Food Microbiology 41 (2014) 27–32

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Combined treatments of high-pressure with the lactoperoxidase system or lactoferrin on the inactivation of *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* O157:H7 in beef carpaccio

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ARTICLE INFO

Article history:
Received 13 May 2013
Received in revised form
23 December 2013
Accepted 4 January 2014
Available online 25 January 2014

Keywords:
Listeria monocytogenes
Salmonella Enteritidis
E. coli O157:H7
Beef carpaccio
High pressure
Lactoperoxidase system
Lactoferrin

ABSTRACT

The effect of high hydrostatic pressure (HHP) treatments in combination with the lactoperoxidase system (LPOS) or activated lactoferrin (ALF) on *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Escherichia coli* O157:H7 was investigated in cured beef carpaccio stored at 8 °C or 22 °C during 7 d. HHP (450 MPa for 5 min) reduced pathogen levels by 1–3 log units and the antimicrobial effect remained during 7 d of storage under temperature abuse conditions at 8 °C and at 22 °C. The individual application of LPOS and ALF did not affect the survival of the three pathogens studied during storage. However, a synergistic bactericidal interaction between LPOS and HHP was observed against *S.* Enteritidis and *E. coli* O157:H7. Combined treatments of HHP with LPOS would be useful to reduce the intensity of pressurization treatments diminishing changes in the quality of meat products.

1. Introduction

Beef carpaccio is a ready to eat product (RTE) that can be contaminated with foodborne pathogens during its processing. Raw or cured pieces of meat usually frozen are sliced, packaged under vacuum or modified atmospheres and marketed at refrigeration temperature. The risk of the product increases when stored at temperature abuse conditions. *Listeria monocytogenes* can persist in the processing environment adhered to stainless steel surfaces which act as reservoir of contamination. *Salmonella* is found in the environment and in the gastrointestinal tract of farmed and wild animals. *Escherichia coli* O157:H7, a verocytotoxin producing *E. coli* (VTEC), associated with both outbreaks and sporadic cases, can be shed in animal faeces and contaminate the surfaces of raw meat during the slaughter, dressing and packaging. The incidence of reported foodborne illness for *L. monocytogenes* and VTEC decreased, whereas for salmonellosis increased since 2006–2008 in the United States (CDC, 2011). In the European Union, cases attributed to *L. monocytogenes* and *Salmonella* have decreased during the last years, but VTEC infections have increased since 2008 (EFSA and ECDC, 2013). RTE meat products stored at temperature abuse conditions can result in spoiled or unsafe food relatively early due to faster multiplication of microorganisms. According to Pal et al. (2008) in the event of contamination of deli meat and poultry food with *L. monocytogenes* even with a lowest detectable level, the pathogen was able to grow especially at 8 and 12 °C.

High hydrostatic pressure (HHP) is a non-thermal technology applied to increase the microbial safety and prolong the shelf-life of foods. In RTE meat products, HHP as a post-processing treatment after packaging provides an additional barrier to assure microbiological safety (Rendueles et al., 2011), and also is considered a novel strategy to alter the structure and quality parameters of meat and meat products (Bajovic et al., 2012). Reductions of *S.* Enteritidis of 3.7–5.9 log units in beef carpaccio pressurized at 450 MPa for 5 and 10 min, respectively, were reported by De Alba et al. (2012), although changes in colour were detected at longer pressurization times. In general, HHP induced colour changes are more intense in fresh red meat than in white meat and cured meat products (Bajovic et al., 2012), that in carpaccio could be attributed to the protective action of nitrite (Szerman et al., 2011; De Alba et al., 2012).

High pressures combined with lysozyme or with some bacteriocins exhibited a synergistic antimicrobial effect against pathogens (Hauben et al., 1996), and were considered a useful strategy to

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http://dx.doi.org/10.1016/j.fm.2014.01.010

reduce intensity of pressurization treatments. The combination of pressurization with other biopreservatives as the lactoperoxidase system (LPOS) or lactoferrin in meat products has not been thoroughly studied. Lactoperoxidase catalyses the oxidation of thiocyanate (SCN^-) by hydrogen peroxide (H_2O_2) to hypothiocyanite ($OSCN^-$), an unstable antimicrobial compound that oxidize exposed sulfhydryl group of enzymes and proteins in the bacterial cell membrane, interfering the transport of nutrients, the DNA and RNA synthesis and the respiratory chain (Pruitt and Reiter, 1985). LPOS has been approved as processing aid for meat and meat products by the Food Standards Australia New Zealand (FSANZ, 2002) and proposed by FAO/WHO (2005) for preventing the microbial growth in milk. Lactoferrin is a single-chain iron-binding glycoprotein that constitutes one of the major antimicrobial systems in milk. Antimicrobial activity is due to the iron-binding properties of the protein. Activated lactoferrin (ALF) is an immobilized lactoferrin patented by Naidu (2001).

The aim of this study was to evaluate the effect of HHP combined with the LPOS or ALF on the survival of inoculated *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 on cured beef carpaccio stored under temperature abuse conditions at 8 °C and at 22 °C during 7 days.

2. Materials and methods

2.1. Microorganisms

L. monocytogenes INIA H66a (from the INIA Culture Collection, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain), *Salmonella enterica* subsp. *enterica* serovar Enteritidis strains CECT 4155, CECT 4300, and CECT 4396, and *E. coli* O157:H7 CECT 4972 (from the Spanish Type Culture Collection, Valencia, Spain) were used. The strains were kept frozen at −80 °C in trypticase soy yeast extract broth (TSYEB) (Biolife, Milano, Italy) with 30% glycerol. Samples were inoculated with an over-night culture grown in TSYEB at 37 °C for 18 h.

2.2. Preparation of samples

Cured beef carpaccio was purchased from a retail market in Madrid (Spain) and kept frozen until used. Slices were aseptically cut into 10 g pieces 2 mm thick and inoculated by spreading 100 μL of diluted over-night cultures of *L. monocytogenes*, *S.* Enteritidis or *E. coli* O157:H7. Inoculated samples were individually vacuum-packed in double bags of BB325 (Cryovac Sealed Air Corporation, Milan, Italy) and held at 4 °C for 18 h until applying the individual or combined treatments.

2.3. HHP processing

High-pressure treatments were performed in a prototype ACIP 6000 (ACB, Nantes, France) of 3.5 L capacity and 600 MPa maximum working pressure. Water was used as pressure transmitting medium. Samples were pressurized at 450 MPa for 5 min at 12 °C. All samples were stored under refrigeration at 8 °C and 22 °C during 7 days. Two independent trials were carried out.

2.4. Application of lactoperoxidase system and activated lactoferrin

Lactoperoxidase from bovine milk (DMV International, Veghel, Holland) was prepared in distilled-deionized water (15 mg/ml) sterilized with 0.22 μm pore size cellulose acetate filters (Millipore, Bedford, MA) and stored at −40 °C. The oxidation of ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (Sigma–Aldrich, Alcobendas, Spain) was used to measure lactoperoxidase activity at 412 nm that was expressed in ABTSU (Shindler et al., 1976). The lactoperoxidase system was activated by adding 2.8 ABTSU/g, 0.03 mg/g potassium thiocyanate (KSCN, Merck, Darmstadt, Germany), 0.16 mg/g glucose and 0.15 U/g glucose oxidase. Activated lactoferrin (ALF) obtained from DMV International was prepared at 20 mg/ml in double distilled water, sterilized with 0.22 μm pore size cellulose acetate filters and stored at −40 °C until used. Inoculated carpaccio samples were treated with 1 mg/g. Antimicrobials were applied by spreading on the carpaccio surface.

2.5. Microbiological analysis

Samples (10 g) were transferred aseptically to a sterile stomacher bags, diluted 10-fold with sterile 0.1% (wt/vol) peptone water solution and homogenized for 90 s in a stomacher 400 (A. J. Seward Ltd., London, UK). Decimal dilutions were prepared in the same solution and spread on duplicate plates. Chromagar Listeria (CHROMagar, Paris, France) for *Listeria*, Salmonella Shigella agar (SSA, Scharlab S.L., Barcelona, Spain) for *Salmonella* and Violet Red Bile agar (VRBA, Oxoid LTD., Basingstoke, Hampshire, England) for *E. coli* O157:H7 were incubated at 37 °C for 24 h.

2.6. Statistical analysis

Data were subjected to analysis of variance (ANOVA) of SPSS program Win 12.0 software (SPSS Inc., Chicago, IL). Significant differences between means were assessed by the Tukey's test ($P < 0.05$).

3. Results

3.1. Effect of treatments on L. monocytogenes population

Survival of *L. monocytogenes* in cured beef carpaccio subjected to HHP at 450 MPa for 5 min, LPOS or ALF and their combinations, and stored at 8 or 22 °C is shown in Fig. 1. Immediately after treatments, pressurization led to mean reductions of 1.1 log cfu/g, whereas no antimicrobial activity was observed when LPOS or ALF were added to carpaccio. Reductions of *L. monocytogenes* achieved by combined treatments of HHP with LPOS or ALF were 1.4 and 1.0 log units, respectively. During the refrigerated storage, *L. monocytogenes* did not grow in control carpaccio during 7 d at 8 °C, with a decrease of 0.2 log cfu/g. Reductions of 1.7 log cfu/g were obtained in pressurized carpaccio, which attained 2.4 and 1.9 log units when HHP and LPOS or ALF were combined, respectively. After 7 d at 22 °C, reduction in counts of *L. monocytogenes* was 2.4 log cfu/g in samples treated by HHP. Counts in carpaccio pressurized in presence of LPOS or ALF were 2.9 and 1.8 log units lower than in control carpaccio. However, no significant differences in *L. monocytogenes* levels were detected between non-pressurized, LPOS or ALF treated carpaccio throughout storage at 8 or 22 °C.

3.2. Effect of treatments on S. Enteritidis population

S. Enteritidis counts in cured beef carpaccio subjected to HHP at 450 MPa, LPOS, ALF or their combinations and stored at 8 or 22 °C are shown in Fig. 2. After treatment, the reduction in the pathogen counts was 2.7 log cfu/g in carpaccio pressurized at 450 MPa for 5 min. Differences in *S.* Enteritidis counts in non-pressurized carpaccio and in samples treated with LPOS or ALF were not significant. When HHP was combined with LPOS or ALF, the inactivation observed for *S.* Enteritidis was 3.6 and 2.7 log units. At the end of the 7 d of refrigeration at 8 °C, counts of *S.* Enteritidis decreased by 0.9 log cfu/g in non-pressurized samples and 3.7 log cfu/g in pressurized samples. The inactivation was higher