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High pressure treatments combined with sodium lactate to inactivate *Escherichia coli* O157:H7 and spoilage microbiota in cured beef *carpaccio*

Marcelo Oscar Masana [a, *], Yanina Ximena Barrio [a, b], Pablo Martín Palladino [a], Ana Maria Sancho [a], Sergio Ramón Vaudagna [a, b, c]

[a] *Instituto Tecnología de Alimentos, Centro de Investigación de Agroindustria, Instituto Nacional de Tecnología Agropecuaria (INTA), CC. 77, B1708WAB, Morón, Pcia. de Buenos Aires, Argentina*
[b] *Departamento de Biotecnología y Tecnología Alimentaria, Facultad de Ingeniería y Ciencias Exactas, Universidad Argentina de la Empresa (UADE), Lima 717, C1073AAO, Ciudad Autónoma de Buenos Aires, Argentina*
[c] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, C1033AAJ, Ciudad Autónoma de Buenos Aires, Argentina*

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ABSTRACT

High-pressure treatments (400 and 600 MPa) combined with the addition of sodium lactate (1 and 3%) were tested to reduce *Escherichia coli* O157:H7 (STEC O157) and spoilage microbiota contamination in a manufactured cured beef *carpaccio* in fresh or frozen conditions. Counts of spoilage microorganisms and STEC O157 were also examined during the curing step to prepare the *carpaccio*.

STEC O157 counts remained almost unchanged through the curing process performed at 1 ± 1 °C for 12 days, with a small decrease in samples with 3% of sodium lactate. High-pressure treatments at 600 MPa for 5 min achieved an immediate reduction of up to 2 logarithmic units of STEC O157 in frozen *carpaccio*, and up to 1.19 log in fresh condition. Counts of spoilage bacteria diminished below detection limits in fresh or frozen *carpaccio* added with sodium lactate by the application of 400 and 600 MPa. Maximum injury on STEC O157 cells was observed at 600 MPa in *carpaccio* in fresh condition without added sodium lactate. Lethality of high-pressure treatments on STEC O157 was enhanced in frozen *carpaccio*, while the addition of sodium lactate at 3% reduced the lethality on STEC O157 in frozen samples, and the degree of injury in fresh *carpaccio*.

1. Introduction

The contamination of beef products with Shiga toxin-producing *Escherichia coli* O157:H7 (STEC O157) is a main concern for the food industry, as STEC O157 has been frequently associated with outbreaks implicating beef, and beef products (Rangel et al., 2005). STEC O157 is considered the most virulent of STEC serotypes due to its ability to cause large outbreaks of severe disease, and to its low infectious dose of 10–100 cells. In Argentina, STEC associated illnesses are a serious public health issue with approximately 400 hemolytic uremic syndrome (HUS) cases reported annually which are predominantly linked (>70%) to the O157 serotype (Rivas et al., 2011).

Preventive interventions at the farm, and at the abattoir, can reduce STEC contamination of beef. Nevertheless, some extent of contamination in carcasses, and derived beef products is practically unavoidable. In Argentina STEC O157 has been found in various beef products such as ground beef, fresh and dry sausages (Chinen et al., 2001), and precooked blood sausages (Oteiza et al., 2006), at retail.

The commercialization of a wide variety of "ready-to-eat" and "ready-to-cook" food products, including fresh marinated or cured meats, has been increasing in domestic and in the international markets. Food safety of these types of foods depends mainly on an adequate combination of safety hurdles, such as pH, temperature and water activity. However, foodborne incidents involving STEC, in dry-fermented sausages (Sartz et al., 2008; Conedera et al., 2007; MacDonald et al., 2004) have shown that such treatment combinations may not be enough to reduce pathogen contamination to acceptable levels, and that, additional processes are needed to ensure food safety objectives.

Non-thermal preservation technologies, such as high-pressure processing (HPP), are alternatives to produce safe and wholesome foods. Application of high-pressure in the range of 300–600 MPa

* Corresponding author. Tel.: +54 11 4621 0457; fax: +54 11 4621 2012.
E-mail address: masana.marcelo@inta.gob.ar (M.O. Masana).

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has proven to be effective to inactivate vegetative cells, while more than 1000 MPa are needed to inactivate bacterial spores, unless HPP is carried out at temperatures in the order of 100 °C. Nevertheless, there is a natural variation of resistance among strains of a bacterial species, as demonstrated in an early work on the effect of high-pressure on STEC O157 strains (Benito et al., 1999). In addition, the growth phase of the microorganisms can influence the effect of HPP, as cells in the stationary phase have been shown to be more resistant (Pagán and Mackey, 2000; Mañas and Mackey, 2004).

HPP has been applied to extend the shelf life of a variety of meat products (Garriga et al., 2004; Diez et al., 2008; Vercammen et al., 2011). Likewise, the inactivation of foodborne pathogens by HPP in several "ready-to-eat" meat products, such as, dry-fermented sausages (Omer et al., 2010), dry-cured ham (Hereu et al., 2012; De Alba et al., 2013), cooked ham (Jofre et al., 2008) and *carpaccio* (Bravo et al., 2014) has also been studied lately. However, the application of HPP to fresh red meats, in the pasteurization ranges (300–600 MPa), can cause an undesirable discoloration (Carlez et al., 1995). This defect can be diminished by the combination of HPP with other treatments such as curing or freezing (Szerman et al., 2011; Vaudagna et al., 2012).

Beef *carpaccio* is traditionally produced as thin slices from frozen raw or cured beef, which are packed under vacuum or modified atmospheres, and kept at refrigeration temperature until consumption. *Carpaccio* is considered a high-risk food because of the possibility of contamination with pathogenic bacteria from the animal reservoir, such as STEC or *Salmonella*, and the minimal process it undergoes. The *Salmonella* Typhimurium DT104 outbreak in Denmark, with imported beef served as *carpaccio* (Ethelberg et al., 2007), has verified the potential risks of this type of meat product.

Sodium lactate (NaL) is a GRAS additive known for its ability to extend the shelf life of cured and uncured meat products (Bloukas et al., 1997; Maca et al., 1999). In addition, NaL has been reported as capable of decreasing the probability of growth of STEC O157 in ground beef (Hwang and Juneja, 2011).

Many factors, such as food components, variation in strain resistance, and processing temperatures can modify the effect of HPP on bacteria. Thus, the development of challenge studies in the food of interest, and the use of pools of strains are recommended to validate HPP. Accordingly, the aim of this work was to study the effectiveness of HPP and NaL addition to inactivate STEC O157 native strains, and to reduce the spoilage microbiota in manufactured cured beef *carpaccio*.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

STEC O157 strains used in this study, included isolates from INTA culture collection (I-105 and I-104), and those kindly provided by Dr. Marta Rivas (FP-14, FP-15) from the ANLIS Dr. Carlos G. Malbrán Institute of Argentina. These STEC O157 strains were originally isolated from abattoirs of Argentina, and have been characterized as $stx_2 - stx_{2c\ (vh\text{-}a)}/eae/ehxA$ (Masana et al., 2010). The strains were maintained in frozen culture at −80 °C, until subcultures were prepared by inoculating a test tube with 10 ml of trypticase soy broth (TSB, Oxoid, UK) with a single colony grown in MacConkey Sorbitol agar (SMAC, Difco, Becton Dickinson, USA), and individually incubated at 37 °C overnight. Cells were harvested by centrifugation at 10,000 rpm (Eppendorf 5417C, Germany), and the pellets washed twice with phosphate-buffered saline (PBS, pH 7.2, Oxoid) and resuspended in PBS to give an approximate concentration of 10^8 CFU/ml. The pool of strains was prepared by mixing equal volumes of each strain in PBS. The STEC pool was appropriately diluted in PBS to serve as inoculum in the assays.

2.2. Carpaccio elaboration

Processing stages for the elaboration of *carpaccio* are presented in Fig. 1. A total of 54 bovine *Semitendinosus* muscles (SM) of pH 5.4–5.7, weighing between 1500 and 1800 g were obtained from a local market 48 h after slaughter. SM were randomly distributed into three curing batches (18 SM each) with formulations differing only in NaL concentration (0, 1 or 3%). SM were subjected to intermittent tumbling (5 rpm–2 min on, 8 min off) for 60 min under vacuum (15 mmHg) and cooling conditions (1 ± 1 °C), in a tumbler (model LT-15, Lance Industries, Allentown, USA).

The basic formulation, as weight of product basis, consisted of sodium chloride 1.2% (Dos Anclas, Argentina), sodium tripolyphosphate 0.1% (Carfosel 991, Tecnoalimenti, Argentina), sodium citrate 0.05% (Polar, Tecnoalimenti, Argentina), sodium nitrite 0.015% (General Chemical, Tecnoalimenti, Argentina), sodium isoascorbate 0.05% (Tate & Lyle, DGM, Brazil). Sodium lactate (PURASAL®S, PURAC, Adama, Argentina) was added to the basic formulation in 0% (control), 1%, or 3% proportion.

After tumbling, 6 SM of each batch were treated and inoculated as described in point 2.3, in order to study the effect of the curing process. Non-inoculated SM (12 per formulation) were then, vacuum-packed in bags (Cryovac BB2800CB, Sealed Air Co., Argentina) and kept in a chill storage chamber (1 ± 1 °C) for 12 days. After the curing process was completed, the SM were frozen at −40 °C in an ultrafreezer (Righi, Argentina). At the time of performing the HPP study the frozen SM were cut in 1.5–2-mm-thick slices with a Berkel 834 slicer (Berkel, Rotterdam, the Netherlands). *Carpaccio* slices were then vacuum-packed in bags (Cryovac BB2800CB, Sealed Air Co., Argentina) in groups of five. In the case of *carpaccio* to be inoculated, slices were individually packed in order to simplify the inoculation procedure.

2.3. Experimental design for the curing process study and SM inoculation procedure

Eighteen SM, 6 per each batch, were used to study the effect of the NaL concentration, and the time of storage, during the curing process on the spoilage microbiota and on STEC O157 counts. Each muscle was divided in half, and three circular sectors (4.90 cm^2 and 3 mm depth) were delimited, with the aid of a sterilized stainless steel cork-borer, in one-half of the SM. Each sector was then inoculated with 100 μl of a dilution of the STEC pool to give a concentration of approximately 10^5 CFU/cm^2, while the other half of the SM remained as a non-inoculated control. SM were then vacuum-packed (Cryovac BB2800CB, Sealed Air Co., Argentina) and kept in a chill storage chamber (1 ± 1 °C). Sampling was performed at days 1 and 12 by analyzing 3 SM for each NaL concentration.

2.4. Experimental design of the HPP study and carpaccio inoculation procedure

A completely randomized design with a 3 × 3 factorial arrangement was applied. The factors to be studied were the concentration of NaL added to the *carpaccio* (0, 1, and 3%) and the pressure level (0.1, 400 and 600 MPa). This design was applied to two conditions of *carpaccio*: fresh or frozen, and repeated for four independent units.

In the case of STEC O157 inoculated *carpaccio*, each unit consisted of a package with one inoculated *carpaccio* slice, while for non-inoculated samples each unit comprised a package containing five *carpaccio* slices.

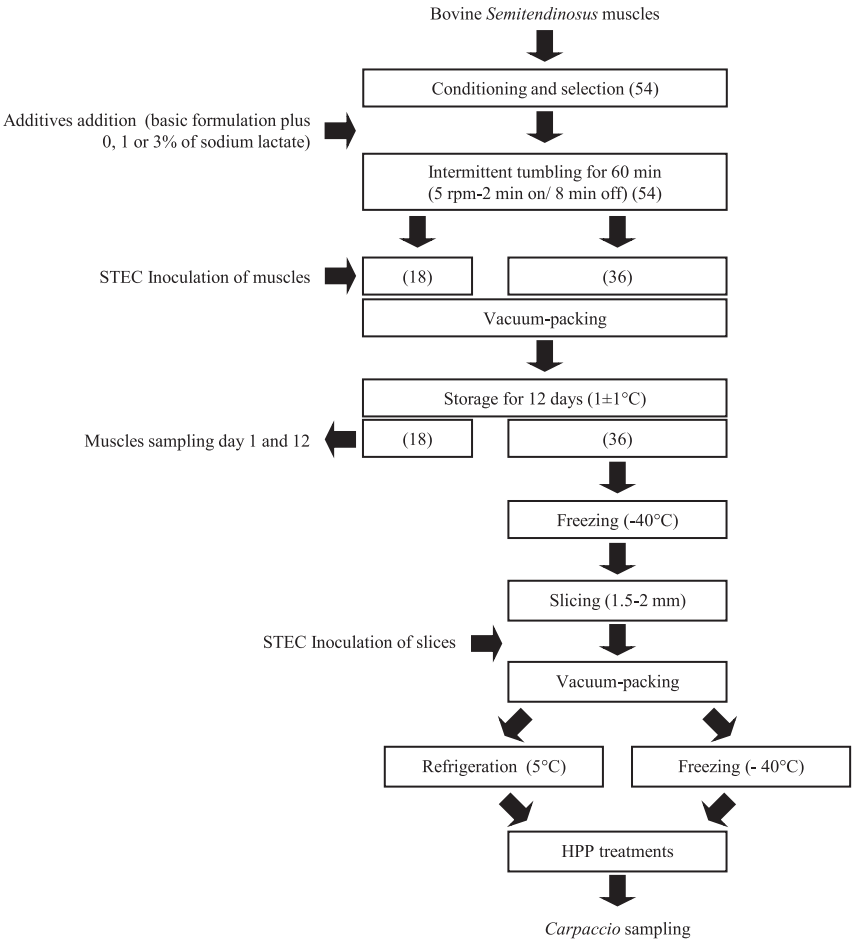

Fig. 1. Flow diagram of *carpaccio* manufacture, inoculation and processing.

For the inoculation procedure *carpaccio* slices (7 ± 1 g and *ca.* 42 cm^2) were aseptically removed from their protective plastic wrapping in a biological safety cabinet (Forma Scientific Inc., USA), and inoculated with 100 μl of inoculum of a pool of STEC O157 strains to give a concentration of approximately 10^6 CFU/g. The inoculated slices were then allowed to dry for 15 min at room temperature in the biological safety cabinet before being individually vacuum-packed (Cryovac BB2800CB, Sealed Air Co., Argentina).

Carpaccio samples were then kept refrigerated at 1 ± 1 °C for 24 h before HPP (fresh condition), or frozen at −40 °C and high-pressure treated in frozen state (frozen condition).

2.5. HPP treatments

Vacuum packed *carpaccio* slices (frozen or fresh) were subjected to 400 or 600 MPa for a holding time of 5 min. Treatments were applied in a High Pressure System Stansted Fluid Power Ltd. model Iso-Lab FPG9400:922 (Stansted, UK), with a vessel working volume of 2 dm^3 and a sample canister with an internal working diameter of 70 mm (maximum working pressure: 900 MPa; temperature range: −20 to 120 °C). A mixture of propylene glycol and water (30:70) was used as compression fluid and temperature-controlling fluid (circulating in the outer jacket of the high pressure vessel). The pressurization rate was 300 MPa min^{-1} and decompression was nearly instantaneous. Conditioning temperature of vessel and initial temperature of compression fluid was 5 °C. The temperature increase of fluid due to adiabatic heating was approximately 2 °C per 100 MPa. Each treatment was carried out in quadruplicate (four independent trials). After high pressure treatments, fresh and frozen *carpaccio* samples were stored overnight at 4.0 ± 1.0 °C until analysis.

2.6. Sample analysis

2.6.1. Microbiological analysis of SM during the curing process

Microbiological analyses of SM samples were performed at the first day of storage (day 1) and at the end of the curing process (day 12). The three circular sectors previously inoculated with STEC O157 were removed, with the aid of a sterile scalpel and tweezers under a laminar flow, and pooled into sterile stomacher bags with filter (Whirl-Pak, Nasco, USA). Samples were stomached (easy Mix, AES, France) for 60 s with sufficient volume of 0.1% buffered peptone water (PW, Oxoid, UK) to obtain an initial 1/10 (w/v) dilution. Likewise, three circular sectors of the same dimensions, from the non-inoculated half of the muscle, were also removed, pooled and analyzed. Microbiological counts were performed by spiral plating 50 μl of the corresponding decimal dilution in decreasing logarithmic mode (Spiral Plater DS+, Interscience, France). STEC O157 was simultaneously counted in two selective media, SMAC agar, and in O157:H7 ID agar (bioMérieux, France), both incubated at 37 °C for 24 h. STEC O157 characteristic colonies were confirmed at random by the latex agglutination test for STEC O157 (*E. coli* O157 Latex Test, Oxoid, UK). For non-inoculated

samples, mesophylic counts were performed on Triptone Soya Agar (TSA Oxoid, UK) incubated at 30 °C for 48–72 h, while psychrotrophic counts were carried out in TSA incubated at 8 °C for 10 days. Enterobacteriaceae were counted on Violet Red Bile Dextrose agar (VRBD, Oxoid, UK) incubated at 37 °C for 24 h, and Lactic acid bacteria (LAB) in Man Rogosa Sharpe Agar (MRS, Oxoid, UK) incubated at 30 °C for 72 h in 10% CO_2 atmosphere (Anaerogen, Oxoid, England). Non-inoculated samples were also analyzed for STEC O157 in SMAC and in O157:H7 ID.

2.6.2. Microbiological analysis of carpaccio samples

Carpaccio samples were analyzed 24 h after HPP was applied. The *carpaccio* slices (five non-inoculated slices, or one inoculated) were aseptically removed from their plastic wrapping, and stomached for 60 s in sterile bags with filter with sufficient volume of 0.1% PW to obtain an initial 1/10 (w/v) dilution. Successive decimal dilutions were then performed and microbial counts performed by spiral plating of the adequate dilution.

STEC O157 was enumerated in TSA supplemented with 0.3% (w/v) yeast extract and 0.1% (w/v) of sodium pyruvate (J. T. Baker, USA) (TSAYEP), as non-selective media, and simultaneously in SMAC and O157:H7 ID as selective media. STEC O157 characteristic colonies were confirmed at random by the latex agglutination test for STEC O157 (*E. coli* O157 Latex Test, Oxoid, UK) in all media. Non-inoculated *carpaccio* slices were analyzed for mesophilic, psychrotrophic, LAB and Enterobacteriaceae counts as explained in the SM microbiological analyses.

2.6.3. Water activity and pH determinations

Water activity (a_w) of *carpaccio* was measured, in duplicate, using a water activity meter (AquaLab 4TE, Decagon Devices Inc., USA). pH measurements of *carpaccio* homogenates (5 g in 25 ml of distilled water standardized at pH 7) were performed in duplicate with a pH-meter (Thermo Orion 710A+, Beverly MA, USA) equipped with a combination pH electrode (model 8102BN ROSS Electrode, Thermo Orion, Beverly MA, USA) and an ATC-Probe (Thermo Orion, Beverly MA, USA).

2.7. Statistical analysis

ANOVA was performed to evaluate significant ($p < 0.05$) effect of factors according to the experimental design described in Sections 2.3 and 2.4. Least Significance Difference (LSD) multiple comparisons test ($p < 0.05$) was applied to analyze statistical differences among means. Statistical analyses were performed using the SPSS-v12 software (SPSS, Chicago, IL., USA).

3. Results

3.1. Effect of the curing process on microbial counts of SM

Most of the curing formulations allowed the growth of mesophilic, psychrotrophic and LAB on SM after 12 days at 1 ± 1 °C (Table 1). Only psychrotrophs showed some decline in counts in SM cured with 3% NaL, while Enterobacteriaceae counts were below the detection limit of 2 log CFU/cm^2 in all of the samples. At day 1 the only significant reduction in counts observed was for LAB by the addition of 3% NaL. At day 12, the addition of 1% NaL significantly ($p < 0.05$) reduced psycrhotrophs and LAB, when compared to the control samples without NaL. Sodium lactate at 3% was able to further diminish significantly ($p < 0.05$) mesophilic and psychrotrophic counts.

On the other hand, STEC O157 inoculated in SM was able to survive the curing process (Table 1). Samples with 3% NaL showed a significant ($p < 0.05$) decrease in STEC O157 counts in SMAC at days 1 and 12, when compared to control samples. At day 12, a tendency to decrease STEC O157 counts, by the increasing NaL concentration was also observed in O157:H7 ID medium although without statistical significance. STEC O157 was not detected in the non-inoculated SM samples.

3.2. Effect of HPP and NaL combinations on microbial counts of carpaccio

The bacterial counts of the spoilage microbiota of *carpaccio* for the treatment combinations are shown in Table 2. It must be noted that, counts for mesophilic, LAB, and most counts for psychrotrophs of *carpaccio* samples treated at 400 or 600 MPa were below the detection limit of 2 log CFU/g. Enterobacteriaceae was not detected as part of the indigenous microbiota of *carpaccio* in this study.

The analysis of the spoilage microorganisms of *carpaccio* showed that, for unpressurized samples (0.1 MPa), mesophilic and LAB counts were higher in fresh samples than in frozen ones. In the case of frozen samples, the addition of NaL had the effect of significantly ($p < 0.05$) diminishing the counts of mesophilic, psychrotrophic and lactic-acid bacteria before pressure was applied (0.1 MPa).

HPP at 400 or 600 MPa was effective to reduce mesophilic and LAB counts bellow the detection limit, in fresh and frozen samples, with or without NaL. The addition of NaL at 1 or 3%, reduced psychrotrophic counts of frozen samples below detection limits when pressure (400 or 600 MPa) was applied.

Logarithmic reductions for STEC O157 in *carpaccio* samples were calculated, for each level of NaL, based on the differences between counts in TSAYEP in pressurized samples and in the non-pressurized control (Table 3).

The increase in pressure level from 400 to 600 MPa was effective to enhance the lethality, in most of the cases in a statistically significant degree ($p < 0.05$). A higher lethality of STEC O157 was observed in frozen samples than in fresh ones. The highest reductions were obtained in frozen samples: 2.01 log (600 MPa–1% NaL) and 1.98 log (600 MPa–0% NaL). Frozen samples with 3% NaL, processed at 600 MPa, presented a lower lethality than those samples with 1% NaL or without it. For fresh samples the highest reductions were 1.19 log (600 MPa–0% NaL) and 0.97 log (600 MPa–3% NaL).

TSAYEP was used as reference medium for maximum recovery of bacterial cells, while STEC O157 counts were also carried out in SMAC, and O157:H7 ID as both media are used for selection, identification, and enumeration of STEC O157 in foods analyses. A degree of injury, for each treatment combination, was estimated from the differences in counts between the non-selective medium and the selective media (Table 4). Counts in O157:H7 ID were in general lower than in SMAC, thus higher logarithmic reductions and injury were found in this medium.

Counts in SMAC and O157:H7 ID showed that, for each NaL concentration, injury was in general significantly increased ($p < 0.05$) when pressure increased, both in fresh as in frozen samples. The highest degree of injury was observed at the 600 MPa–0% NaL treatment in fresh samples: 2.27 log of injured cells in SMAC, and 2.69 log in O157:H7 ID equivalent to a 98.6% and 99.5% of injured cells respectively.

It must be noted, that SMAC and O157:H7 ID media were inhibitory to STEC O157 cells even when no pressure was applied (0.1 MPa), in that case a tendency was observed to lower injury in samples with 3% in comparison to 1% NaL. Also in fresh samples treated at 600 MPa there was a significant ($p < 0.05$) injury decrease with 3% NaL. In pressure-treated frozen samples, the concentration of NaL did not change significantly the degree of injury.

Table 1
Microbial counts (log CFU/cm^2) in bovine *Semitendinosus* muscle during the curing process for *carpaccio* elaboration.

Time of storage (days)	Sodium Lactate (%)	Mesophiles	Psychrotrophs	Lactic acid bacteria	Enterobacteriaceae	STEC O157	
						SMAC	O157:H7 ID
1	0	2.70 (0.16)^{ns}	3.44 (0.16)^{ns}	3.13 (0.07)^{a}	ND*	4.80 (0.08)^{a}	4.15 (0.40)^{b}
	1	2.66 (0.05)^{ns}	3.24 (0.26)^{ns}	2.81 (0.26)^{a}	ND	4.94 (0.05)^{a}	4.87 (0.03)^{a}
	3	2.57 (0.03)^{ns}	3.30 (0.02)^{ns}	2.07 (0.43)^{b}	ND	4.21 (0.30)^{b}	4.75 (0.17)^{a}
12	0	5.83 (0.51)^{a}	7.02 (0.52)^{a}	5.73 (0.18)^{a}	ND	4.92 (0.13)^{a}	4.99 (0.07)^{ns}
	1	5.00 (0.53)^{a}	3.79 (0.01)^{b}	4.13 (0.12)^{b}	ND	4.75 (0.06)^{a}	4.61 (0.31)^{ns}
	3	3.21 (0.08)^{b}	3.09 (0.08)^{c}	4.12 (0.99)^{b}	ND	4.08 (0.16)^{b}	4.56 (0.22)^{ns}

Values expressed as mean and standard deviation of three replicates.
^{abc}For each time of storage, means in the same column with different superscripts differ significantly ($p < 0.05$) according to LSD test.
^{ns}Not significant.
*ND: not detected, counts were below the limit of detection of 2 log CFU/cm^2.

3.3. Effect of HPP and NaL combinations on water activity and pH of carpaccio samples

Table 5 shows the a_w and pH values of *carpaccio* for the NaL and HPP combinations assayed. Regardless of the pressure applied, the addition of 3% NaL significantly reduced a_w ($p < 0.05$), in fresh and frozen *carpaccio* as could be expected from the water-binding capacity of the NaL salt. Application of pressure at 400 and 600 MPa resulted in significantly higher pH values ($p < 0.05$) in frozen *carpaccio*, with or without added NaL. Likewise, in fresh *carpaccio* a similar trend was observed. This increase in pH of pressurized samples could be explained by a loss of free protons due to a redistribution of ions facilitated by the increased ionization that occurs at elevated pressures (Macfarlane et al., 1984).

Table 2
Microbial counts (log CFU/g) of spoilage microbiota of *carpaccio* samples under different treatment combinations.

Condition	Pressure (MPa)	Microbial counts	Sodium Lactate		
			0%	1%	3%
Fresh	0.1	Mesophiles	6.04 (0.08)^{a}	5.65 (0.51)^{b}	5.88 (0.31)^{ab}
		Psychrotrophs	6.12 (0.09)^{ns}	5.63 (0.17)^{ns}	5.86 (0.33)^{ns}
		LAB	6.04 (0.07)^{a}	5.49 (0.06)^{c}	5.82 (0.16)^{b}
		Enterobacteriaceae	ND*	ND	ND
	400	Mesophiles	ND	ND	ND
		Psychrotrophs	ND	ND	ND
		LAB	ND	ND	ND
		Enterobacteriaceae	ND	ND	ND
	600	Mesophiles	ND	ND	ND
		Psychrotrophs	ND	ND	ND
		LAB	ND	ND	ND
		Enterobacteriaceae	ND	ND	ND
Frozen	0.1	Mesophiles	4.91 (0.48)^{a}	4.25 (0.25)^{b}	3.25 (0.36)^{c}
		Psychrotrophs	6.13 (0.07)^{Aa}	4.63 (0.37)^{b}	3.96 (0.04)^{b}
		LAB	4.78 (0.15)^{a}	4.26 (0.28)^{b}	3.39 (0.04)^{c}
		Enterobacteriaceae	ND	ND	ND
	400	Mesophiles	ND	ND	ND
		Psychrotrophs	3.49 (0.35)^{B}	ND	ND
		LAB	ND	ND	ND
		Enterobacteriaceae	ND	ND	ND
	600	Mesophiles	ND	ND	ND
		Psychrotrophs	3.77 (0.54)^{B}	ND	ND
		LAB	ND	ND	ND
		Enterobacteriaceae	ND	ND	ND

Values expressed as mean and standard deviation of four replicates.
*ND: not detected, counts were below the limit of detection of 2 log CFU/cm^2.
^{a,b,c}For each condition, means in the same row with different lowercase letters differ significantly ($p < 0.05$) according to LSD test.
^{AB}For frozen condition, means in the same column with different uppercase letters differ significantly ($p < 0.05$) according to LSD test.
^{ns}Not significant.

On the other hand, pH values of fresh *carpaccio* showed an increase ($p < 0.05$) with 1% NaL, and a further decrease ($p < 0.05$) with 3% NaL, when compared to control samples (0% NaL). In agreement, Byrne et al. (2002) have reported that 4% NaL in beefburger patties resulted in a significant reduction in the mean pH values.

4. Discussion

The aim of this study was to assess the effect of several high-pressure and NaL combinations on STEC O157, and on the spoilage microbiota of cured beef *carpaccio* immediately after processing. A pool of native STEC O157 strains was employed to challenge the HPP treatments applied to fresh and frozen *carpaccio* manufactured with bovine *Semitendinosus* muscles cured with different formulations that included NaL. For the purpose of the challenge a high inoculum of STEC O157 (*ca.* 10^6 CFU) was used. Although, this condition is most unlikely to occur in naturally contaminated foods, it was selected in order to compare the reductions obtained by the different treatments.

The curing process of SM was carried out in vacuum packages during 12 days at 1 ± 1 °C. These conditions were chosen as they were previously found to ensure the homogenous distribution of the curing salts through the muscles (Szerman et al., 2011), and could minimize the chance of pathogen growth. The observed growth of mesopohilic, psycrotrohic and LAB was compatible with those environmental conditions. Only for psychrotrophic counts, with no added NaL, counts exceeded 7 log CFU/cm^2 after 12 days of storage.

Lactate salts are among GRAS additives, which have been effectively used to extend the shelf life of meat products, through its ability of increasing lag times, mainly of gram positive bacteria (De Wit and Rombouts, 1990). Lactate–diacetate salts have also been used to increase the shelf life in cooked ham treated by HPP (Marcos et al., 2008). In this study, the addition of NaL in the curing

Table 3
Microbial counts (log CFU/g) of STEC O157 in *carpaccio* samples under different treatment combinations on TSAYEP medium.

Condition	Pressure (MPa)	Sodium Lactate		
		0%	1%	3%
Fresh	0.1	6.98 (0.05)^{abc}	7.17 (0.17)^{a}	7.06 (0.17)^{ab}
	400	6.48 (0.05)^{d}	6.90 (0.06)^{abc}	6.79 (0.13)^{bc}
	600	5.79 (0.39)^{f}	6.70 (0.10)^{cd}	6.09 (0.28)^{e}
Frozen	0.1	7.11 (0.13)^{a}	7.11 (0.03)^{a}	7.07 (0.07)^{a}
	400	5.95 (0.10)^{b}	5.56 (0.08)^{d}	5.76 (0.19)^{c}
	600	5.13 (0.12)^{e}	5.10 (0.11)^{e}	5.63 (0.20)^{cd}

Values expressed as mean and standard deviation of four replicates.
^{abcdef}For each condition, means with different superscripts differ significantly ($p < 0.05$) according to LSD test.

Table 4
Microbial counts (log CFU/g) of STEC O157 in *carpaccio* samples under different treatment combinations on SMAC and O157:H7 ID media.

Condition	Pressure (MPa)	Sodium Lactate					
		0%	1%	3%	0%	1%	3%
		SMAC			O157:H7 ID		
Fresh	0.1	6.85 (0.15)^{ab}	6.93 (0.29)^{a}	7.02 (0.18)^{a}	6.66 (0.09)^{ab}	6.49 (0.40)^{ab}	6.90 (0.09)^{a}
	400	5.87 (0.11)^{d}	6.53 (0.06)^{cd}	6.44 (0.42)^{c}	5.22 (0.47)^{de}	6.19 (0.13)^{bc}	5.65 (0.12)^{cd}
	600	3.52 (0.32)^{f}	5.30 (0.18)^{e}	5.52 (0.06)^{e}	3.09 (0.83)^{g}	4.19 (0.61)^{f}	4.98 (0.25)^{e}
Frozen	0.1	7.04 (0.08)^{a}	7.08 (0.10)^{a}	7.07 (0.07)^{a}	6.96 (0.18)^{a}	6.87 (0.10)^{a}	6.99 (0.08)^{a}
	400	5.67 (0.15)^{b}	5.19 (0.05)^{c}	5.48 (0.22)^{b}	5.49 (0.29)^{b}	5.04 (0.34)^{bc}	5.48 (0.71)^{b}
	600	4.79 (0.21)^{d}	4.66 (0.45)^{d}	5.14 (0.10)^{c}	4.80 (0.22)^{cd}	4.38 (0.49)^{d}	4.93 (0.17)^{c}

Values expressed as mean and standard deviation of four replicates.
^{abcdefg}For each condition, means with different superscripts differ significantly ($p < 0.05$) according to LSD test.

mixture was effective to reduce the microbial load of spoilage microorganisms at the end of the curing process.

STEC O157 was not expected to growth under the curing process conditions (1 ± 1 °C). In contrast, a trend to diminish STEC O157 counts was observed with the addition of NaL. Lactate salts have been reported to provide another hurdle to increase food safety in ground beef by diminishing the probability of growth of STEC O157 when stored at 10 °C (Hwang and Juneja, 2011). Other authors (Byrne et al., 2002) have reported that the addition of 4% NaL acted synergistically with frozen storage in reducing STEC O157 by 0.5 log in frozen patties before heat processing.

The results of the curing assay verified that, if beef becomes contaminated with STEC O157 at the abattoir, it could survive the curing process with a risk of contaminating *carpaccio* with STEC O157 strains from the animal reservoir. The curing assay also showed that, the addition of NaL was able to decrease, even in a small degree, STEC O157 counts aiding to the safety of the product.

HPP at constant pressure in the range of 300–600 MPa (industrial scale) for short times (<10 min) at moderate temperatures are an alternative to inactivate vegetative microorganisms and enzymes, without significantly modifying the nutritional properties and sensory attributes of the food. However, in the case of fresh beef, and fresh-marinated/cured beef, HPP technology has not been successful because of the discoloration that occurs in the range of pressures required to inactivate pathogenic and spoilage vegetative microorganisms (i.e., higher than 300 MPa). The addition of antioxidant compounds, such as sodium nitrite (Carlez et al., 1995), and the application of HPP at subzero temperatures (Fernández et al., 2007; Vaudagna et al., 2012), have been some of the alternatives evaluated to minimize the effect on beef color. HPP at subzero temperatures can minimize the effect of pressure on the chromatic parameters, but it is not as effective to reduce the spoilage microorganisms as HPP at room, or refrigeration temperatures (Szerman et al., 2011; Vaudagna et al., 2012). In addition, subzero temperatures are not attainable in an industrial scale HPP system. Alternatively, in the present study *carpaccio* was HPP treated at 5 °C under two conditions (frozen or fresh) in order to compare its effect on the microbiota and to minimize sensory alterations. Under these conditions, a visual inspection was carried on the appearance of *carpaccio* samples. For the frozen condition there were no evident differences in color between unpressurized and pressurized samples. However, pressurized fresh *carpaccio* showed a noticeable discoloration through pressure processing.

In fresh *carpaccio*, the HPP treatments at 400 and 600 MPa were effective to inactivate all groups of spoilage microorganisms tested (mesophiles, psycrotrophs and LAB) from a maximum of 6 log CFU/g to below detection limits. HPP at 600 MPa has been successfully applied on marinated beef for extending its shelf life under refrigeration by several weeks (Garriga et al., 2004). This was achieved by an immediate decrease in microbial counts of aerobic total, psycrotrophs and LAB from 6 log CFU/g to less than 2 log CFU/g. In the present study, a similar degree of reductions was achieved at 400 MPa and at 600 MPa, which indicates that shelf life would be also extended.

Addition of NaL in the curing mixtures reduced the initial load of the spoilage microbiota of frozen *carpaccio* before the HPP (0.1 MPa), and increased the lethal effect of HPP on psychrotrophs in the frozen samples. The spoilage microbiota of *carpaccio* was mostly originated from microorganisms that had grown during the curing process, and were transferred to *carpaccio* surface during the slicing of the frozen SM. *Carpaccio* slices were then kept under refrigeration, or frozen, before HPP was applied. Differences in initial microbial counts, between fresh and frozen *carpaccio* microbiota, may be due to some inactivation by freezing/thawing of *carpaccio* slices, and to some growth that may occur in fresh slices before HPP.

Table 5
Water activity and pH of *carpaccio* samples under different treatments combinations.

Condition	Pressure (MPa)	Sodium Lactate					
		0%	1%	3%	0%	1%	3%
		a_w			pH		
Fresh	0.1	0.987 (0.001)^{bc}	0.993 (0.001)^{a}	0.974 (0.001)^{de}	5.96 (0.04)^{c}	6.32 (0.03)^{a}	5.58 (0.09)^{d}
	400	0.991 (0.001)^{ab}	0.987 (0.001)^{c}	0.972 (0.002)^{e}	6.12 (0.06)^{b}	6.41 (0.07)^{a}	5.69 (0.10)^{d}
	600	0.992 (0.001)^{a}	0.989 (0.001)^{abc}	0.977 (0.001)^{d}	6.10 (0.11)^{b}	6.44 (0.02)^{a}	5.71 (0.01)^{d}
Frozen	0.1	0.998 (0.001)^{a}	0.988 (0.005)^{ab}	0.979 (0.006)^{bc}	5.93 (0.02)^{b}	5.95 (0.04)^{b}	5.97 (0.09)^{b}
	400	0.997 (0.001)^{a}	0.996 (0.001)^{a}	0.968 (0.001)^{c}	6.07 (0.05)^{a}	6.05 (0.09)^{a}	6.04 (0.05)^{a}
	600	0.995 (0.001)^{a}	0.992 (0.002)^{a}	0.978 (0.001)^{bc}	6.07 (0.08)^{a}	6.08 (0.02)^{a}	6.09 (0.02)^{a}

Values expressed as mean and standard deviation of two replicates.
^{abcd}For each condition, means with different superscripts differ significantly ($p < 0.05$) according to LSD test.

STEC O157 strains used in this study were isolated from cow carcasses, and heifer feces in beef abattoirs of Argentina (Masana et al., 2010). These strains were characterized as Shiga toxin 2 producers, with $stx_2 - stx_{2c\ (vh\text{-}a)}$/*eae*/*ehx*A genotype which is the predominant genotype isolated from cattle, and from human infections in Argentina (D'Astek et al., 2012). A preliminary trial showed that these strains were able to withstand 400 MPa for 5 min in PBS with a reduction of less than 2.5 log (data not shown).

Under the conditions of this study, the lethality on STEC O157 increased as the pressure increased for each treatment combination, being the maximum inactivation achieved of two logarithmic cycles in the frozen *carpaccio* treated at 600 MPa (0% or 1% NaL). The comparison of inactivation rates among different HPP studies is difficult because of variations in strain resistance, food composition, processing conditions, and microbial analyses of samples. STEC strains have shown a high variability with respect to its resistance to HPP, with some of the most resistant strains being STEC O157 isolates from food outbreaks. Some of those STEC O157 strains were able to survive treatments at 500 or 600 MPa for more than 5 min, with a limited decrease in viable counts (Benito et al., 1999; Whitney et al., 2007; Alvarez-Ordóñez et al., 2013).

Nevertheless, the lethality values found in this work can be contrasted to STEC resistance reported in different meat products. In dry-cured ham, an immediate 1.81 log reduction for STEC O157 was reported at 500 MPa for 10 min at 12 °C (De Alba et al., 2013). In dry-fermented sausage processed at 600 MPa for 10 min at 25 °C, almost 3 log reductions were reported for a STEC O103:H25 strain (Omer et al., 2010). Higher inactivation rates, with reductions of 3 log CFU/g and 1 log CFU/g at day 0, were reported in ground beef processed at 400 MPa for 10 min at 20 °C and at −5 °C respectively (Black et al., 2010).

SMAC and O157:H7 ID media are commonly used for isolation and enumeration of STEC O157 in different types of foods, and environmental samples. In this study we report the use of O157 ID and SMAC media to assess the degree of injury on STEC O157 cells by the effect of HPP. Both media contain bile salts, at the same concentration (1.5 g/l), that are known to impede the recovery of injured cells with damage in their outer membrane (Espina et al., 2010). In comparison, it was observed a tendency to obtain lower counts on O157:H7 ID than in SMAC for the same treatment. The results showed that their use to enumerate STEC O157 in HPP foods could lead to an underestimation of the real contamination, with a risk that injured cells could recover viability, given a situation of temperature abuse.

The effect of HPP on the inactivation of bacteria depends on the processing temperature, among others factors. In this study, the temperature of the compression fluid applied during HPP of frozen and fresh *carpaccio* was the same (5 °C). However, a higher lethality was observed in frozen *carpaccio* than in fresh samples. On the contrary, injury was higher in fresh *carpaccio*, than in frozen samples. Hence, injured cells may have been further stressed by freezing, resulting in a higher lethality. The combined effect of HPP and freezing has been reported during storage of HPP processed foods (Black et al., 2010).

In this study, the effect of NaL on STEC O157 inactivation was dependent on the *carpaccio* condition, and the pressure level applied. In fresh *carpaccio*, the addition of NaL does not seem to modify significantly the lethality values. In comparison, in thermal processing, Huang and Juneja (2003) reported that concentrations of NaL up to 4.5% in ground beef did not affect the thermal resistance of STEC O157 cells. However, in frozen *carpaccio* treated at 600 MPa, the addition of NaL at 3% significantly reduced ($p < 0.05$) the lethality on STEC O157. For fresh *carpaccio* treated at 600 MPa the addition of 3% NaL also increased STEC O157 counts on SMAC and on O157:H7 ID showing a protective effect of NaL on injury.

As seen in Table 5 the addition of 3% NaL was able to significantly diminish the a_w of the manufactured *carpaccio*. Some authors have also reported that lowering the a_w of foods, such as cooked ham (Jofre et al., 2008) can decrease the inactivation of microorganisms by HPP. Likewise, a screening study that tested the effect of different additives in HPP in PBS, showed a protective effect of NaL (1%) in *Salmonella enteritidis* inactivation at 250 MPa at 25 °C (Ogihara et al., 2009).

5. Conclusions

This study is a first contribution to examine the effectiveness of HPP to inactivate STEC O157 strains, isolated from the bovine reservoir of Argentina, in a "ready-to-eat" meat product. The results showed that STEC O157 native strains have a degree of baroresistance consistent with other reports in the literature. The maximum lethality achieved immediately after HPP was equal to 2 logarithmic reductions in frozen *carpaccio* treated at 600 MPa for 5 min. Lethality of HPP treatments on STEC O157 was enhanced by the combination with freezing, while the addition of NaL at 3% could provide some protective action by reducing the number of injured cells. None of the high-pressure combined treatments tested was able to attain an immediate reduction of STEC O157 counts enough to ensure the safety of *carpaccio*. This fact validates the need to search for extra hurdles to obtain higher safety levels.

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