
Effect of salting and cold-smoking process on the culturability, viability and virulence of *listeria monocytogenes* strain scott a

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Abstract:

The aim of the present study was to determine the effect of the different steps of the cold-smoking process and vacuum storage on the culturability and viability of *Listeria monocytogenes* strain Scott A inoculated in sterile salmon samples. Additionally, the virulence of *L. monocytogenes* cells was assessed by intravenous inoculation of immunocompetent mice. Salmon (*Salmo salar*) portions were inoculated with *L. monocytogenes* at a level of 6 log CFU/g and were then dry salted (5.9 %), smoked (0.74 mg phenol/100g), partially frozen (-7°C), vacuum-packed and stored for 10 days at 4°C followed by 18 days at 8°C. Salting represented the only step of the process with a weak but significant listericidal effect (0.6 log reduction). Although the other processing steps had no immediate reduction effect on *L. monocytogenes*, combination of them significantly lowered by 1.6 log CFU/g the number of *L. monocytogenes*. The culturable count remained less than 7 log CFU/g until the end of the storage period whereas in unprocessed samples (control) the culturable counts reached values up to 9 log CFU/g. To mimic a post-process contamination, salmon portions were also inoculated with *L. monocytogenes* after being cold-smoke processed. A reduction of the culturable count during the 2 first weeks of storage was observed but then growth occurred and identical values observed for pre-process contamination were reached at the end of the storage. A viable but non culturable (VBNC) state transition of strain Scott A was not observed and the cold-smoking process did not affect the virulence of bacteria isolated at the beginning and end of the storage.

Key words: *Listeria monocytogenes*, smoked salmon, salt, VBNC state, virulence

During the last two decades *Listeria monocytogenes* has been associated with the occurrence of several foodborne illness outbreaks (31). Psychrotolerance of *L. monocytogenes* makes this pathogen capable of surviving and multiplying at temperatures ranging from -2°C to 42°C (3). Traditionally used by food industry as preserving methods, salting and smoking are nowadays applied for sensorial advantages aiming to satisfy consumers taste that prefer slightly processed products (14). *L. monocytogenes* has been often isolated in cold-smoked salmon at the manufacturing level as well as in vacuum-packaged products at the retail level (7, 24, 34) showing that salt and smoke are insufficient to hinder *Listeria* proliferation in the product. Growth of *L. monocytogenes* in cold-smoked salmon has been reported by many authors (8, 13, 21) and different predictive microbiology models have been evaluated to predict growth *in situ* (6, 16, 25, 32). Those studies were conducted with *L. monocytogenes* inoculated in the final product, mimicking the case for post-process contamination. However, contamination can also occur before processing, via raw material or during slaughtering (10, 24) and there are few studies dealing with the behavior of *L. monocytogenes* during the processing and its subsequent capacity to grow in the final product. This is probably due to difficulties of processing raw salmon inoculated with a pathogenic bacterium, which could contaminate smoking facilities. The aim of the present study was to determine the viability and culturability of a virulent *L. monocytogenes* strain (Scott A) during the processing and storage of cold-smoked salmon under laboratory conditions. The presence of cells in a viable but non culturable (VBNC) state was investigated as a recent study showed the existence of a VBNC state in *L. monocytogenes* (4) which cannot be detected by the conventional cultural methods used in microbiology. Furthermore, the virulence of bacteria was studied at critical steps like the end of manufacturing and the end of storage (shelf-life). Indeed, although frequently contaminated by *L. monocytogenes* no documented cases of listeriosis have been associated with the consumption of cold-smoked salmon (23). *L. monocytogenes* subtypes present in the smoked fish industry have been shown to have attenuated ability to cause human disease but little is known about the effect of the food matrix on the virulence of this pathogen (20). Therefore, this study should allow a better understanding of the effects of both cold-smoking process and vacuum storage on the survival and pathogenicity of *L. monocytogenes* in artificially contaminated salmon samples.

1. Materials and methods

Preparation of bacterial inoculum. For all experiments, strain Scott A of *L. monocytogenes*, provided by the culture collection of the Institut Pasteur (Paris, France) was used. Stock cultures were maintained in cryovials containing a suspension of Brain Heart Broth (BHB) (Merck, Darmstadt, Germany) and 10% of glycerol (v/v) (Merck) stored at -20°C. Bacteria were grown in BHB for 48 h at 15°C. Bacteria were harvested by centrifuging 20 ml of the previously described culture ($10\ 000 \times g$, at 4°C for 15 min). The supernatant was discarded and the cell pellet was washed in sterile physiological water (0.85% NaCl, pH 7.2) and re-suspended in 20 ml of sterile physiological water before inoculation.

Salmon samples. Three fresh 3-4 kg salmon (*Salmo salar*) from Norway were purchased from the market. After filleting, each fillet was cut in two pieces having a thickness of 1.3 cm and Petri dish-shaped portions were manufactured. Each fillet enabled us to manufacture an average of eight salmon disks of 66.1 ± 1.7 g. Portions were put in Petri dishes, packed in polyamide polyethylene PAPE bags (Atelier des Landes, Plelo, France), vacuum-packed and frozen at -20°C. The frozen portions were sterilized by gamma radiations (2 kGy) in a plant equipped with electron beam facilities (Radiant Ouest, Berric, France). The sterile samples were stored at -20°C.

Preparation of the cold-smoked salmon. Samples were thawed overnight at 4°C and kept inside the Petri dishes during the subsequent steps. Each salmon disk was aseptically dried-salted with 3.9 g of previously sterilized NaCl and stored 2 h at 12°C for salt penetration

(salting step). Autoclaved liquid smoke L1165 (Lutecia Jaeger, Arnouilles les Gonesses, France) was sprayed on each sample at a level of 0.6 % (v/w) with a sprayhead (Paasches V, Paasche Airbrush Company, Illinois, USA) connected to a compressor and samples were stored for 1 h at 12°C (smoking step). Afterwards, samples were dried by storing the Petri dishes open for 2 h at 20°C under the laminar flow hood and stored for 24 h at 4°C (maturation step). Samples were held at -80°C for 25 min in order to reach -7°C in the flesh (common in processing plants in order to obtain a harder tissue before slicing) (frozen step) and vacuum-packaged in PAPE bags. Finally, the packaged processed salmon samples were stored 10 days at 4°C followed by 18 days at 8°C as specified in the French standard for shelf-life validation of perishable and refrigerated food (19).

Inoculation of the salmon. In order to simulate a contamination of the raw product, a group of 27 raw sterile samples was inoculated with *L. monocytogenes* at a desired level of 6 log CFU/g in the flesh. Inoculation was done by spraying 0.6 ml of a dilution of the bacterial inoculum containing 8 log CFU/ml (the bacterial concentration was assessed by direct microscopic counts with the 4',6-diamidino-2-phenylindole, dilactate (DAPI) staining) with the sprayhead used for liquid smoke. Samples were stored 1 h at room temperature before being processed and stored as previously described.

Contamination of the final product was simulated by spraying 0.6 ml of a dilution of the bacterial inoculum containing 8 log CFU/ml on 15 cold-smoke processed samples after the frozen step at an identical level of 6 log CFU/g in the flesh. Samples were vacuum-packaged and stored for 28 days at the same temperature conditions described for the first trial.

Finally, a control was prepared by inoculating at a level of 6 log CFU/g in the flesh 18 samples that were not subsequently processed but kept at 4°C for 29 h (processing time), vacuum-packaged and stored.

Enumeration of culturable *L. monocytogenes* cells. The culturability of *L. monocytogenes* in samples contaminated before the salting, smoking, maturation and frozen steps was analyzed after each step of the process and weekly during storage. In the case of samples contaminated after being cold-smoke processed, the culturability was assessed after inoculation (before packaging) and weekly during storage. The control trial was checked for culturability after inoculation, before packaging and weekly during storage. Each salmon sample was homogenized in 264 ml of physiological water (dilution factor of 1:5) and held 30 min at room temperature for resuscitation. Culturability was quantified by spread plate counts of appropriate dilutions on Plate Count Agar (PCA) (Merck) and Palcam-Listeria-Selective Agar (Merck). Plates were incubated for 48 h at 37°C. The assessment of culturability was systematically based on the results of three independent samples.

Assessment of the viability of *L. monocytogenes* cells. Bacteria contained in 2 ml of the diluted suspension described above were first separated by the use of the Listerscreen immuno-magnetic-capture kit (AES, Combourg, France). The viability of the captured cells was investigated by the cyanoditylotetrazolium chloride (CTC) - 4',6-diamidino-2-phenylindole, dilactate (DAPI) double staining technique as described by Besnard et al. (4). Cells showing red CTC formazan crystals were taken as viable cells. DAPI blue-stained cells without red CTC formazan crystals were taken as non viable cells. The total cell counts were performed by adding the number of viable cells to the number of non viable cells. For each of the three samples, ten microscope fields were randomly observed. Viable and total cell counts were assessed at the same times as culturability.

Virulence of *L. monocytogenes* cells. Virulence was assessed before packaging (beginning of the 28 day storage) and at the end the of 28 day storage. *L. monocytogenes* colonies from PCA plates used to quantify culturability were taken to prepare a suspension in physiological water at a desired concentration of 4.0 log CFU/ml. A volume of 100 µl of this suspension was intravenously injected into five-week old OF1 female mice (Iffa Credo, France). Two days after inoculation, mice were sacrificed by cervical dislocation and their spleens were aseptically removed. Spleens were weighed and homogenized with physiological water by manual compression (dilution factor of 1:10). Dilutions of the obtained suspension ranging between 10⁻¹ and 10⁻⁵ were plated on PCA. Three samples were systematically analyzed and groups of five mice were used to test each sample. Two

additional groups of five mice were inoculated with physiological water and a suspension of exponential phase Scott A cells to perform respectively a negative and a positive control.

Chemical analysis. Sodium chloride was measured with a Chloride Analyzer 926 (Corning, Halstead, England). Total phenolic compounds were quantified by the method described in the French Standard for smoked salmon (18).

Statistical analysis. All results are expressed as mean of 3 triplicates \pm 95% Confidence Interval (CI = $1.96 \times \sqrt{\text{standard deviation}^2/3}$). *L. monocytogenes* means were compared by one-way variance analysis with Fischer test at the 0.05 level of probability, performed on Excel software (Excel 2000, Microsoft).

2. Results

Chemical composition of processed products. The content of salt and total phenols in the processed samples were 5.9 ± 0.3 g/100 g and 0.74 ± 0.09 mg/100 g, respectively.

Enumeration of culturable *L. monocytogenes* cells. Figure 1 shows the culturable counts of contaminated samples during the process. Values correspond to the average of culturability obtained with PCA spread counts. No difference was observed with Palcam-Listeria-Selective Agar count (data not shown). Raw salmon samples were inoculated at a level of 6.3 ± 0.2 log CFU/g. The salting step had a weak but significant effect on *L. monocytogenes* culturability assessed on PCA, reducing by 0.6 log CFU/g the average population. Then, smoking the salmon disks had a similar effect, reducing again the number of *L. monocytogenes* by 0.6 log CFU/g, but this difference was not statistically significant due to a lower repeatability of the triplicates. During maturation, the effect of salt and smoke continued and the average number of *L. monocytogenes* was reduced by 0.5 log CFU/g (not significant). Finally, although each processing step did not show individually a significant effect on *L. monocytogenes*, combination of the 3 steps significantly reduced by 1.6 log CFU/g the number of *L. monocytogenes* and culturability gradually decreased from the initial inoculation level of 6.3 ± 0.2 log CFU/g to 4.7 ± 0.5 log CFU/g after maturation. A slight but not significant increase was observed after samples were partially frozen.

Figure 2 shows culturability assessed on PCA agar during the four weeks of storage of control (not processed salmon) and trials inoculated before and after the process. Results obtained with the control samples showed that no significant growth occurred during the 29 h of storage at 4°C (processing time), the level of *L. monocytogenes* before vacuum-packaging (6.4 ± 0.2 log CFU/g) being identical to the inoculation level. During the first seven days of storage, corresponding to storage at 4°C *L. monocytogenes* reached 8.9 ± 0.02 log CFU/g. During the three subsequent weeks of storage culturability remained at the same level. In the case of samples contaminated before the process, the culturable count after the frozen step just before packaging (5.1 ± 0.5 log CFU/g) increased by 1.3 log CFU/g during the first week of storage, reaching 6.4 ± 0.7 log CFU/g. This count then remained at a quite constant level over the course of the experiment. *L. monocytogenes* culturable count in the product contaminated after the process followed a quite different evolution during storage. A gradual decrease of 1.2 log CFU/g, from 6.6 log CFU/g to 5.4 log CFU/g, was observed between the time of inoculation and 14 days of storage. Between the second and the third weeks of storage, the culturability increased significantly by 1.7 log CFU/g, reaching a mean value of 7.0 log CFU/g, and then remained quite constant until the end of storage, showing a value of 7.2 log CFU/g at the end of the experiment.

Assessment of the viability versus culturability of *L. monocytogenes* cells. Figure 3 shows the evolution of the viable and total counts of *L. monocytogenes* of samples contaminated before the process. After salting, the viable count was significantly reduced by 1.4 log bacteria/g showing a value of 4.6 log bacteria/g. Due to a lower repeatability of the triplicates, no significant difference in the viable count was then observed until the freezing step with a value of 4.3 log CFU/g. Viable count decreased significantly to 3.9 log CFU/g

after the first 7 days of the storage and then, although an increase to 5.2 log CFU/g was noticed, it was not statistically significant.

Between inoculation and smoking, there was no significant difference between total and viable counts of *L. monocytogenes*, indicating that there was no immediate listericidal effect of salting and smoking steps. After the 24 h maturation period, the viable count (4.3 log CFU/g) became significantly lower than the total count (5.4 log CFU/g), corresponding to 92% of mortality. The mortality rate remained constant over the storage but differences between the total and viable counts were statistically significant only until the first week.

In general, no significant differences were observed between the culturable counts and the viable counts during the process. However, during storage, culturable counts became higher than viable counts and significant differences were observed with a maximum of 2.5 log CFU/g after the first week of the storage.

Virulence of *L. monocytogenes* cells. Table 1 shows the level of virulence of *L. monocytogenes* Scott A assessed by the intravenous route in mice in terms of log of CFU/spleen. *L. monocytogenes* were not detected in the spleens of the negative control (mice inoculated with physiological water). The positive control (mice inoculated with exponential phase Scott A cells) showed a value of 7.6 ± 0.4 log CFU/spleen. With strains inoculated in salmon, the numbers of log CFU/spleen at the end of the storage were systematically slightly lower than those obtained at the beginning of the storage, whatever the trial considered (inoculation before and after the process and unprocessed control). However, these differences were not statistically significant at the 0.05 level of confidence.

3. Discussion

Culturability assessed with PCA and Palcam-Listeria-Selective Agar were similar. This applies to the study of control samples as well as to the study of processed samples (data not shown), indicating that recovery of cells stressed by salt and/or smoke was not affected by the use of a selective medium. Our results are in agreement with those obtained by Duffy et al. (9) working with meat products. According to these authors, the recovery of *L. monocytogenes* was not significantly affected by the use of selective and non-selective enrichment broths.

Salting salmon had an immediate effect on culturable count, reducing by 0.6 log CFU/g the number of *L. monocytogenes*, which corresponded to a 4-fold reduction. To our knowledge, experiments *in situ* had only been done by Guyer and Jemmi (12), which showed no effect of salting whatever the inoculation level of *L. monocytogenes* tested, ranging from 1.4 log to 4.4 log MPN/g. However, the salt concentration in the flesh was unknown in this study, but probably lower than in our experiments because product had been marinated for 24 h in a 6% brine. In the present study, a 6% (total flesh) concentration was used. In another set of experiments (unpublished data), we worked with salmon salted at 3% and similarly to those authors no effect on *L. monocytogenes* was observed.

A weak, but not significant effect of liquid smoke, reducing by 0.6 log CFU/g the number of *L. monocytogenes*, which corresponded to a 4-fold reduction was observed. The anti-*L. monocytogenes* effect of liquid smoke products was first demonstrated in 1988 by Messina et al. working with beef franks (17). These authors used different types of liquid smoke and showed that they had different antimicrobial properties regarding *L. monocytogenes* probably as a result of a different phenol content. Likewise, Suñen et al. (29) showed different capacity of four liquid smokes to inhibit *L. monocytogenes* growth in broth media. Those results were then confirmed in brined rainbow trout inoculated with 2.7 ± 0.3 log CFU *L. monocytogenes* (30). In this study, two liquid smoke, whose total phenol derivatives were 1.5 and 1.8 % led to a reduction immediately after smoking and all over the 21 day storage at 4°C (count under the detection threshold but presence reported in 30 g). On the other hand, two other liquid smokes with 0.2 % of total phenol derivatives showed no effect on *L. monocytogenes*. Liquid smoke used in our laboratory had a similar 0.3 % total phenol derivatives and showed a very

weak inhibitory effect, which indicates that inhibition is highly correlated with total phenolic concentration. However, results are difficult to compare because total phenolic compounds in the flesh are not mentioned in the study of Suñen et al. (30). Moreover, they have not worked with sterile fish and possible inhibitory effect of naturally contaminating flora can not be dissociated. In France, the use of liquid smoke is not common for fish smoking, but some manufacturers are highly interested by replacing the conventional salmon smoking process by the use of smoke flavoring, sometimes simultaneously with salt in brine (5). This liquid smoking technique has been used in our study instead of conventional smoking because it is easier to apply when one works with the pathogen *L. monocytogenes* in laboratory facilities. Liquid smoke L1165 from Lutecia Jaeger had been chosen among different liquid smokes as it enables the production of smoked salmon samples with sensory characteristics and total phenol content very similar to traditional products (26). In our set of experiments, the phenol content in the flesh was 0.74 mg 100/g which is in the average of smoke content measured in traditionally smoked European products (15). Thus it is believed that the experimental smoking process developed in our laboratory gives results comparable to traditional smoking process. Some authors, using a traditional smoking kiln and inoculating previously brined salmon samples have also shown that the effect of smoking was very weak against *L. monocytogenes* (10,12). Guyer and Jemmi (12) observed no reduction of *L. monocytogenes* with a processing time of 6 h at 26-30°C. It is possible that a slight inhibitory effect of smoke was balanced by the period at 26-30°C which can be favorable for *Listeria* growth. Indeed, Eklund et al. (10) showed that the inhibitory effect of traditional smoking at 17-21°C (10-25 – fold reduction), observed on three strains of *L. monocytogenes* including Scott A, was lowered to a 3-fold reduction when samples were smoked at higher temperatures (22-30°C). Although not quantified, the effect of smoking on naturally contaminating *L. monocytogenes* has been clearly stated by Aase and Rørwick (1). Among 200 samples taken in four smokehouses just before smoking, 54% were *L. monocytogenes* positive whereas only 9.5% positive samples were detected after smoking.

Salting and smoking had a relatively weak immediate effect against *L. monocytogenes*. However, combination of these steps allowed a 44-fold reduction of the initial culturable number of *L. monocytogenes* after a 24 h maturation time. During chilled vacuum storage, the effect of those two parameters continued and the final count (6.7 ± 0.6 log CFU) was always maintained at a level close to the inoculation level whereas in unprocessed samples, the count reached 8.9 ± 0.02 log CFU, which corresponded to a 160-fold increase. During the first week of vacuum storage at 4°C, a slight growth of *L. monocytogenes* in samples contaminated before process was observed; then culturable count remained quite constant although temperature raised from 4°C to 8°C at the 11th day of storage. Growth of *L. monocytogenes* in cold-smoked salmon stored at 4°C has been reported by many authors and most of them report an accelerated growth when working at abuse temperature close to 8-12°C (8, 13, 32) which was not the case in our study.

Unprocessed salmon contamination and post-processing contamination with strain Scott A at a level of 6 log CFU/g resulted in identical culturable counts after 28 days of storage. However, during storage culturability showed a different evolution. The reduction in culturability observed after the first 14 days of storage for samples inoculated after the process could reflect the mortality of bacteria less adapted to the chemical compounds used in the process (salt and phenolic compounds).

In the present study we were also interested in the detection of *L. monocytogenes* viability in order to search for possible VBNC cells. The VBNC state of *L. monocytogenes* was previously shown using a microcosm water model consisting of sterile, distilled and filtered water in which *L. monocytogenes* cells of several strains including Scott A were placed at a level of 6 log CFU/ml (4). As far as we know, a possible transition of bacteria to a VBNC state has never been investigated using a food model. Due to the high detection limit of the viability method (10^4 bacteria/ml) we were forced to choose a high inoculation level which has been fortunately never reported in natural conditions. Moreover, the present study showed that the use of immuno-magnetic separation did not allow us to obtain a satisfying recovery rate (cultural counts were higher than total and viable counts). Low recovery rates

associated to high concentrations of bacteria and immuno-magnetic separation have been reported before (27, 33). Recently, Geng et al. (11) were able to demonstrate that the expression of *L. monocytogenes* cellular antigens was affected by both osmotic and cold stresses, which could also explain the poor recovery rate of the immuno-magnetic separation kit used in our experiments. Due to this technical limitation, possible VBNC state transition of strain Scott A during the cold-smoking process of salmon samples could not be detected in this study. However, assuming that the ratio total/viable cells was conserved after the immuno-magnetic separation, we still used the Listerscreen kit to estimate the listericidal effect of the smoking process on *L. monocytogenes*.

The assessment of the virulence of *L. monocytogenes* by the intravenous inoculation of immunocompetent mice is a useful model for characterizing its pathogenic aptitude that has been used by several authors (2, 22, 28). *L. monocytogenes* isolated at the beginning of storage from the three set samples (control and inoculation before and after the process) was still capable of colonizing the spleen of Swiss OF1 female mice. Moreover, the virulence of strain Scott A cells did not change significantly between the beginning and the end of storage. Virulence of those cells ranged between 6.6 ± 1 log/spleen and of 8.0 ± 0.3 log CFU/spleen and was found similar to virulence obtained with the suspension of exponential phase cells inoculated to mice (positive control). Using the scale proposed by Roche et al (22) these values correspond to virulent *L. monocytogenes* strains and demonstrate that even submitted to several technological stresses, strain Scott A was able to conserve its infectious ability.

The major finding of this study is that the salting and cold-smoking process of salmon can reduce the numbers of culturable *L. monocytogenes* without affecting the pathogenic ability of this organism.

Acknowledgements

This work was supported by a PhD fellowship within the POCTI Program administered by the National Board for Scientific and Technological Investigation (MCES, Portugal) and by the European SEAFOODplus project. We would also like to thank Florence Jugiau for her excellent technical support.

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Tables

TABLE 1. Virulence of *Listeria monocytogenes* Scott A isolated from salmon inoculated before or after the cold-smoking process and from control (unprocessed) at the beginning and at the end of the 28 days storage (10 days at 4°C and 18 days at 8°C). Values are expressed in log CFU/spleen. The data represent mean \pm confidence interval of three independent experiments.

3.1. Sample	4. Virulence (log CFU/spleen)	
	beginning of storage	end of storage
4.1. Contamination before process	7.5 \pm 0.6	6.6 \pm 1.0
4.2. Post-processing contamination	7.1 \pm 0.3	6.7 \pm 0.4
Control (unprocessed product)	8.0 \pm 0.3	7.4 \pm 0.6
Negative control (physiological water suspension)		NCD*
Positive control (exponential phase of <i>L. monocytogenes</i> suspension)		7.6 \pm 0.4

*NCD = no colony detected

Figures

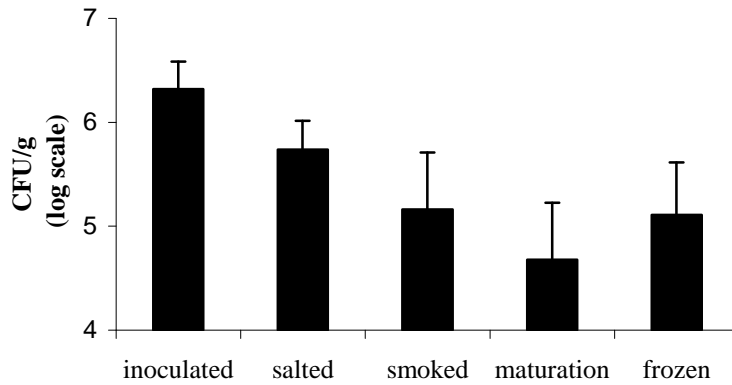


FIGURE 1. Effect of the salting, smoking, maturation and frozen steps of the cold-smoke process on the culturability of *Listeria monocytogenes* Scott A inoculated in sterile salmon samples. The data represent mean \pm confidence interval of three independent experiments.

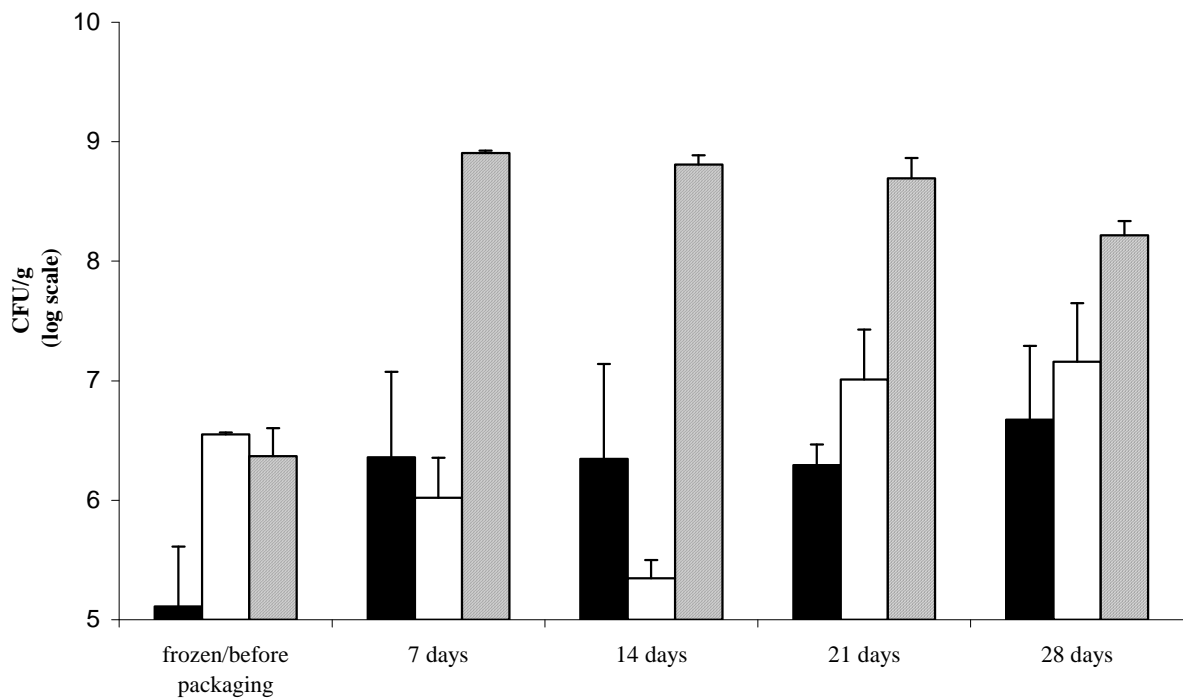


FIGURE 2. Evolution of the culturability of *Listeria monocytogenes* Scott A inoculated in sterile salmon weekly assessed during the 28 days storage (10 days at 4°C and 18 days at 8°C). Solid bars: contamination before the process; open bars: contamination after the process; hatched bars: control (unprocessed samples). The data represent mean \pm confidence interval of three independent experiments.

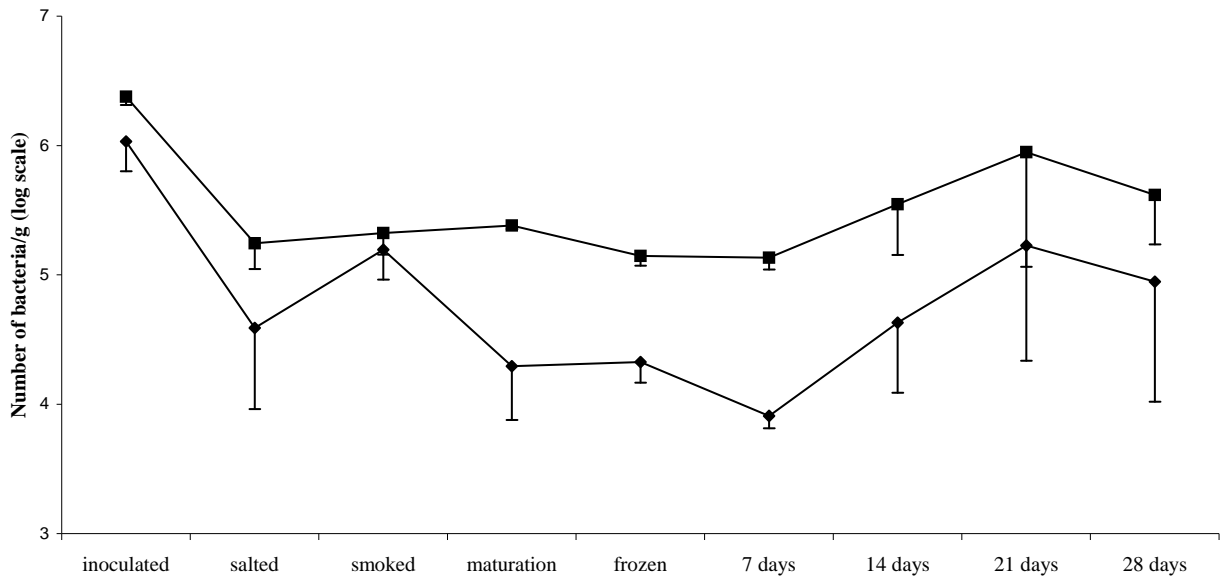


FIGURE 3. Evolution of the total (■) and viable (◆) counts estimated by the CTC-DAPI double staining method of *Listeria monocytogenes* Scott A inoculated in sterile salmon samples during cold-smoke process and vacuum storage (10 days at 4°C and 18 days at 8°C). The data represent mean \pm confidence interval of three independent experiments.