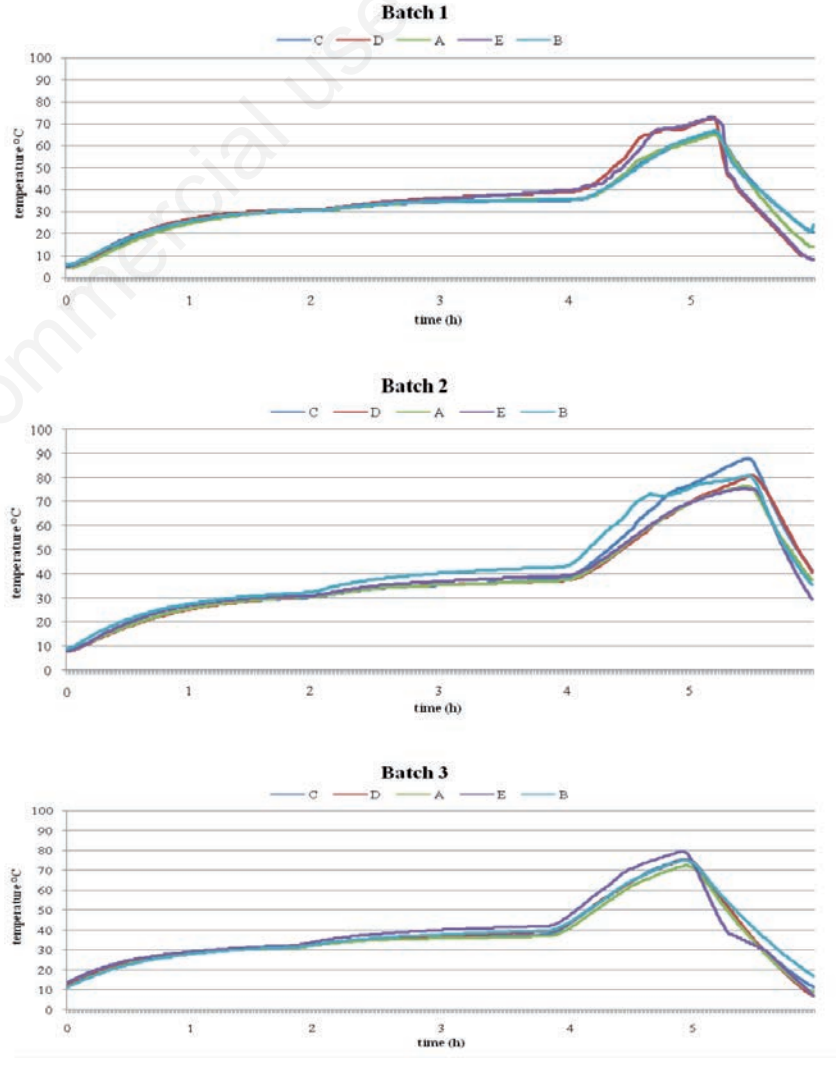

Figure 2. Thermal profile logged in each batch during the smoking process.

um-packed smoked tench were maintained at 8°C for 7 days, and at 12°C for the remaining storage time (23 days) (EUCRL, 2008).

The physicochemical determinations of smoked tench were conducted at 0, 10, 20 and 30 days of storage time in one-unit samples of each batch (CTRIZS); for the CTR group, analysis was done at 0 and 30 days. The pH measurements were made using a puncture electrode probe connected to a portable pH meter (Mettler Toledo Inc., Columbus, OH, USA). The salt content was determined as described by Branciari *et al.* (2014). Water activity (a_w) was measured at 25°C with an AquaLab series 3 model TE a_w recorder (Decagon Devices Inc., Pullman, WA, USA).

The detection and enumeration of *L. monocytogenes* were conducted at 0, 10, 20 and 30 days of storage time in all unit samples of each batch according to Annex I of Regulation No. 2073/2005 (European Commission, 2005), using the validated method AFNOR BIO 12/11-03/04 (AFNOR, 2010) for detection and EN ISO 11290-2 (ISO, 1996), the reference method for enumeration.

Results and Discussion

After the smoking process, no *L. innocua* was detected in any of the inoculated samples regardless of batch or position of the tench in the smoking chamber. It has been demonstrated that a smoking process using a temperature of approximately of 30°C (cold smoking process) is not sufficient to inactivate this pathogen in smoked salmon (Swaminathan *et al.*, 2007; Porsby *et al.*, 2008). Hence, the chance that *L. monocytogenes* survives and proliferates after the smoking process is rather high. Swaminathan *et al.* (2007) showed that for *L. monocytogenes* to be inactivated, products must receive heat treatment above 50°C and the addition of smoke, which has an antilisterial effect because of its phenolic content.

As shown in Figure 3, software revealed that the experimental treatments were equivalent to a treatment of 70°C applied for 2.732 min and they were able to reduce *L. monocytogenes* by 5.25 log CFU g^{-1}. The most unfavourable thermal shoulder determined by the software using the temperatures logged is reported in Figure 4. In this worst case scenario, the temperature did not reach 70°C; nevertheless, the products were at least heated up to 50 min over 50°C, and this treatment in combination with liquid smoke and brine represented sufficient hurdles to alter the homeostasis of the bacte-

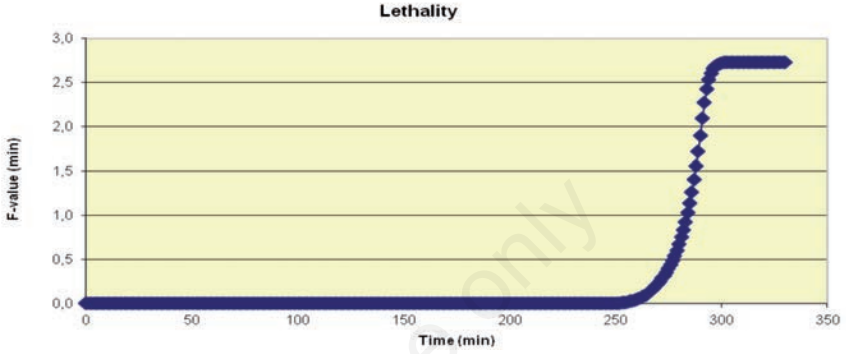

Figure 3. Inactivation of *Listeria monocytogenes*, thermal and temperature conditions.

Table 1. pH, water activity and sodium chloride of smoked tench during storage.

		Days of storage			
		0	10	20	30
CTR	pH	6.02±0.14	nd	nd	5.95±0.08
	a_w	0.970±0.01	nd	nd	0.960±0.01
	NaCl	2.62±0.35	nd	nd	2.97±0.73
CTRIZS	pH	5.98±0.08	6.07±0.13	6.08±0.08	5.97±0.07
	a_w	0.973±0.013	0.71±0.01	0.97±0.01	0.963±0.01
	NaCl	2.53±0.36	2.58±0.53	2.61±0.35	3.02±0.75

CTR, third group; CTRIZS, second group; a_w, water activity; NaCl, sodium chloride; nd, not detected. Data represent the average values±standard deviation of three replicate samples for three smoked process replicates.

Table 2. Calculation of growth potential of *Listeria monocytogenes*.

Batch	Day	Concentration	SD	Median	Growth potential (δ) per batch	Highest δ among the 3 batches
1	0	2.91 2.88 2.78	0.07	2.88	8.20-2.88=5.32	
	30	8.26 8.20 8.11	0.07	8.20		
2	0	2.90 2.40 2.43	0.28	2.43	8.11-2.43=5.68	5.68
	30	8.11 8.13 8.04	0.05	8.11		
3	0	2.58 2.88 2.52	0.19	2.58	6.65-2.58=4.07	
	30	6.62 6.65 7.15	0.29	6.65		

SD, standard deviation. Values are expressed as log CFU g^{-1}.

ria resulting in *Listeria* inactivation.

The physicochemical characteristics of the smoked tench contaminated after the smoking process (challenge test) and during storage are reported in Table 1. No difference in pH, a_w or NaCl was detected among batches, between the two different sample units (CTRIZS and CTR) or during the storage time.

The results of the behaviour of *L. monocytogenes* during storage are reported in Figure 5. The average pathogen counts in contaminated samples are shown in Table 2. No *L. monocytogenes* was found in the control sample. During the storage of vacuum-packed smoked tench, *L. monocytogenes* significantly increased in the inoculated samples. At day 0, the median concentrations of *L. monocytogenes* were log 2.88 CFU g^{-1} (range log 2.78-2.91 CFU g^{-1}), log 2.43 CFU g^{-1} (range 2.40-2.90 log CFU g^{-1}) and log 2.58 CFU g^{-1} (range 2.52-2.88 log CFU g^{-1}) for batches 1, 2 and 3, respectively. The standard deviations between the three results at day 0 were 0.23 log CFU g^{-1} (batch 1, 0.07 log CFU g^{-1}; batch 2, 0.28 log CFU g^{-1}; batch 3, 0.19 log CFU g^{-1}). The standard deviation result obtained was always below the value 0.3 log CFU g^{-1}, set by the technical guidance document (EUCRL, 2008) as the limit to consider the challenge test acceptable due to measurement uncertainty and contamination heterogeneity. The growth potential of *L. monocytogenes* was 5.32 log CFU g^{-1} in batch 1, 5.68 log CFU g^{-1} in batch 2, and 4.07 log CFU g^{-1} in batch 3. The maximal difference between the median concentration at day 30 and the median concentration at day 0 (log CFU g^{-1}) of each batch was: =5.68 log CFU g^{-1}. Considering the results of the present study based on values that were higher than 0.5 log CFU g^{-1}, it is possible to establish that smoked tench belongs to the category of RTE foods able to support the growth of *L. monocytogenes* in compliance with EC Regulation No 2073/2005 (European Commission, 2005). The multiplication of *L. monocytogenes* showed that refrigeration at 4 to 10°C does not prevent the growth of the pathogen in vacuum-packed smoked tench. Other authors indicated that *L. monocytogenes* was also able to grow on various fish products at refrigeration temperatures, and a similar increase was also observed in smoked salmon (Kang *et al.*, 2012; Ferreira *et al.*, 2014).

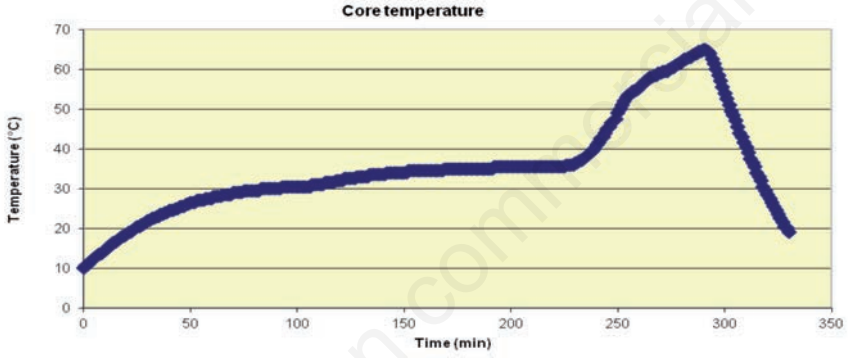

Figure 4. The most unfavourable thermal shoulder determined by the software.

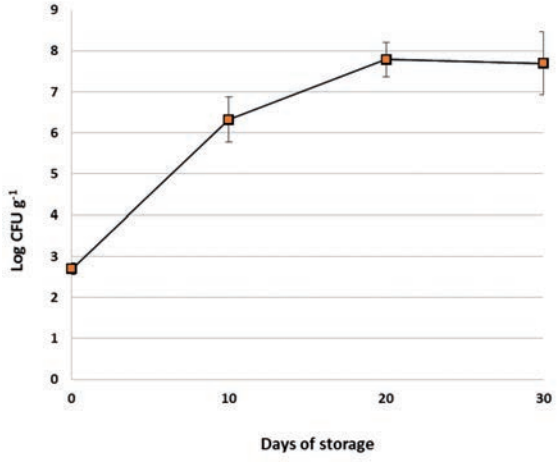

Figure 5. Behaviour of *Listeria monocytogenes* during storage.

Conclusions

The study confirmed that thermal inactivation of *Listeria* occurred during the hot smoking process using an appropriate combination of time and temperature. Post-process contamination could be a risk for consumers, as the food is suitable for *Listeria* multiplication. Thus, product recontamination should be prevented by means of appropriate application of hygiene measures.

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