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Effect of chitosan, and bacteriocin – Producing *Carnobacterium maltaromaticum* on survival of *Escherichia coli* and *Salmonella* Typhimurium on beef

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ABSTRACT

The aim of this study was to investigate the synergistic effect of chitosan and bacteriocins against *Escherichia coli* and *Salmonella* in media and in lean beef. The inhibitory effects of chitosan and bacteriocins against *E. coli* AW1.7 and *S. enterica* Typhimurium in media were determined by a critical dilution assay. The efficacy a bacteriocin-producing strain of *Carnobacterium maltaromaticum* and high molecular weight chitosan (HMWC) in inactivation of *E. coli* AW1.7 and *S.* Typhimurium was evaluated on beef. Current interventions applied in the beef industry, steaming coupled with lactic acid, were used as reference. HMWC demonstrated higher antibacterial activity than water soluble chitosan (WSC) or chitosan oligosaccharides (COS) in media, and the addition of partially purified bacteriocins from *C. maltaromaticum* UAL307 increased the activity of the chitosan *in vitro*. The hurdle combinations associated with HMWC inactivated *E. coli* AW1.7 and *S. enterica* Typhimurium more effectively on lean beef when compared to steam or steam coupled with lactic acid. When used on beef, addition of bacteriocins and chitosan did not increase the antibacterial efficacy. Cell counts of *S. enterica* were further reduced during storage in presence of *C. maltaromaticum* and chitosan; however, this decrease was not dependent on bacteriocin production. In conclusion, addition of chitosan alone or in combination with *C. maltaromaticum* UAL 307 as protective culture significantly reduces cell counts of *E. coli* and *Salmonella* on beef. Results will be useful to improve pathogen intervention treatments in beef processing.

1. Introduction

Salmonella enterica and virulent strains of *Escherichia coli*, especially Shiga-toxin producing *E. coli* (STEC), are foodborne zoonotic agents associated with outbreaks worldwide and pose a threat to public health (EFSA, 2010; Nguyen and Sperandio, 2012). Cattle are a main vehicle for transmission of STEC but they also transmit *Salmonella* (Nguyen and Sperandio, 2012; Wingstrand and Aabo, 2014). Contamination of muscle tissues occurs primarily with the dehiding and evisceration steps during the beef slaughter process (Aslam et al., 2004; Barkocy-Gallagher et al., 2001). In North America, beef carcasses are routinely decontaminated by pasteurization with steam or hot water, and by spraying with lactic acid and/or peroxyacetic acid (Gill, 2009). Despite multiple pathogen intervention technologies *E. coli* and *Salmonella* continue to cause outbreaks associated with beef (CDC, 2014). The continued presence of *Salmonella* and STEC on fresh beef may relate to recontamination of carcasses during handling and cutting (Gill, 2009), or to strain-to-strain variation of the resistance of *E. coli* and *Salmonella* to heat and acid (Dlusskaya et al., 2011; Foster, 2004; Liu et al., 2015, Mercer et al., 2017). The burden of foodborne disease caused by STEC and *Salmonella* necessitates novel tools to ensure the safety of beef and beef products.

Chitosan, poly-(β-(1 → 4)-glucosamine, is a partially or fully deacetylated derivative of chitin and exhibits antimicrobial activity when the amino group is protonated, *i.e.* at a pH below the pK_A of 6.2–7.0 (Devlieghere et al., 2004; Tsai and Su, 1999). The antimicrobial activity of chitosan relates to its polycationic properties, which enable electrostatic interactions with negatively charged structures of the cell envelope, including the cytoplasmic membrane and the lipopolysaccharide (LPS) in the outer membrane of Gram negative organisms (Devlieghere et al., 2004; Helander et al., 2001; Mellegård et al., 2011). Chitosan has GRAS approval in the U.S.A. (FDA, 2011) and is an effective preservative in meat or meat products when applied at concentrations of 1–10 g/L (Kanatt et al., 2013; Sagoo et al., 2002;

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Surendran-Nair et al., 2016). Chitosan seems particularly effective when used in combination with other preservative agents including heat, antimicrobial phenolic compounds (Surendran-Nair et al., 2016), or citrus extracts (Vardaka et al., 2016). The outer-membrane permabilizing activity of chitosan may also support synergistic activity of chitosan with bacteriocins of lactic acid bacteria.

Bacteriocins produced by lactic acid bacteria (LAB) are ribosomally synthesized peptides that have antimicrobial activity in nanomolar concentrations (Drider et al., 2006). Bacteriocins are classified into Class I peptides, which undergo post-translational modifications, and unmodified Class II peptides (Alvarez-Sieiro et al., 2016). Class I bacteriocins include lantibiotics, *e.g.* nisin, and cyclic bacteriocins, *e.g.* carnocyclin A; Class II bacteriocins include the pediocin-like bacteriocins that exhibit activity against *Listeria monocytogenes* (Alvarez-Sieiro et al., 2016). Food applications of purified compounds or food-grade bacteriocin producing protective cultures inhibit foodborne pathogens as well as spoilage organisms (Drider et al., 2006; Perez et al., 2014). However, bacteriocins of lactic acid bacteria are inactive against Gram-negative bacteria because the outer membrane prevents access to the cellular target, the cytoplasmic membrane (Gänzle et al., 1999a; Stevens et al., 1991). Chemical or physical treatments that disrupt the outer membrane may allow the use of bacteriocins for control of Gram-negative pathogens in food (Cutter and Siragusa, 1995; Martin-Visscher et al., 2011). The outer-membrane permeabilizing activity of chitosan sensitizes *E. coli* and *Salmonella* to nisin (Cai et al., 2010); however, this synergistic effect has not been validated in food applications, and was not verified for bacteriocins other than nisin.

The aim of this study was to determine the single and combined antimicrobial activity of chitosan and bacteriocins in media, and to verify the activity in a model meat system mimicking pathogen intervention technologies that used in beef processing. The heat resistant *E. coli* AW1.7 and *Salmonella enterica* Typhimurium TA2442 were used as target organisms; nisin and bacteriocin cocktails purified from two strains of *Carnobacterium maltaromaticum* were evaluated to represent Class I and Class II bacteriocins.

2. Material and methods

2.1. Bacterial strains and culture conditions

Escherichia coli AW1.7, a heat resistant beef isolate (Dlusskaya et al., 2011) and *Salmonella. enterica* Typhimurium TA2442, obtained from the *Salmonella* genetic stock centre (Calgary, AB, Canada) were aerobically grown in Luria-Bertani broth (LB; Difco; Becton, Dickinson and Company, Sparks, MD, USA) at 37 °C for 18 h. *E. coli* AW1.7 and *S.* Typhimurium were enumerated on LB agar (Difco) to detect all viable cells, or on violet red bile agar (VRBA, Difco) to enumerate cells of *E. coli* AW1.7 and *S.* Typhimurium cells without sublethal injury. *Carnobacterium divergens* LV13, a bacteriocin sensitive indicator strain, *C. maltaromaticum* UAL307, a strain used in commercial biopreservatives and producing piscicolin 126, carnobacteriocin BM1, and carnocyclin A (Martin-Visscher et al., 2008), and *C. maltaromaticum* UAL8 producing carnobacteriocin A, BM1 and B2 (Allison et al., 1995) were routinely grown in All Purpose Tween (APT) broth (Difco) at 25 °C. APT agar was used to enumerate viable carnobacteria. For purification of bacteriocins from cultures of *C. maltaromaticum* UAL307, the strain was cultured in Casamino Acid (CAA) medium containing the following per litre: 15 g casamino acid; 5 g yeast extract; 2 g K_2HPO_4; 2 g $C_6H_{14}N_2O_7$; 0.1 g $MgSO_4$; 0.05 g $MnSO_4$; pH = 6.5 at 25 °C for 21 to 24 h.

2.2. Chemicals and preparation

High molecular weight chitosan (HMWC) was supplied by Yuhan Ocean Biochemistry Co. Ltd. (Tauzhou, China). The degree of deacetylation and molecular weight of HMWC were 92% and 210 kDa, respectively. Water soluble chitosan (WSC) was prepared by enzymatic hydrolysis of HMWC with neutral protease from Ningxia Xiasheng Industry Co. Ltd. (Ningxia, China). The degree of deacetylation (DD) of WSC was 92% as determined by titration (Tolaimate et al., 2000). The degree of polymerization (DP) as determined by size exclusion chromatography on a Superdex Peptide column (GE Healthcare) ranged from 4- to 50 units. Chitosan oligosaccharides (COS) with a degree of deacetylation of 100% and a DP of 2–6 were obtained from GlycoBio (Dalian, China). HMWC, WSC or COS were dissolved in 1% (w/v) acetic acid (Fisher Scientific, Canada), the pH was adjusted to 5.4 with 10 M NaOH, and the concentration was adjusted to 1% (w/v). HMWC stock solution with pH 5.4 was stored at 4 °C for use within one week; WSC or COS stock solutions were prepared on the day or use.

A nisin preparation containing 2.5% nisin and 97.5% NaCl and milk solids was obtained from MP Biomedicals (Montreal, Canada). A nisin stock solution containing 125 mg/L nisin was prepared by dissolving 25 mg commercial nisin preparation and 37.5 mg NaCl in 4.8–4.85 mL 0.02 M HCl (Sigma-Aldrich, USA), followed by adjustment of the pH to 5.4 with NaOH solution and adjustment of the total volume to 5 mL with water. The nisin solution was sterilized by filtration.

2.3. Partial purification of bacteriocins and determination of bacteriocin activity

The bacteriocins produced by *C. maltaromaticum* UAL307 were purified as described (Balay et al., 2017) with some modifications. *C. maltaromaticum* UAL307 was grown in 1 L of Casamino Acid (CAA) medium. After 21 to 24 h of incubation, the culture including cells and supernatant was applied to a column (2.5 × 50 cm) containing 60 g/L of Amberlite XAD-16 N resin (Sigma-Aldrich®, Saint Louis, MO, USA), equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA), at a flow rate of 5 mL/min at 6 °C. The column was successively washed with 500 mL of H_2O, 500 mL of 20% (v/v) ethanol, and 500 mL of 40% (v/v) ethanol all at 10 mL/min. Bacteriocins were eluted with 1 L of 70% isopropyl alcohol, acidified to pH 2 at 5 mL/min. This fraction was concentrated to around 24 mL using a Buchi® rotary evaporator (Brinkman Instruments, Westbury, NY, USA) at 30 °C under vacuum and loaded onto three Water-Pak 12 cc C18 cartridges. The three cartridges were each washed with 20 mL H_2O, 20 mL 30% (v/v) ethanol, 20 mL 20% (v/v) isopropanol at a flow rate of 5 mL/min. Bacteriocins were eluted from each cartridge with 40 mL of 70% (v/v) isopropanol, pH 2. The active fractions collected from each of the 3 cartridges were combined and concentrated under vacuum to a volume of about 5 mL. All fractions were assayed for antimicrobial activity with *C. divergens* LV13 as the indicator strain. The activity was determined by a critical dilution assay (Eloff, 1998) with some modification. In brief, serial two-fold dilutions of each fraction with APT broth were prepared on 96-well microtiter plates (Corning, USA). Overnight cultures of *C. divergens* LV13 in APT broth were subcultured and incubated at 25 °C for 12 h, diluted ten-fold and used to inoculate the microtiter plates. After incubation of the plates for 18 h, 40 µL of a 0.2 g/L p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich) solution in water was added to each well and the plate was incubated for 3 h at 25 °C. The wells without bacterial growth remained colorless; one activity unit (AU) was defined as the highest dilution of each fraction that inhibited growth of *C. divergens*.

2.4. Determination of inhibitory activity of different antimicrobials against E. coli AW1.7 and S. Typhimurium

The inhibitory effects of chitosan, nisin, or purified bacteriocins against *E. coli* AW1.7 and *S.* Typhimurium were determined by a critical dilution assay as described (Gänzle et al., 1999a) with some modifications. In brief, two-fold serial dilutions of HMWC WSC, or COS were prepared with MES-buffered nutrient broth (NB-MES) in 96-well microtiter plates (Corning, USA); 2D “checkerboard” dilutions to determine the combined activity of chitosan and bacteriocins were prepared as described (Gänzle et al., 1999a). *E. coli* AW1.7 and *S.*