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.

Key words: chitosan; bacteriocin; nisin; *Carnobacterium maltaromaticum*; beef.

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; Mellegard 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; 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 et al., 1995; Martin-Visscher et al., 2011). The outer-membrane permeabilizing activity of chitosan sensitises *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

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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., 2011), 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 ℃ 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 liter 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 liter 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.* Typhimurium were sub-cultured twice in nutrient broth (NB) and incubated at 37 °C for 8-10 h and 12 h, respectively. The cultures were diluted ten-fold with NB-MES, and 50 µl of these diluted cultures were added to the microtitre plates. The plates were incubated for 16–20 h at 37 ℃, the optical density was measured at 630 nm using a microtiter reader (Varioskan Flash, Thermo Electron Corporation, Canada), and the MIC of chitosan, nisin, or purified bacteriocins was assessed as concentration in mg/L or AU/mL inhibiting growth of the indicator strains by 50%.

2.6 Preparation of meat samples

Frozen lean beef was obtained as vacuum packaged and frozen bulk product. To obtain aseptic cuts of beef, frozen beef was tempered at 4°C for 12 h and cut into 2.5 cm and 7.5 cm steaks. These steaks were flamed with ethanol to sterilize the surface, triple wrapped in plastic bags and stored at -20°C. To prepare meat cylinders, frozen steaks were tempered at room temperature for 1 to 2 h. A sterilized circular corer with a diameter of 2.0 cm (surface area of 3.14 cm^2) was hammered into the partially frozen meat. The core of meat was aseptically sliced into cylinders around 5 mm thick. Meat cylinders were stored at -20°C until use. Total cell counts and coliform cell counts of the meat cylinders were enumerated on LB agar and VRBA; both cell counts were below the detection limit of 100 CFU/g.

2.7 Establishment of bench-top steaming apparatus and steaming procedures

The steaming apparatus (Figure 1) consisted of a glass flat bottom flask that was placed on a magnetic heater to generate stream. A foil-insulated custom-made glass nozzle conducted the stream to the meat sample. The distance between the steam outlet and the surface of the meat samples was 2.2 cm.

2.8 Different treatments and microbiological analysis of samples

Meat cylinders were thawed at room temperature for 1 h. The meat surface was inoculated with 100 µl of cultures of *E. coli* AW1.7 or *S.* Typhimurium and the surface was air dried 20 °C for 15 min; uninoculated samples without treatment were used as negative control. Positive controls were inoculated but did not receive any treatment; other samples were steamed for 8 s. Steamed samples were also treated by adding 200 µL of one or two of the following solutions or organisms: 8% lactic acid, 1% acetic acid, 1% HMWC solution in 1% acetic acid, bacteriocins partly purified from cultures of *C. maltaromaticum* UAL307, culture of *C. maltaromaticum* UAL8 culture, or culture of *C. maltaromaticum* UAL307. When combination treatments of two solutions were used, 100µL of each of the two solutions was added.

After treatment, samples were air dried and incubated for 4 h. Total cell counts, cell counts of coliform bacteria, and cell counts of carnobacteria were determined by surface plating of appropriate dilutions on LB agar, VRBA, and APT agar, respectively. Observation of a uniform colony morphology verified that the colony morphology of carnobacteria enumerated after refrigerated storage matched the colony morphology of the inocula.

2.9 Microbiological analysis of samples during vacuum-packaged and refrigerated storage.

A second experiment employed the most efficient treatments to observe the antimicrobial efficacy during 4 weeks of refrigerated storage. Samples inoculated with 100 µl of *E. coli* AW1.7 or *S. enterica* Typhimurium cultures (around 10^8 CFU/cm^2) were treated as described above, vacuum-packaged and stored for 32 days (d) at 4°C. Uninoculated and untreated inoculated controls were also prepared as described above. The plate counts of samples were determined at 4 h and 1, 4, 8, 16, 24 and 32 d.

2.10 Statistical analysis.

All data are expressed as means ± SD. Differences among treatments were tested for significance by one-way ANOVA with Least Significant Difference (LSD) test using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) for Windows 8.1. Significance was assessed at an error probability of 5% ($p \leq 0.05$).

3. Results

3.1 Single and combined activity of bacteriocins or chitosan in media.

To assess the activity of bacteriocins, the MIC of nisin and a bacteriocin preparation from *C. maltaromaticum* UAL307 were determined with *E. coli* and *S.* Typhimurium as indicator strains. At pH 5.4, the MIC of nisin against *E. coli* AW1.7 was 10 mg/L whereas *S.* Typhimurium was resistant to nisin at a concentration of 20 mg/L. A single chromatographic step achieved partial purification of bactericoins produced by *C. maltaromaticum* UAL307 (Balay et al., 2017). Elution of the column with 70% isopropanol eluted peptides with antimicrobial activity while all other fractions obtained in the purification procedure exhibited no activity. The activity of the final bacteriocin preparation was 20480 AU/ml. Assaying the antimicrobial activity of the preparation against *E. coli* and *S.* Typhimurium demonstrated that these two Gram-negative organisms were about 100 times less sensitive than *C. divergens* (Fig 1). The MIC of chitosan oligosaccharides (COS), water soluble chitosan (WSC) and high molecular weight chitosan (HMWC) against *E. coli* ranged from 14 to 42 mg/L (Figure 2 and 3); the HMWC was the most active of the three chitosan preparations. The MIC of COS, WSC and HMWC against *S.* Typhimurium ranged from 30 to 69 mg/L; again, again, HMWC was the most active compound (Fig 2 and 3).

The combined activity of bacteriocins and chitosan preparations is shown in Figures 2 and 3. Nisin did not increase the susceptibility of *E.coli* AW1.7 and *S.* Typhimurium to chitosan (Fig 2); however, a synergistic effect was observed for HMWC and bacteriocins from *C. maltaromaticum*

UAL307; this synergistic effect was weaker or absent for the COS or WSC (Fig 3). These results indicate that high molecular weight chitosan permeabilizes the outer membrane of *E. coli* and *S.* Typhimurium to bacteriocins from *C. maltaromaticum* UAL7.

3.2 Screening the efficient treatments in inactivating *E.coli* AW1.7 and *S. enterica* Typhimurium on fresh lean beef.

An initial experiment explored the effect of steam and lactic acid alone, in combination with chitosan, or in combination with chitosan and bacteriocin-producing carnobacteria or bacteriocins. Based on the *in vitro* screening, HMWC and bacteriocins from *C. maltaromaticum* UAL307 were selected to determine their single and combined antimicrobial effects on meat. Surviving cells of *E. coli* and *S.* Typhimurium were enumerated on LB agar and VRBA to quantify viable and sublethally injured cells. After inoculation, cell counts on the surface of lean beef cylinders ranged from 6.2 to 6.9 log(CFU/cm^2) (Fig 4). Steaming reduced cell counts of *S.* Typhimurium by approximately 1 log(CFU/cm^2) (Fig. 4) while no significant cell reduction of *E.coli* was observed after steaming. Treatment with lactic acid after steaming had no additional antimicrobial effect (Fig. 4). Likewise, treatments of meat with cultures of *C. maltaromaticum* or purified bacteriocins produced from *C. maltaromaticum* UAL307 were as effective as treatments with steam only (data not shown). Treatments of meat with chitosan after steaming additionally reduced cell counts of *E. coli* and *S.* Typhimurium by approximately 1 log(CFU/cm^2) (Fig. 4). The antimicrobial effect of steam plus chitosan treatment was not increased by addition of bacteriocin-producing carnobacteria, or bacteriocins purified from *C. maltaromaticum* UAL307 (Fig. 4). Different from *in vitro* results (Fig. 3), chitosan and bacteriocins displayed no synergistic activity; however, chitosan addition to meat substantially enhanced the antimicrobial effect of steam treatment.

3.3. Effect of treatment with steam and chitosan on meat microbiota during refrigerated storage.

Subsequent experiments aimed to determine the influence of intervention treatments with steam and chitosan on the viability of *E. coli* and *Salmonella* during refrigerated storage. Meat was additionally inoculated with carnobacteria to assess the impact of intervention treatments on non-pathogenic meat microbiota. Results obtained with *E. coli* AW1.7 are shown in Fig 5. Cell counts of *E. coli* were reduced by 1 – 2 log(CFU/cm^2) during refrigerated storage; this reduction was particularly apparent for cell counts on VRBA, which exclude sublethally injured cells (Fig. 5A and 5C). The effect of streaming on cell counts of *E. coli* during storage was generally not significant; likewise, addition of acetic or lactic acids did not influence cell counts after treatment or after treatment and storage (Fig. 5A and 5C). Treatment with chitosan reduced cell counts by 1 log(CFU/g) and this difference to the steam treated control remained throughout the 32 d of storage (Fig. 5A and 5C). Inoculation of meat with carnobacteria did not affect cell counts of *E. coli* during refrigerated storage (Fig. 5B and 5D); however, chitosan was also effective in presence of carnobacteria (Fig. 5B and 5D). The overall reduction of cell counts that was achieved by steam and lactic acid intervention treatments, chitosan addition and refrigerated storage exceeded 3 log(CFU/cm^2) (Fig. 5).

The cell counts of *S.* Typhimurium during refrigerated storage are shown in Figure 6. Comparable to *E. coli*, chitosan reduced cell counts by about 1 log(CFU/cm^2) while treatments with organic acids were ineffective (Fig. 6). Different from *E. coli*, steam treatment significantly reduced cell counts of *Salmonella* by about 1 log(CFU/cm^2), and cell counts of *Salmonella* remained stable throughout refrigerated storage unless carnobacteria and chitosan were both present. In presence of chitosan and any of the two strains of *C. maltaromaticum*, cell counts were reduced by 1 – 2 log(CFU/cm^2) during refrigerated storage (Fig. 6B and D). The overall reduction of cell counts

267 achieved by steam treatment followed by addition of chitosan and carnobacteria exceeded 3
268 log(CFU/cm^2).

269 Because the presence of carnobacteria influenced survival of *Salmonella* during refrigerated
270 storage of beef when chitosan was present, cell counts of carnobacteria were additionally
271 monitored during refrigerated storage. Cell counts of co-cultures with *Salmonella* are shown in
272 Figure 7; cell counts of co-cultures with *E. coli* were essentially identical (data not shown). The
273 two strains of *C. maltaromaticum* also showed a comparable response to treatment and refrigerated
274 storage (Figure 7 and data not shown). In the absence of chitosan, carnobacteria grew from about
275 6 log(CFU/cm^2) to 7 log(CFU/cm^2) (Fig. 7). Chitosan initially reduced cell counts of carnobacteria
276 by about 99%; however, during refrigerated storage, the surviving cells grew to high cell counts
277 even in presence of chitosan.

278 4. Discussion

279 This study assessed the activity of chitosan in combination with steam pasteurization, acid
280 interventions, and bacteriocins or bacteriocin producing cultures to reduce beef contamination with
281 *Salmonella* and *E. coli*. The North American beef industry applies steam pasteurization or hot
282 water washes in combination with application of lactic acid or peroxyacetic acid to reduce carcass
283 contamination. Steam pasteurization reduces the numbers of *E. coli* on meat by 0.05 to 2 log
284 (cfu/cm^2) (Corantin et al., 2005; Gill, 2009; McCann et al., 2006; Minihan et al., 2003). The
285 variable effect of steam or hot water interventions may relate to variations in the intensity of
286 thermal treatments, differences between lean and adipose tissue, or to strain-to-strain variation of
287 heat resistance (Dlusskaya et al., 2011). The variable effect of thermal interventions necessitates
288 improved intervention technologies to reduce the burden of foodborne disease associated with beef.
289 The present study implemented a lab-scale steam treatment to heat the surface of the meat to >95°C

for several seconds, thus matching conditions that are typically employed in beef processing (Gill, 2009). *E. coli* AW1.7 is a heat resistant beef isolate (Dlusskaya et al., 2011) and heat resistance of the strain is mediated by the locus of heat resistance (LHR) (Mercer et al., 2015). LHR-mediated heat resistance is observed in approximately 2% of all *E. coli* and in 4% of *E. coli* isolated from beef processing plants (Mercer et al., 2015); LHR-mediated heat resistance also occurs in *Salmonella* but with a much lower frequency (Mercer et al., 2017). The bactericidal effect of steam treatment on *E. coli* AW1.7 and *S.* Typhimurium corresponded to the differential heat resistance of the two organisms. Steam treatment is effective only on the surface of the tissue, therefore, stream treatments reduced cell counts of the heat sensitive *Salmonella* by less than 2 log(cfu/cm^2) (Fig. 4 and 7). Interventions with lactic or acetic acids had no effect on cell counts of *E. coli* or *Salmonella*, reflecting the acid resistance of these organisms (Foster, 2004) and the high buffering capacity of lean tissue.

Bacteriocins from lactic acid bacteria alone or in combination with chitosan may increase the bactericidal effect of pathogen intervention technologies in beef processing. Bacteriocins from *C. maltaromaticum* and nisin inhibited *E.coli* AW1.7 and *S. enterica* Typhimurium in media with pH 5.4, in keeping with prior observations that a low pH increases sensitivity of Gram-negative bacteria (Gänzle et al., 1999b; Martin-Visscher et al., 2011). High proton concentrations, corresponding to a low pH, displace divalent cations from the LPS binding sites; the resulting increase in permeability of the outer membrane renders cells more susceptible to hydrophobic inhibitors including bacteriocins (Vaara, 1992, Gänzle et al., 1999b). The net charge density of chitosan and the intensity of electrostatic interactions between chitosan and cell surface are crucial to antibacterial activity; therefore, chitosan is active only when the ambient pH is below its pK_A of 6.5 (Gerasimenko et al., 2004; Kong et al., 2010; Mellegard et al., 2011; Zheng and Zhu, 2003).

313 High molecular weight chitosan generally exhibits a higher antibacterial activity than chitosan
314 oligosaccharides (Mellegard et al., 2011), which was confirmed in the present study. Chitosan with
315 higher activity also leads to a more intense disruption of outer membrane (OM) of *E.coli*.
316 (Mellegard et al., 2011). Perturbation of the outer membrane permeability barrier by chitosan
317 (Eaton et al., 2008; Helander et al., 2001; Kong et al., 2010) may increase the sensitivity to outer-
318 membrane impermeant inhibitors such as bacteriocins. Synergistic activity of chitosan and nisin
319 has previously been described *in vitro* (Cai et al., 2010) but has not been employed to inhibit Gram-
320 negative organisms in food. This study employed NB broth to determine the *in vitro* synergistic
321 activity; the low protein content of this medium minimizes interactions of chitosan with media
322 components. Synergistic activity of chitosan was observed with high molecular weight chitosan
323 and bactericiocins from *C maltaromaticum*, in keeping with prior observation that outer membrane
324 perburbation sensitizes *E. coli* to carnocyclin A (Martin-Visscher et al., 2011). However,
325 inconsistent with prior reports (Cai et al., 2010), synergistic activity was not observed with nisin
326 and chitosan. We employed commercial nisin containing 2.5 % nisin with NaCl and milk proteins.
327 These ingredients may decrease chitosan activity by neutralizing the positive charges of chitosan
328 (Devlieghere et al., 2004).
329 In this study, addition of HMWC after steaming reduced *E.coli* or *Salmonella* by around 1
330 log(cfu/cm^2) while treatments with lactic or acetic acids had no additional effect. The overall
331 bactericidal effect of chitosan on meat, which reduced cell counts by 90%, matched the reduction
332 of cell count of *Salmonella* in chicken skin by application of 0.5% chitosan (Menconi et al., 2013)
333 and the effect of addition of 2% chitosan of cell counts of *E. coli* in kabab (Kanatt et al., 2013).
334 Carnobacteria were more sensitive to chitosan application on meat than *E. coli* or *Salmonella* (Fig.

335 5, 6, and 7); however, chitosan did not prevent growth of carnobacteria to high cell counts during
336 refrigerated storage.
337 The application of chitosan in meat was particularly effective in hurdle applications that combined
338 chitosan with heat and additional antimicrobial agents. Chitosan addition at a level of 0.1% did not
339 affect survival of enterohaemorrhagic *E. coli* during refrigerated storage of ground beef; however,
340 chitosan showed synergistic effects with rutin and resveratrol during cooking of beef patties
341 (Surendran-Nair et al., 2016). The use of citrus extract in combination with low molecular weight
342 chitosan showed an additive effect against *E. coli* and *S. enterica* populations in fresh turkey meat
343 stored under vacuum at 4°C or 10°C (Vardaka et al., 2016). A potential synergistic effect of
344 bacteriocins and chitosan on meat, however, remains unknown. Nisin in raw meat is inactivated
345 by addition of glutathione (GSH) (Rose et al., 1999); moreover, nisin exhibited no synergistic
346 activity with chitosan. Meat applications combining chitosan and bacteriocins thus focused on
347 bacteriocins of *C. maltaromaticum* and application of bacteriocin-producing cultures on meat. Cell
348 counts on LB and VRBA differed by less than 1 log(cfu/cm^2) after treatment of meat with steam
349 and chitosan, indicating that outer membrane perturbation by chitosan, which was demonstrated
350 *in vitro* (Helander et al., 2001), is not observed on meat. Accordingly, the application of
351 bacteriocins did not reduce cell counts of *E. coli* and *Salmonella*, and did not enhance the
352 bactericidal effect of chitosan (Fig. 4, 5, and 7).
353 The present study evaluated the use of the bacteriocin-producing cultures *C. maltaromaticum*
354 UAL8 and UAL307 as an alternative strategy to control enteric pathogens in combination with
355 chitosan. Application of 2% chitosan reduced cell counts of *E. coli* and *Salmonella* during
356 refrigerated storage of vacuum packaged turkey meat (Vardaka et al., 2016), but the effect of
357 spoilage microbiota was not considered. Remarkably, refrigerated storage differentially affected

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358 *E. coli* and *Salmonella*, chitosan, and protective cultures. Cell counts of *E. coli* were reduced during
359 refrigerated storage; the reduction was irrespective of the presence of chitosan or carnobacteria. In
360 contrast, cell counts of *Salmonella* remained stable during storage unless carnobacteria and
361 chitosan were both present (Fig. 5, 6 and 7). In both cases the combined bactericidal effect of steam
362 treatment, chitosan, and protective cultures reduced cell counts by 3 log(cfu/cm^2). This represents
363 a substantial improvement to current or proposed intervention technologies (Gill, 2009; Surendran-
364 Nair et al., 2016). It remains unknown whether the effect of carnobacteria relates to competition
365 for nutrients and acid formation, or to a specific effect of the bacteriocins that are produced during
366 storage (Holzapfel et al., 1995).
367 In conclusion, chitosan exhibited bactericidal activity against *Salmonella* and *E. coli* on beef.
368 Chitosan exhibited no synergistic activity with bacteriocins on meat, however, chitosan together
369 with bacteriocin-producing protective cultures reduced cell counts of *Salmonella*. The use of
370 chitosan and protective cultures in addition to steam treatment was significantly more effective
371 than the use of steam alone or in combination with lactic acid, and thus may provide novel solutions
372 for improved meat safety. The application is particularly promising for production of ground beef
373 and mechanically tenderized beef, where internal contamination with pathogenic bacteria may
374 occur (Gill et al., 2005; Phebus et al. 2000).

375 **Acknowledgements**

376 The Alberta Livestock and Meat Agency is acknowledged for funding (Grant No. 2013F030R).
377 Ziyi Hu and Michael Gänzle acknowledge financial support from the China Scholarship Council
378 and the Canada Research Chairs Program, respectively.

379

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Figure 1. Schematic diagram of the bench-top steaming apparatus used in this work.

Figure 2. Effect of nisin on the activity of chitosan-oligosaccharides (▲), water-soluble chitosan (■) and high-molecular weight chitosan (●) against *E.coli* AW1.7 (A) and *S. enterica* Typhimurium (B). Error bars indicate the means ± standard deviation of triplicate independent experiments.

Figure 3. Effect of bacteriocins produced by *C. maltaromaticum* UAL 307 on the activity of chitosan-oligosaccharides (▲), water-soluble chitosan (■) and high-molecular weight chitosan (●) against *E.coli* AW1.7 (A) and *S. enterica* Typhimurium (B). Data to the right of the axis break indicate the MIC of bacteriocins in absence of any chitosan preparation. Data are shown as means ± standard deviation of triplicate independent experiments.

Figure 4. Cell counts of lean, aseptic beef cylinders inoculated with *E. coli* (Panel A) or *Salmonella* (Panel B) after different pathogen intervention treatments as indicated. Cell counts were enumerated on LB agar (white bars) and VRBA agar (grey bar). Steam, treatment for 8 sec, lactic acid, application of 8% lactic acid; chitosan, surface application of 1% high molecular weight chitosan in 1% acetic acid; UAL307, inoculation with *C. maltaromaticum* UAL307 after steaming; inoculation with *C. maltaromaticum* UAL8 after steaming; Bacteriocin, purified bacteriocins produced by *C. maltaromaticum* UAL307 (1280 AU/mL). Data indicate means ± standard deviation of two or three independent experiments. Cell counts that are different from the cell counts of samples treated with only steam are indicated by an asterisk (*$P<0.05$*).

Figure 5. Cell counts of *E.coli* on vacuum packaged lean beef cylinders during storage at 4 °C. The counts of *E.coli* were enumerated on LB agar (Panels A and B) or on VRBA (Panels C and D). Beef cylinders shown in Panels A and C were inoculated only with *E. coli*; samples shown in panels B and D were inoculated with *C. maltaromaticum* UAL307 (□) or UAL8 (○) after steaming.

Panels A, C: Before packaging, beef cylinders were not treated (control, Δ), or treated with steam for 8 sec (○) in combination with the following additions: acetic acid (●); lactic acid (■); or 1% HMWC (▲). Panels B, D: Treatment with steam for 8 sec, followed by inoculation with *C. maltaromaticum* UAL307 (□); *C. maltaromaticum* UAL8 (■); *C. maltaromaticum* UAL307 with 1% HMWC (○); or *C. maltaromaticum* UAL8 with 1% HMWC (●). Data indicate means ± standard deviation of two or three independent experiments. For treatments that were significantly more lethal than steam and storage for the same time ($P<0.05$), the corresponding symbol is indicated at the upper x-axis.

Figure 6. Cell counts of *S. enterica* on vacuum packaged lean beef cylinders during storage at 4 °C. The counts of *S. enterica* were enumerated on LB agar (Panels A and B) or on VRB agar (Panels C and D). Beef cylinders shown in Panels A and C were inoculated only with *S. enterica*; samples shown in panels B and D were inoculated with *C. maltaromaticum* UAL307 (□) or UAL8 (○) after steaming. Panels A, C: Before packaging, beef cylinders were not treated (control, Δ), or treated with steam for 8 sec (○) in combination with the following additions: acetic acid (●); lactic acid (■); or 1% HMWC (▲). Panels B, D: Treatment with steam for 8 sec, followed by inoculation with *C. maltaromaticum* UAL307 (□); *C. maltaromaticum* UAL8 (■); *C. maltaromaticum* UAL307 with 1% HMWC (○); or *C. maltaromaticum* UAL8 with 1% HMWC (●).Data indicate means ± standard deviation of at least two independent experiments. For treatments that were significantly more lethal than steaming and storage for the same time ($P<0.05$), the corresponding symbol is indicated at the upper x-axis.

Figure 7. Cell counts of *Carnobacterium* on vacuum packaged lean beef cylinders inoculated with *S. enterica* and *C. maltaromaticum* UAL307 or *C. maltaromaticum* UAL8 during storage at 4 °C for 32 days. Carnobacteria were selectively enumerated on APT agar. Beef cylinders were

553 inoculated with *S. enterica,* treated with HMWC (black symbols) or not (open symbols), steamed
554 for 8 sec, followed by inoculation with *C. maltaromaticum* UAL307 (□,■) or *C. maltaromaticum*
555 UAL8 (○,●). Data indicate means ± standard deviation of at least two independent experiments.
556 Comparable cell counts of carnobacteria were obtained from beef cylinders inoculated with *E. coli*
557 (data not shown).

42

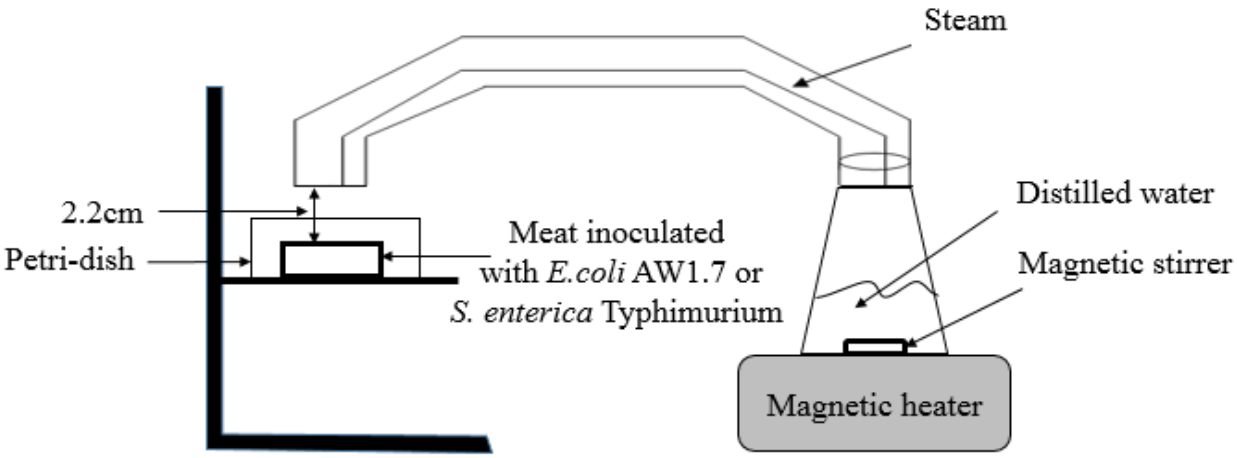
Steam
Distilled water
Magnetic stirrer
2.2cm
Petri-dish
Meat inoculated
with *E.coli* AW1.7 or
S. enterica Typhimurium
Magnetic heater

Figure 1

42

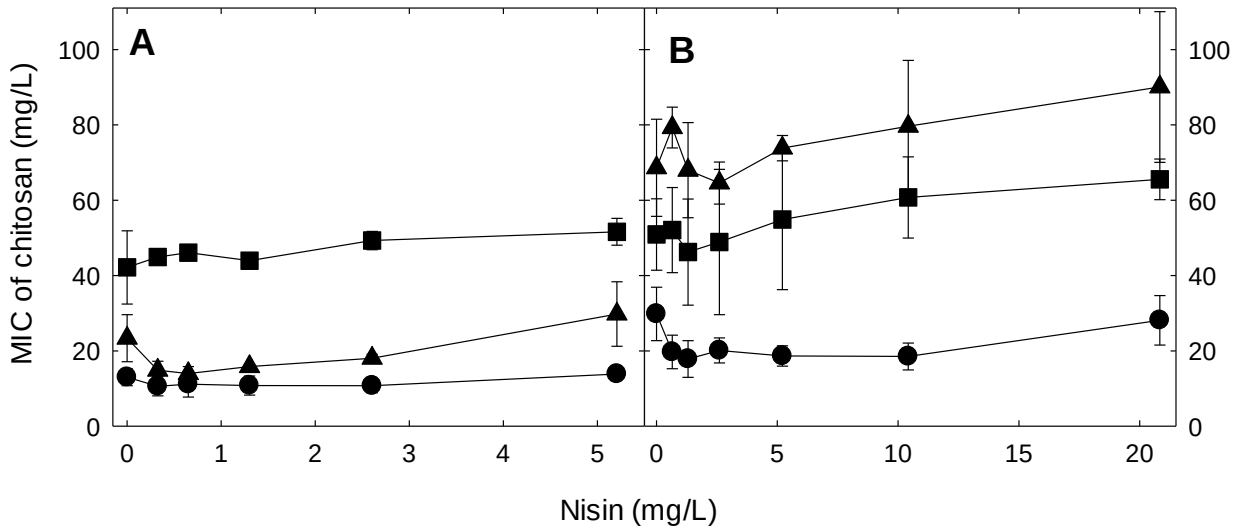
A
B
MIC of chitosan (mg/L)
Nisin (mg/L)
0
20
40
60
80
100
0
1
2
3
4
5
0
5
10
15
20

Figure 2

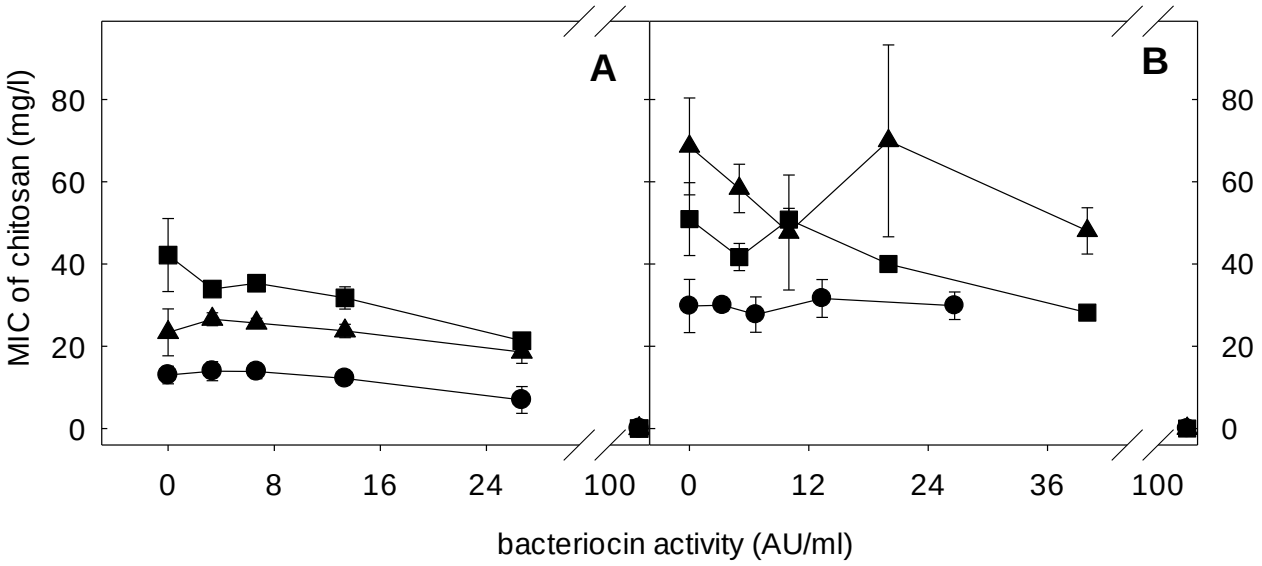
A
B
MIC of chitosan (mg/l)
0
20
40
60
80
0
8
16
24
100
0
12
24
36
100
bacteriocin activity (AU/ml)

Figure 3.

Figure 4.

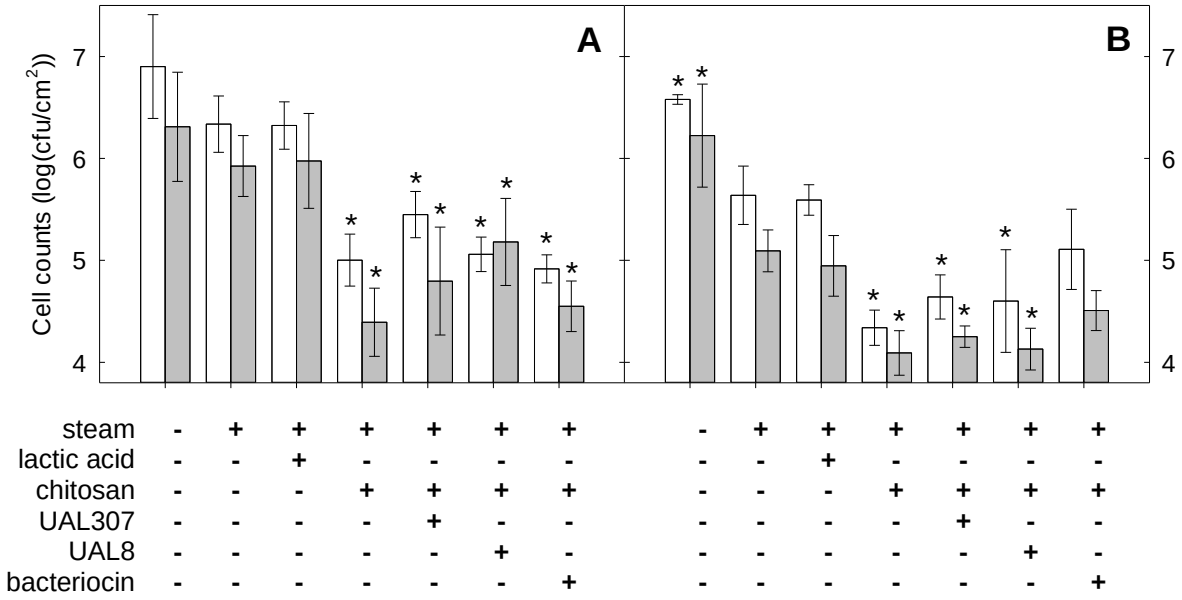
A
B
Cell counts (log(cfu/cm^2))
7
6
5
4
steam
lactic acid
chitosan
UAL307
UAL8
bacteriocin

Figure 5.

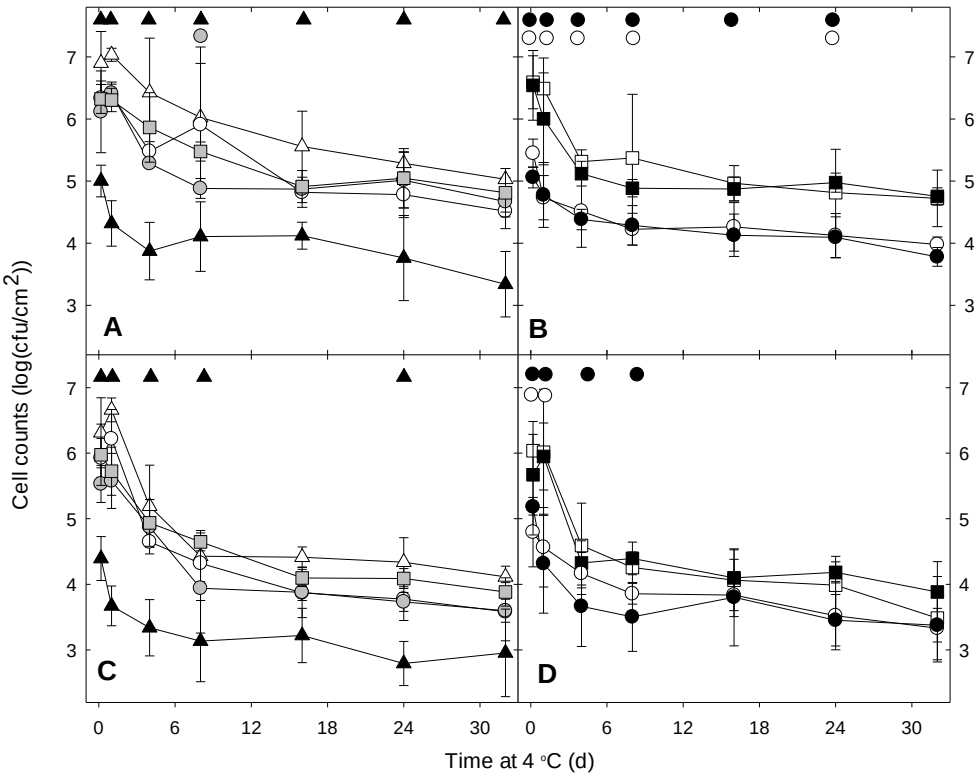

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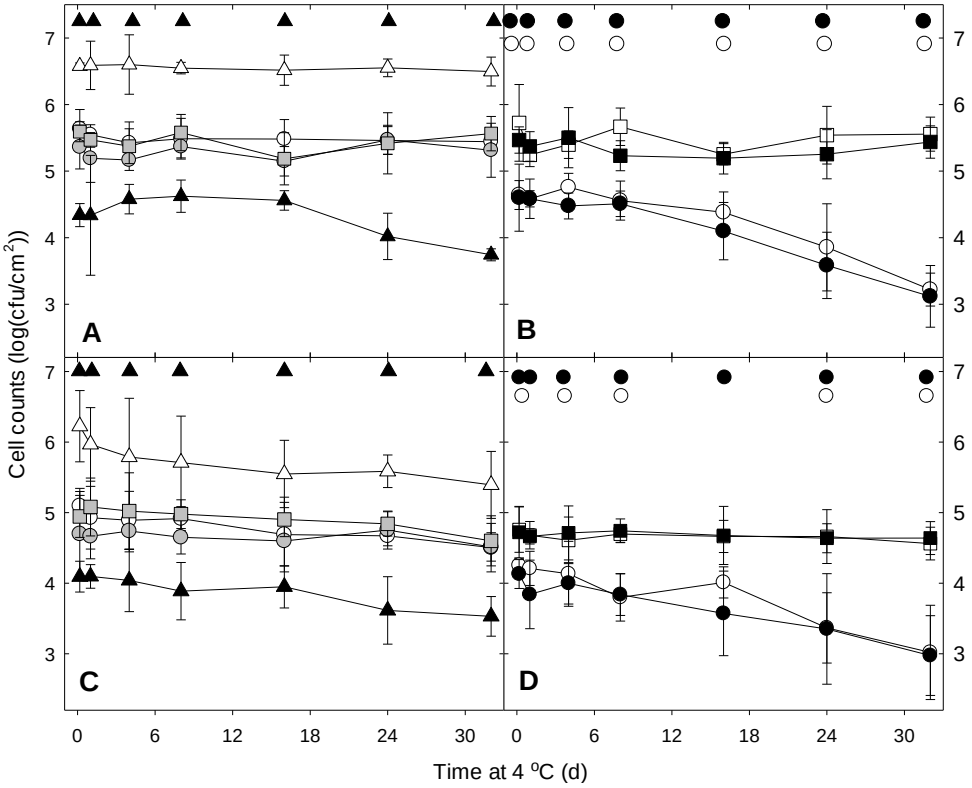
Figure 6.



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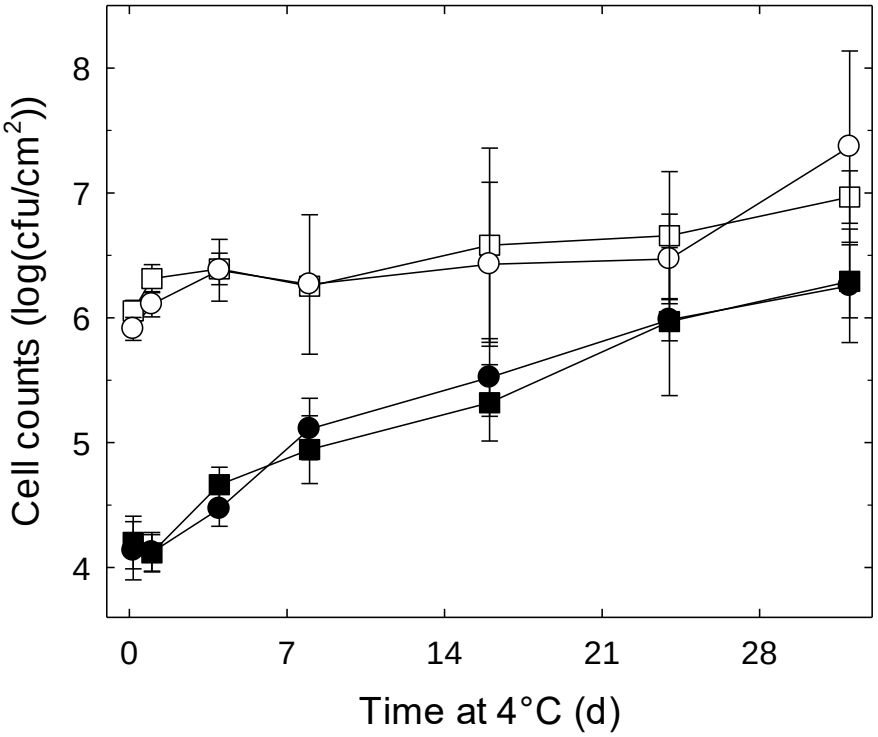

Figure 7.