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Potentially active spoilage bacteria community during the storage of vacuum packaged beefsteaks treated with aqueous ozone and electrolyzed water

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ABSTRACT

The microbial contamination that occurs during the slaughtering process and during handling of the meat results in a shortening of the shelf-life of meat. In this study, which has had the aim of extending the shelf life of beefsteaks, pilot-scale treatments were carried out with aqueous ozone (AO) and electrolyzed water (EW) before vacuum packaging (VP). The development of the potentially active microbiota and the associated volatilome were followed over 15 days of storage under refrigerated conditions (4 °C), in order to define the potential long-term effects of the treatments and storage condition on microbiota.

The targeted RNA-based amplicon sequencing identified *Pseudomonas fragi* as the most frequent species before and after the treatments with AO and EW, as well as in the untreated control. The tested treatments did not reduce the overall presence of this species, but they affected the intra-species distribution of its oligotypes, albeit slightly. With the progression of the refrigerated storage and the reduction of the oxygen availability, *Lactobacillus sakei*, *Leuconostoc gasicomitatum* and *Lactococcus piscium* became the dominant, potentially active, beef microbiota, as confirmed by microbiological data. When the OTU abundances and volatilome were coupled, a significant association was observed between the organic acids, esters and aldehydes and these lactic acid bacteria species.

In spite of the limited effectiveness of the treatments over the short and long term, this study has provided a detailed view of beef spoilage using RNA as the sequencing target, strengthening and confirming the current knowledge based on DNA-amplicon sequencing.

1. Introduction

Apart from abiotic factors (e.g. oxygen and UV radiation) and endogenous autolytic enzymatic reactions, the spoilage of meat is mainly caused by complex microbial dynamics that encompass heterogeneous bacterial taxa, of which the most common are *Pseudomonas* sp., *Enterobacteriaceae*, *Brochothrix thermosphacta* and psychrotrophic lactic acid bacteria (LAB), all of which are capable of surviving and proliferating in a cold environment (Doulgeraki et al., 2012a, 2012b; Doulgeraki et al., 2010; Ercolini et al., 2009; Pothakos et al., 2015). It is well known that several different bacterial groups contaminate meat during the slaughtering processes, although the complexity of the microbiota of meat is reduced when it is sold, due to the selective pressure determined by the storage temperature, the packaging atmospheres and the initial antimicrobial treatments (de Filippis et al., 2013; La Storia et al., 2012; Stellato et al., 2016). The main problem faced by the meat processing industry is the necessity of efficiently contrasting the development of species capable of producing the volatile organic compounds (VOCs) that are associated with unpleasant odors (Argyri et al., 2015; Casaburi et al., 2015; Casaburi et al., 2011).

Accordingly, the treatment of meat with adequate preservation technologies before the being packaged may represent a feasible solution to extend its shelf-life, and thus to avoid product losses. Several non-thermal treatments have been considered and developed for the sanitization of ready-to-eat portions of meat, and promising results have been achieved through the utilization of supercritical CO_2, gamma radiation, and ultraviolet light (Buckow et al., 2017; Jermann et al., 2015; Sommers et al., 2017). In this frame, low levels of aqueous ozone

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(AO) and electrolyzed water (EW) may represent economically convenient, environmentally friendly and safe approaches for the sanitization of meat at the end of the slaughtering process, as well as of the slaughter environments, since they are broad-spectrum disinfectants and leave the treated food free of residues. AO and EW have long been known to be detrimental to the bacterial cells that result from the destructive oxidation of membrane-bound respiratory enzymes and lipids, the perturbation of cellular electrical charge maintenance, proteins and peptidoglycan in spore coats and virus capsids (Huang et al., 2008; Miller et al., 2013; Veasey and Muriana, 2016). To date, the decontaminant efficacy of these oxidative agents has only been tested at low concentrations and at a pilot-scale level by spraying or dipping beef and poultry, without observing deterioration of the organoleptic characteristics due to lipid oxidation or irreversible color modification, while the viable counts of several microbial group have been found to be reduced (Duan et al., 2016; Kalchayanand et al., 2008; Pohlman et al., 2002; Veasey and Muriana, 2016). However, the studies carried out so far on beef sprayed with AO and/or EW have been limited to observing the microbial reduction after treatment or the decontaminating effect toward deliberately introduced pathogens, without considering the complex dynamics of spoilage microbiota and the associated volatilome that may develop after the treatments and during storage. So far, the treatment of other food has also followed similar approaches, with attention being focused only on the short term effect of AO and EW (Pinto et al., 2015; Segat et al., 2014). Only recently has the post-treatment effect of aqueous ozone been investigated on wine grapes by means of culture-independent techniques applied during winemaking, and a significant perturbation of the yeast population of the final wine volatilome has been revealed (Cravero et al., 2016).

Nowadays, such ecological studies, aimed at unraveling the composition and dynamics of food microbiota, cannot be dealt without the use of high-throughput amplicon target sequencing (HTS) approaches, which may be oriented either toward DNA or RNA to explain the total microbial community (Ercolini, 2013; Ferrocino and Cocolin, 2017; Li et al., 2016). Notably, RNA-based amplicon sequencing is susceptible to biases depending on PCR process and presence of rRNA beyond the life cycle of the cells (Rosselli et al., 2016). The decay of rRNA after bacterial death is not generally predictable (Ceuppens et al., 2014), however rRNA remains the most suitable target to detect microbial phylotypes with potentially metabolic activities in the food matrix (Yang et al., 2017). This approach may result in a more reliable correlation between taxa and volatile compounds related to meat spoilage (De Angelis et al., 2015; De Pasquale et al., 2016).

Therefore, the aim of this work was to investigate the effect of AO and EW treatments on the complexity and dynamics of the potential active microbiota of beefsteaks, and their associated volatilome, during storage at 4 °C and in vacuum packaging conditions.

2. Materials and methods

2.1. Treatments with aqueous (AO) ozone and electrolyzed water (EW)

The studied steaks, weighing about 200 g each, were obtained from three different batches of tender boneless beef, 24 h after slaughtering. Each batch of beefsteaks was divided equally into four parts (7 beefsteaks per part) and treated with (EW) electrolyzed water, (AO) aqueous ozone and (W) water, while a fourth untreated part was used as a control (C) (Fig. 1). The AO was produced using a C32-AG O_3 generator (De Nora S.P.A, Milano, Italia) equipped with an oxygen concentrator, with/which has a nominal production capacity of 32 g O_3/h, and using/considering pure oxygen as an/the input gas. The AO treatment was performed with water containing 6.00 ± 0.25 mg/L. EW was produced from salt (KCl) diluted in tap water using an Eva System 100 (De Nora S.P.A.). The system produced EW of approximately 4 g/L free chlorine, pH 9 and 1% residual KCl. The treatments were performed with diluted EW at 100 mg/L of free chloride. The water treated samples (W) were treated in the same way using the same time frame and the same type of water used to produce EW and AO, in order to highlight any effect due to the water itself without oxidizing agents. All the treatments were carried out by homogeneously spraying each side of the beefsteaks, placed on a still grid in a dedicated sanitized room of a local slaughterhouse, for 90 s (Cuneo, Italy). The spraying treatments were performed with a distance of 20 cm between the meat and nozzles, and pumping tap water at 4 °C at a constant flux. The treated beefsteaks (AO, EW, W) were left to dry for 20 min on the grids and, together with the untreated control beefsteaks (C), were packed singly in linear low-density polyethylene (LLDPE; oxygen transmission, 0.83 $cm^3 \cdot m^{-2} \cdot h^{-1}$ at 23 °C, 30 cm × 30 cm) and vacuum packed.

The samplings were performed before the treatments, for each treatment and each batch, on the first day and after 5, 9 and 15 days of storage at 4 °C.

2.2. Microbiological analysis

The packages were aseptically opened on each sampling day. Five surface portions of 1 cm^2 were cut from each side of the beefsteaks, using a sterile scalpel and a cork borer (about 10 g of meat each sample), and were homogenized in 90 mL of Ringer's solution (Oxoid, Basingstoke, Hampshire, UK) for 2 min using a Stomacher® 400 Circulator (LAB blender 400; PBI, Milan, Italy). Decimal dilutions were prepared, and aliquots of the appropriate dilutions were spread in triplicate on the following media: (i) plate count agar (PCA, Lab M, Heywood, Lancashire, UK) to establish the total aerobic bacteria incubated for 48 to 72 h at 30 °C; (ii) De Man Rogosa and Sharpe agar (MRS, LabM) to establish the total LAB population, incubated at 30 °C for 48 h; (iii) violet red bile agar (VRBGA, LabM) to establish the *Enterobacteriaceae*, incubated at 30 °C for 24–48 h; (iv) malt extract agar (MEA, LabM) plus tetracycline (0.05 g/L; Sigma-Aldrich, St. Louis, USA) to establish the yeasts and molds/molds incubated at 25 °C for five days. The results were calculated as the means of log colony forming units per cm^2 (log CFU/cm^2) of the beefsteak surface for the three batches (± standard error mean).

In parallel, two 25 cm^2 surface pieces (about 25 g, one for each beefsteak surface) were cut and minced, and the pH was measured with a pH-meter (Crison, Modena, Italy).

ANOVA (One way-Analysis of Variance), coupled with Tukey's post-hoc test and the Kruskal–Wallis non-parametric test, were used to assess the overall variation and differences between the multiple groups. Statistical analyses were performed with Statistica, ver. 7.0 (StatSoft Inc., Tulsa, OK, USA).

2.3. GC–MS analysis of the volatile compounds (VOCs)

Chemical analyses were performed before the treatment and after 1 and 15 days of storage. A static headspace solid-phase microextraction analysis was carried out as described by Argyri et al. (2015), with some minor modifications. Briefly, in parallel with the microbiological samplings, 3 g of surface pieces (one for each side of the beefsteak) were cut from the beefsteak and then cut into small pieces using a sterile knife. Then, 1 g of minced meat sample was placed in a 20 mL glass vial and mixed with 2 mL of 25% NaCl solution and 10 µL of internal standard (3-octanol, final concentration of 97 µg/kg).

After an equilibration time of 5 min at 40 °C, the extraction was performed, with stirring (250 rpm), adopting the same temperature for 30 min with a 50/30 µm DVB/CAR/PDMS fiber (Supelco, Milan, Italy) using an SPME autosampler (PAL System, CombiPAL, Switzerland). The fiber was desorbed at 260 °C for 1 min in splitless mode. A GC/MS analysis was performed with a Shimadzu GC-2010 gas chromatograph, equipped with a Shimadzu QP-2010 Plus quadruple mass spectrometer (Shimadzu Corporation, Kyoto, Japan) and a DB-WAXETR capillary column (30 m × 0.25 mm, 0.25 µm film thickness, J & W Scientific Inc., Folsom, CA). The carrier gas (He) flow rate was 1 mL/min. The