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Antimicrobial effect of different peroxyacetic acid and hydrogen peroxide formats against spores of *Clostridium estertheticum*

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ABSTRACT

"Blown pack" spoilage is primarily caused by *Clostridium estertheticum*. The primary source of contamination is probably pelts, faeces and soil during opening cuts and de-hiding. Peroxyacetic acid (POAA) based fogs are commonly included in an abattoir's routine cleaning process. Hydrogen peroxide (H_2O_2) is a powerful oxidizing agent that penetrates microbe cell walls causing cell death. In this study, we compared the ability of H_2O_2 and OXYSAN ZS (POAA containing 1-hydroxyethylidine-1,1-diphosphonic acid as a stabilizer) in different formats to inactivate *C. estertheticum* spores. Hydrogen peroxide treatment using Phytagel™ gel as carrier was effective on fleece against both naturally contaminating microflora and *C. estertheticum* spores. This is the first time an antimicrobial treatment has been shown to inactivate *C. estertheticum* spores on such a complex and highly contaminated matrix. Both H_2O_2 and OXYSAN ZS treatments inactivated *C. estertheticum* spores on stainless steel indicating their potential use as an in-plant decontamination procedure or inclusion in routine in-process cleaning.

1. Introduction

Premature spoilage of vacuum packaged red meat by psychrophilic and psychrotrophic *Clostridium* spp. can result in significant economic losses. At present, the meat processing industry relies on hygienic dressing to reduce this contamination, along with stringent management of the cold-chain (Mills, Donnison, & Brightwell, 2014).

Blown pack spoilage (BPS) of vacuum packaged chilled meat products is commonly attributed to psychrophilic clostridia, the main causative agent being *Clostridium estertheticum* (Collins, Rodrigues, Dainty, Edwards, & Roberts, 1992). BPS is usually associated with an unpleasant odour and pack distention due to the production of large volumes of gas (Adam, Flint, & Brightwell, 2010; Broda, DeLacy, Bell, Braggins, & Cook, 1996; Dainty, Edwards, & Hibbard, 1989).

The primary source of meat contamination by *C. estertheticum* spores is likely to be animal skins, faeces and soil during opening cuts and de-hiding (Brightwell, Broda, & Boerema, 2009). These spores are resistant to heat, freezing, many chemicals and harsh environments (Broda, 2007). The most common chemical interventions to control BPS in New Zealand are peroxyacetic acid (POAA) based fogs applied during an abattoir's routine boning room cleaning process. However, POAA-based carcass rinses have not been shown to be highly effective at reducing the onset of BPS in artificially inoculated vacuum-packed chilled beef (Boerema, Broda, Penney, & Brightwell, 2007).

Hydrogen peroxide (H_2O_2) is both colourless and odourless and has been shown to inactivate spores of bacteria and fungi (Hall, Otter, Chewins, & Wengenack, 2008; Rij & Forney, 1995; Rogers et al., 2005). It is a powerful oxidizing agent that penetrates microbe cell walls and causes cell death. Studies using hydrogen peroxide vapour systems have been shown to eliminate *C. difficile* from surfaces (100% reduction) (Barbut, Menuet, Verachten, & Girou, 2009; Boyce et al., 2008). Currently the use of hydrogen peroxide is permitted as a bleaching, washing and peeling agent for use in foods (FSIS Directive 7120.1 Rev. 46).

Previously we observed that hydrogen peroxide treatment effectively inactivated *C. estertheticum* spores suspended in an aqueous solution (unpublished). Biocidal activity of chemical antimicrobials can be enhanced by if used in a vapour phase or carried within a semi-solid carrier. This study looked at the feasibility and effectiveness (compared with a commercially available biocide, OXYSAN ZS) of using different formats (vapour and gel) of hydrogen peroxide as an antimicrobial against *C. estertheticum* spores on fleece and meat processing surfaces.

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2. Materials and methods

2.1. Bacterial culture, spore preparation, enumeration and PCR confirmation

C. estertheticum DSM 8809^T type strain was retrieved from −80 °C storage, grown and checked for purity on plates of Columbia blood agar (CBA; Oxoid CM331, Oxoid Ltd., Basingstoke, UK) containing 5% sheep blood. Spore suspensions of *C. estertheticum* were prepared as described by Adam (2012). Enumeration of spore suspensions and treatment "survivors" was determined from serial dilution of heat-treated (80 °C) samples prepared in Phosphate Buffered Saline (PBS) (pH 7.3, 0.01 M) or pre-reduced Maximum Recovery Diluent (MRD)(Fort Richard, Auckland). Duplicate volumes (0.1 mL) of spore suspensions were spread onto the surface of pre-reduced CBA plates (cooled in anaerobic chamber (Forma Scientific) operating with an anaerobic gas mixture of 10%, v/v H_2; 5%, v/v CO_2; 85%, v/v N_2), the plates were then incubated anaerobically in a refrigerated incubator at 10 °C for 21 days following which, the means of duplicate colony counts were calculated. Cold enrichment of *C. estertheticum* was in pre-reduced peptone yeast extract glucose starch (PYGS; Lund, Graham, George, & Brown, 1990) broth for 3 weeks at 10 °C, followed by plating on CBA as described above. Representative colonies identified as *C. estertheticum* were confirmed by the Real-Time PCR assay designed to detect *C. estertheticum*-like bacteria described by Brightwell and Clemens (2012). To confirm the presence of *C. estertheticum* in enrichment broths prior to plating, DNA was extracted from one mL of a PYGS enrichment using the HiPure DNA template kit (Roche Diagnostics) as described by Broda, Boerema, and Bell (2003). Each DNA template was then subjected to the *C. estertheticum* Real-Time PCR assay described above.

2.2. Antimicrobial treatment regimes

The individual OXYZAB ZS and hydrogen peroxide treatment regimes are outlined in Table 1.

2.3. OXYSAN ZS treatment

OXYSAN ZS treatment was carried out using a proprietary product, OXYSAN ZS (Ecolab, USA). Working solutions of 0.125% were prepared and tested using a POAA test kit (Ecolab). The active component in the 0.125% working solutions ranged between 183 and 199 ppm. OXYSAN ZS at working concentrations also contains HEPD (1-hydroxyethylidine-1,1-diphosphonic acid) at 7-24 ppm as a stabilising component.

2.3.1. Stainless steel (treatment 1)

C. estertheticum (DSM 8809^T) spore suspension was pipetted onto the surface of 6 small stainless steel coupons (12 mm diameter) (3.04 $Log_{10}CFU/cm^2$) and left to air-dry for approximately 1 h. Two additional coupons were set aside as un-inoculated controls and three inoculated coupons were not treated. The inoculated test coupons were sprayed with OXYSAN ZS (185 ppm) and left for 1 min. Coupons were cultured either by plate count or cold enrichment. For plate count, the bacteria were removed from the coupon by vortexing in 10 mL MRD before enumeration on CBA. For cold enrichment, the coupon was placed into pre-reduced PYGS for 3 weeks before assessing growth on CBA and by Real-Time PCR.

2.3.2. Fleece (treatment 2)

Fresh ovine fleece obtained from a local abattoir was cut into eight 2 × 5 cm sections (10 cm^2 surface area). Six of the fleece samples were each inoculated with *C. estertheticum* (3.49 $Log_{10}CFU/cm^2$). Two control fleece samples were inoculated with an equivalent volume of PBS. A spreader was used to work inoculum into the fleece and then the samples were left at room temperature for 10 min. A fresh preparation of OXYSAN ZS (199 ppm) was sprayed onto three of the spore-inoculated fleece samples and left at room temperature for 1 min. Treated fleece and control fleece were then transferred into a Whirlpak bag (SigmaAldrich, USA) with 100 mL of ice-cold PBS. Survivor bacteria were enumerated and representative colonies confirmed by Real-Time PCR.

Table 1
OXYSAN ZS and hydrogen peroxide treatment regimes performed on *C. estertheticum* spores to determine efficacy of antimicrobial activity.

Treatment	Conc.	Spore Count	Control	Test	Exp. time	Heat treatment	CBA plates	PYGS
Treatment 1. OXYSAN ZS spray on Stainless Steel coupons	183 ppm	3.04 $Log_{10}CFU/cm^2$	Uninoculated coupon Inoculated not treated coupons	OXYSAN ZS treated inoculated coupons	1 min	80 °C 10mins	Y 3 weeks 10 °C	Y 3 weeks 10 °c
Treatment 2. OXYSAN ZS spray on fleece	199 ppm	3.49 $Log_{10}CFU/cm^2$	PBS treated fleece Inoculated untreated fleece	OXYSAN ZS treated inoculated fleece	1 min	80 °C 10mins	Y 3 weeks 10 °C	N
Treatment 3. Bioquell HPV treatmentof stainless steel coupons	35%	2.36 $Log_{10}CFU/cm^2$	Uninoculated coupon (swabbed 0 h) Uninoculated treated coupons (swabbed 24 h) Uninoculated treated coupons (PYGS)	Inoculated treated coupons (swabbed 24 h) Inoculated treated coupons (PYGS)	150 min		Y 3 weeks 10 °C	Y 3 weeks 10°c
Treatment 4. H_2O_2 treatment of fleece in *Aloe Vera* as the carrier	70% gel in PBS	3.48 $Log_{10}CFU/cm^2$	PBS treatment of fleece uninoculated fleece 0% H202 (in gel) treatment of inoculated fleece	Inoculated fleece treated with H_2O_2 9%, 5%, 3%, 1.5% in aloe vera gel	10 min	60 °C 10 min	Y 2 + 7 weeks 10 °C	N
Treatment 5. H_2O_2 treatment of fleece in Phytagel™ as the carrier	0.7% gel in H_2O	3.48 $Log_{10}CFU/cm^2$	Uninoculated (and un-washed) fleece treated with 0% H_2O_2 in PBS Uninoculated (and un-washed) fleece treated with 15% H_2O_2 in PBS Inoculated (and un-washed) fleece treated with 0% H_2O_2 in Phytagel™ Inoculated (pre-washed) fleece treated with 0% H_2O_2 in Phytagel™	Inoculated (and un-washed) fleece treated with 15% H_2O_2 in PBS Inoculated (pre-washed) fleece treated with 15% H_2O_2 in Phytagel™	10 min	60 °C 10 min	Y 2 + 7 weeks 10 °C	N