Effect of sodium chloride and chitosan on the inactivation of heat resistant or Shiga-toxin producing *Escherichia coli* during grilling of burger patties

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ABSTRACT

Cattle are a reservoir for enterohemorrhagic *Escherichia coli* (EHEC), and ground beef is a major vehicle for human infection with EHEC. Heat resistance of *E. coli*, including EHEC, is impacted by NaCl and other additives. This study aimed to evaluate the effect of NaCl and other additives on the heat resistance of *E. coli* in beef patties. *E. coli* AW1.7ΔpHR1(pLHR) with the locus of heat resistance (LHR), *E. coli* AW1.7ΔpHR1(pRK767) without LHR, or a 5-strain cocktail of EHEC were inoculated (10^7-10^8 CFU/g) into ground beef (15% fat) with NaCl (0 – 3%), marinade, carvacrol (0.1%), potassium lactate (3%) or chitosan (0.1%) following different protocols. Patties were grilled immediately, or stored in sterile bags for two days at 4 °C prior to grilling to a core temperature of 71 °C. Cell counts of LHR-positive *E. coli* AW1.7ΔpHR1(pLHR) were higher than that of the isogenic LHR-negative *E. coli* AW1.7ΔpHR1(pRK767) by more than 3 $\log_{10}$ (CFU/g) after cooking. Addition of 3% NaCl increased survival of *E. coli* AW1.7ΔpHR1(pRK767) and the EHEC cocktail while cell counts of the heat resistant strains were not changed. A protective effect of NaCl was not observed with *E. coli* AW1.7ΔpHR1(pRK767) or EHEC if cells of *E. coli* were cooled to 4 °C prior to mixing with cold meat and NaCl, indicating that the response of *E. coli* to osmotic shock contributes to this effect. Chitosan enhanced the thermal destruction of LHR-positive *E. coli* AW1.7ΔpHR1(pLHR) in ground beef stored at 4 °C for 2 days, while marinade, carvacrol, or potassium lactate had no such effect, indicating that chitosan can be characterized as an effective hurdle concept to reduce the potential risk of LHR-positive pathogen to meat safety.

Key words: Locus of heat resistance (LHR); Verotoxin producing *Escherichia coli*; cooking; EHEC; NaCl; chitosan; ground beef;

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) can cause substantial morbidity and mortality even after ingestion of less than 10 cells (Croxen et al., 2013; Paton et al., 1996; Tilden et al., 1996) and are thus a significant concern for public health. Ruminants are a main reservoir of EHEC and cattle are an important source for food and environmental contamination (Low et al., 2005). Pathogen intervention during beef processing reduces meat contamination with pathogenic *E. coli*, however, despite these interventions, 0.5 – 2% of ground beef samples in North America are contaminated with EHEC (Aslam et al., 2004; Ferens and Hovde, 2011; Gill, 2009). Accordingly, food-borne infections with EHEC remain linked to the consumption of undercooked ground beef patties (Rhee et al., 2003; WHO, 2018).

Cooking of meat to an internal core temperature of 71 °C is recommended to eliminate food-borne pathogens including EHEC in ground beef (Health Canada, 2015). However, the heat resistance of some *E. coli* strains questions the safety of this recommended cooking temperature (Dlusskaya et al., 2011; Jin et al., 2008; Liu, 2015). In *E. coli* and other *Enterobacteriaceae*, heat resistance is conferred by a 14 or 19 kb genomic island termed locus of heat resistance (LHR) (Boll et al., 2017; Mercer et al., 2015). While *E. coli* generally have $D_{60°C}$ values of less than 1 min, LHR-positive *E. coli* have $D_{60°C}$ values of 15 to more than 71 min (Dlusskaya et al., 2011) and resist cooking in beef patties to an internal temperature of 71 °C with a reduction of cell counts by less than 2 log10 (CFU/g) (Mercer et al., 2017). The LHR occurs in about 2% of strains of *E. coli* and is transferred between strains of *E. coli* (Boll et al., 2017; Mercer et al., 2015). Two Shiga-toxin

producing *E. coli* (STEC) out of 613 clinical isolates of *E. coli* were also found to be positive for the LHR (Ma and Chui, 2017).

Addition of 2-4 % NaCl increases the heat resistance of strains of *E. coli* irrespective of the presence of the LHR (Mercer et al., 2017; Pleitner et al., 2012). NaCl also increased the heat resistance of *E. coli* O157:H7 in ground beef containing 2.7 % NaCl (Juneja et al., 2015). NaCl alone or in conjunction with marinades is applied to improve the taste and texture of meat products (Verbeke et al, 2010; Vlahova-Vangelova and Dragoev, 2014). However, effects of NaCl or marinade on the heat resistance of LHR-positive *E. coli* and LHR-negative *E. coli* in ground beef have not been compared. Additionally, improving the safety of ground beef products necessitates the development of more effective interventions. Carvacrol and chitosan are two membrane active compounds that are derived from biological systems, have generally recognized as safe (GRAS) approval in the U.S.A. (FDA, 2011, 2018) and enhanced thermal destruction of EHEC in burger patties (Hu and Gänzle, 2019; Juneja and Friedman, 2008; Surendran Nair et al., 2016). Potassium lactate is also an effective preservative in extending shelf life of meat products (Sofos and Geornaras, 2010). However, effects of these compounds on survival of heat resistant *E. coli* in beef patties and the interaction with other ingredients, particularly NaCl, has not been studied. Therefore, this study investigated the effect of NaCl and other different additives on heat resistance of EHEC as well LHR-positive and LHR-negative *E. coli* strains in ground beef, and determined whether chitosan or carvacrol potentiate the heat inactivation of *E. coli* in ground beef.

2. Materials and methods

2.1 Bacterial strains and culture conditions.

Bacterial strains used in this study are listed in Table 1. *E. coli* AW1.7ΔpHR1 is an LHR-negative derivative of *E. coli* AW1.7 (Mercer et al., 2015; Pleitner et al., 2012). Isogenic LHR-positive and LHR-negative derivatives of *E. coli* AW1.7ΔpHR1 were generated by transformation with pLHR or the control plasmid pRK767 (Mercer et al., 2015). Strains of *E. coli* were cultured at 37 °C in Luria-Bertani broth (LB, Difco™, Becton Dickinson, Sparks, MD, USA) media. *E. coli* AW1.7ΔpHR1(pLHR) and *E. coli* AW1.7ΔpHR1(pRK767) were cultured in LB with 15 mg/L tetracycline-HCl to ensure plasmid maintenance.

2.2 Meat products, marinades and chemicals.

Lean ground beef (15% fat) was purchased from local supermarket and stored at -20 °C in portions of 200 g until use. Cell counts of un-inoculated ground beef were enumerated on All Purpose Tween (APT) Agar (Difco) and Violet Red Bile (VRB) agar (Difco, Sparks, MD US), respectively. APT agar was chosen because the most abundant bacteria on vacuum-packaged beef, *Carnobacterium* spp., are cultured on APT but not on plate count agar or tryptic soy agar. The plates were incubated at 20 and 37 °C, respectively, for 24 h. Cell counts on APT agar and VRB agar were 3.5 ± 0.9 $\log_{10}$ (CFU/g) and 2.3 ± 0.3 $\log_{10}$ (CFU/g), respectively.

Chitosan with high molecular weight (210kDa) was supplied by Yuhan Ocean Biochemistry Co. Ltd. (Tauzhou, China). The deacetylation degree as determined by titration (Tolaimate et al., 2000) was 92 %. Chitosan was dissolved in 1 % (w/v) acetic acid (Fisher Scientific, Canada) and pH of the chitosan solution was adjusted to 5.4 with 10 M NaOH. Chitosan solutions were prepared on the day of use.

Commercial teriyaki marinade was provided by Griffith Laboratories and contained sugar, salt, soy sauce powder (soy sauce from wheat and soybeans, corn maltodextrin), sodium phosphates, flavor, caramel, garlic powder, onion powder, spices, xanthan gum, monounsaturated vegetable oil, and sulphites. Carvacrol (analytical grade) was purchased from Fisher Scientific (New Jersey, USA). A 60 % potassium L-lactate solution (0.6 g lactate / g) was obtained from Sigma-Aldrich (St. Louis, USA).

2.3 Protocols for inoculation and heat treatment of ground beef.

E. coli were streaked onto LB agar and incubated at 37 °C for 18 h, and sub-cultured on LB agar, inoculated in LB broth and incubated at 37 °C for 18 h with agitation (200 rpm). EHEC cocktails were created by mixing equal volumes of stationary cultures of each strain. Five different protocols were used to sequentially mix ground beef and NaCl with bacterial cultures (Fig. 1). Cells were washed by centrifugation of cultures at 5311 x g and re-suspension in 0.1% peptone water. Washing was performed at 20°C (protocol B) or at 4 °C (protocols D and E). Mixing of cells with meat and NaCl was performed in a Stomacher without pre-cooling of cultures (A, B and C). In protocols D and E, NaCl was added after mixing of cultures with meat and 12 h of refrigerated storage (Fig. 1). Beef patties were heated after mixing with cells and NaCl and holding for 30 min at ambient temperature (protocols A, B and D), or after storage for 2 d at 4 °C (protocol C and E). Control patties were prepared by the same protocols but were not heated. The temperature of the meat after inoculation was monitored by insertion of a thermometer (Tinytag, Interworld Electronics Inc., Markham, ON, Canada) into the geometric centre of the beef patty.

2.4 Effect of other additives on the heat resistance of *E. coli* in ground beef.

132 The effects of other additives on the heat resistance of *E. coli* in ground beef were assessed
133 with the protocol A (Fig. 1). Teriyaki powder was mixed with sterile water at the ratio of
134 5.7:11 by weight to created marinade solution; this marinade solution was then mixed with
135 10 mL of stationary culture and 200 g ground beef. The composition of the final raw burger
136 was 83.3 % meat, 11 % water, and 5.7 % teriyaki powder by weight. The effect of carvacrol
137 and potassium lactate was investigated by inoculation of 200 g ground beef with 10 mL of
138 stationary culture. Carvacrol was dissolved in ethanol at the ratio of 1:1 by volume,
139 dispersed in teriyaki marinade, and the carvacrol / teriyaki marinade then mixed with
140 inoculated ground beef at a ratio of 16.7:83.3 (marinade solution: meat). The concentration
141 of additives was chosen on the basis of their current commercial use (potassium lactate,
142 marinade) or their *in vitro* MIC against *Escherichia coli* (chitosan, carvacrol). The resulting
143 final concentration of carvacrol was 0.10 %. Potassium L-lactate solution was mixed with
144 inoculated ground beef at a ratio of 3:100 (v/w). Chitosan solution (1%) was mixed with
145 200 g ground beef, followed by mixing with 10 mL of stationary culture. Mixing of ground
146 beef, cells, and additives was achieved by stomaching for 2 min; after 35 minutes at 23-
147 25 °C, beef patties were grilled as described below.

148 **2.5 Effect of chitosan on survival of *E. coli* in ground beef during 2 d storage at 4 °C.**

149 The effect of chitosan on heat resistance of *E. coli* in beef patties was additionally assessed
150 with protocol E (Fig. 1). Stationary cultures (20 ml) were washed with cold peptone water,
151 mixed with 200 g ground beef, and stored at 4 °C for 12 h. After storage, the inoculated
152 ground beef was mixed with 20 ml of 1% chitosan solution, stomached for 2 min at 23-
153 25 °C, and stored at 4 °C for 48 h. After storage, a 10 g of raw meat was sampled and
154 enumerated (cell counts at 2 day).

155 **2.6 Sampling, grilling and enumeration of *E. coli*.**

156 To determine the cell counts of samples, inoculated beef was sampled before storage
157 (protocols C and E) and before grilling by sampling 10 g samples, followed by mixing with
158 20 mL of 0.1 % peptone water in a stomacher. The homogenate was serially diluted, and
159 viable cell counts were determined by plating appropriate dilutions on LB and VRB agars
160 with a spiral plater, followed by incubation at 37 °C for 24 h.

161 Prior to grilling, ground beef was shaped into a ball, rolled onto the burger press covered
162 by aluminium foil, and pressed to form a patty. Patties were cooked on a clamshell grill
163 (Cuisinart 5-in-1 griddler, Woodbridge, ON, Canada) that was preheated to medium heat
164 for at least 20 min. Monitoring of the internal temperature of the patty during the grilling
165 process, sampling and enumeration after the complete grilling were conducted as follows:
166 (1) In initial experiments to assess the effect of NaCl on survival of different heat resistant
167 and heat sensitive *E. coli* strains, the internal temperature of the patty was monitored with
168 one thermocouple (Tinytag, Interworld Electronics Inc., Markham, ON, Canada) inserted
169 into the geometric centre of the patty. Once the core temperature reached 71 °C, the burger
170 was placed in 200 mL of iced buffered peptone water and stomached for 2 min. The
171 homogenate was serially diluted and the appropriate dilutions were plated on LB agar by
172 spiral plater and incubated overnight at 37 °C for 24 h. Treatment lethality was calculated
173 as follows:

174 $$reduction\ of\ cell\ counts = \log(N_0) - \log(N_t),$$

175 where N_t refers to the cell counts of treated patties and N_0 refers to the cell counts of
176 untreated patties that were prepared with the same culture or culture-cocktail on the same
177 day. Cell counts on control patties ranged from 10^7 – 10^8 cfu / g.

(2) Subsequent experiments to assess the effect of NaCl on survival of isogenic *E. coli* strains and EHEC in cooked beef patties, and to study the effect of other additives (teriyaki, carvacrol, potassium lactate and chitosan) on heat resistance of single isogenic *E. coli* strains in ground beef were monitored with two thermocouples that were inserted approximately 1 cm to the left and to the right of the geometric centre of the patty. The temperature profile of beef patties monitored by two thermometers during grilling is shown in Fig. 2. After both thermocouples indicated a temperature of 71 °C, 10 g of meat between the two thermocouples was sampled with a corer. The cored meat was put into a filter stomacher bag filled with 20 ml of iced peptone water and stomached for 2 min; viable cell counts were determined by surface plating as indicated above.

2.7 Statistical analysis.

Experiments were performed in biological triplicates. All data are expressed as means ± SD. Differences between variables were tested for significance by one-way or two-way ANOVA with Least Significant Difference (LSD) test using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) for Windows 8.1. Differences at $P \leq 0.05$ were considered to be significant.

3. Results

3.1 Effect of NaCl on survival of *E. coli* after grilling of beef patties

The effect of NaCl on survival of *E. coli* was assessed in beef patties containing different NaCl levels that were cooked to a core temperature of 71 °C. In burger patties without NaCl, LHR-positive strains of *E. coli* were more resistant than LHR-negative *E. coli*. Addition of 3 % NaCl did not alter the heat resistance of LHR-positive *E. coli* (Fig. 3 A), however, NaCl addition increased the survival of LHR-negative *E. coli* and STEC and the

survival of LHR-negative STEC in patties with 3 % NaCl was equivalent to that of LHR-positive strains (Fig. 3 B**).**

3.2 Effect of NaCl on survival of *E. coli* stored or treated at different temperatures prior to grilling.

Initial experiments mixed cells of *E. coli* with NaCl and meat at a temperature of 15 – 20 °C, i.e. at a temperature that supports a physiological response of *E. coli*. To determine whether an alteration of the sequence NaCl addition, cooling and refrigerated storage impacts heat resistance, patties were inoculated with different protocols (Fig. 1). Temperature profiles of meat handled with protocols B and D are shown in Fig. 4. Simultaneous addition of non-refrigerated cultures and NaCl to refrigerated meat resulted in a temperature of about 20 °C (Fig. 4A). In contrast, refrigeration cultures of *E. coli* prior to mixing with beef and separate addition of NaCl maintained the temperature below 15 °C (Fig. 4B). To minimize the temperature variation during grilling of patties, the temperature was monitored with two thermocouples. Grilling of burgers reduced cell counts of *E. coli* AW1.7ΔpHR1(pLHR) by 3.5 $\log_{10}$ (CFU/g) irrespective of the presence of NaCl or the protocol used for inoculation (Table 2). In contrast, surviving cells of *E. coli* AW1.7ΔpHR1(pRK767) were not detectable after grilling of burgers without salt, regardless of the protocol used for inoculation. Addition of NaCl protected *E. coli* AW1.7ΔpHR1(pRK767) only in protocols A, B and C, i.e. when cells were kept at a temperature of more than 15°C during or after addition of NaCl (Table 2).

3.3 Effect of NaCl on survival of EHEC in grilled burger patties

To assess effect of NaCl on the heat resistance of EHEC in ground beef, a 5-strain cocktail of EHEC was inoculated into ground beef with 0 % or 3 % NaCl using protocol A (cells at

~ 20 °C at NaCl addition) or protocol D (cells at < 10 °C at NaCl addition) (Fig. 5). Similar to LHR-negative non-pathogenic *E. coli*, addition of 3 % NaCl did not decrease the thermal inactivation of EHEC unless cells, NaCl and meat were mixed at a temperature of more than 15 °C (Fig. 5). Cooling cells to 4 °C in raw meat for 12 h prior to NaCl addition eliminated protective effect of NaCl against heat (Fig. 5).

3.4 Effect of other additives on the heat resistance of *E. coli* in burger patties.

To determine whether other additives have a comparable effect as NaCl (marinade, potassium lactate) or can enhance thermal inactivation (carvacrol, chitosan), survival of *E. coli* in cooked patties was also evaluated using protocol A with *E. coli* AW1.7ΔpHR1(pLHR) and AW1.7ΔpHR1(pRK767) (Fig. 6A and B). Chitosan enhanced thermal inactivation of heat resistant strains (P<0.05, Fig. 6A) while other additives had no effect on survival (Fig. 6A). Cell counts of *E. coli* AW1.7ΔpHR1(pRK767) were reduced to levels below the detection limit in cooked patties (Fig. 6B); however, after addition of Teriyaki marinade, cell counts of this strain was reduced only by 5 – 6 $\log_{10}$(CFU/g).

3.5 Effect of chitosan on the heat resistance of *E. coli* in ground beef patties during 2 d cold storage.

To further validate the effect of chitosan on survival of *E. coli* in beef patties, LHR positive and negative isogenic strains of *E. coli* were inoculated into beef patties with or without chitosan, followed by refrigerated storage and cooking (protocol E). Cell counts remained essentially stable throughout the 2 d of storage irrespective of the presence of the LHR, or the addition of chitosan. Grilling reduced cell counts of *E. coli* AW1.7ΔpHR1(pRK767) to levels below the detection limit irrespective of the presence of chitosan (Fig. 7). Cell counts

247 of *E. coli* AW1.7ΔpHR1(pLHR) remained at about 4 $\log_{10}$(cfu/g) after cooking to 71°C;
248 the addition of chitosan reduced cell counts of this strain by 0.5 to 1 $\log_{10}$(CFU/g) (Fig. 7).

249 **4. Discussion**

250 Addition of 3% NaCl increased heat resistance in LHR-negative strain of *E. coli* strain and
251 EHEC cocktails if bacterial cells were combined with NaCl and meat at more than 15 °C.
252 The resulting inactivation of EHEC was less than 5 $\log_{10}$(CFU/g) after cooking to
253 recommended temperatures. To eliminate the risk of EHEC, a full lethality by 5
254 $\log_{10}$(CFU/g) cell reduction is required by the Canadian Food Inspection Agency (CFIA)
255 (CFIA, 2015). Increased heat resistance of *E. coli* was previously reported in laboratory
256 model systems, or in beef heated to 55.0-62.5 °C (Juneja et al., 2015; Mercer et al., 2017;
257 Pleitner et al., 2012). Our study validates the effect of NaCl on survival of *E. coli* including
258 EHEC in meat cooked according to the recommended cooking guidelines.

259 Accumulation of compatible solutes in response to hyperosmotic conditions, such as the
260 presence of NaCl, increases resistance of bacteria to multiple environmental insults
261 (Pleitner et al., 2012; Sleator and Hill, 2010). Compatible solutes protect bacterial proteins
262 and ribosomes against heat denaturation (Herberhold et al., 2004; Pleitner et al., 2012;
263 Ruan et al., 2003). Osmolytes that are accumulated in response to osmotic stress are
264 preferentially excluded from the hydration shell of proteins, and increase the stability of
265 proteins (Parsegian et al., 2000; Timasheff, 2002). The protective effect of NaCl against
266 heat-induced inactivation of *E. coli* including EHEC in ground beef may be attributed to
267 the accumulation of compatible solutes in response to an increased osmotic stress.
268 Remarkably, the LHR enhanced the effect of osmotic stress caused by addition of 3 %
269 NaCl in protecting cells from thermal inactivation in media (Mercer et al., 2017; Pleitner

et al., 2012) but not in ground beef (this study), indicating that NaCl and the accumulation of compatible solutes provide no incremental protection if LHR-positive strains are heated in a protective meat matrix. The protective effect of NaCl was observed only if bacterial cells adapted to the osmotic upshock at a temperatures above 15 °C, highlighting that a physiological response of *E. coli* to osmotic shock is required for improved survival of *E. coli* in meat after cooking. Low temperature likely decreases or eliminates the capacity of *E. coli* to respond to NaCl stress by accumulation of compatible solutes (Jones and Inouye 1994), hence eliminating the protective effect of NaCl against heat. Adaptation of *E. coli* to temperatures below 15 °C also alters membrane properties and consequently decreases the heat resistance of *E. coli* (Cao-Hoang et al., 2010; Katsui et al., 1981). These findings may additionally explain why the protective effect of NaCl against heat was not observed for the cold-shocked cells in this study.

Teriyaki marinade exhibited a similar protective effects against heat in as NaCl, in keeping with the assumption that osmotic shock relates to heat resistance. Addition of 1.8% (w/w) potassium lactate did not alter the thermal inactivation of *E. coli*. Likewise, addition of lactate at the level of 1.8%-4.5% did not affect survival of *E. coli* in meat cooked by 55-65 °C (Huang and Juneja, 2003; Mukherjee et al., 2008). Meat with a low pH of 5.5, such as raw ground beef, already contains about 0.9% of lactic acid (Pothast and Hamm, 1976). Carvacrol at the level of 0.5 to 1.0% (v/w) increased inactivation of *E. coli* O157:H7 in ground beef (Juneja and Friedman, 2008), while carvacrol at 0.1% (v/w) has no effect (this study). The dose-dependent bactericidal effect of essential oils needs to be balanced with their impact on food flavour (Jayasena and Jo, 2013).

Chitosan potentiated the thermal injury and inactivation of LHR-positive *E. coli* irrespective of the protocol used for application of NaCl, cells, and chitosan. Chitosan with molecular weight of 1.5 kDa also enhanced thermal inactivation of EHEC in ground beef patties stored at 4 °C for 5 days by around 2 $\log_{10}$ (CFU/g), and the combination of rutin or resveratrol with chitosan was more effective, enhancing thermal destruction of EHEC by 5 $\log_{10}$(CFU/g) (Surendran Nair et al., 2016). Chitosan is protonated when the ambient pH is below its pKa of 6.2-7.0 (Tsai and Su, 1999). Polycationic chitosan disrupts the integrity of negatively charged cell envelope of *E. coli*, including lipopolysaccharides (LPS) and cytoplasmic membrane, through electrostatic interactions (Helander et al. 2001, Liu et al. 2004; Mellegård et al. 2011). This study demonstrated an effect of high molecular weight chitosan on thermal destruction of LHR-positive *E. coli* in ground beef, characterizing chitosan as an effective hurdle concept that can be combined with the cooking process recommended currently to reduce the potential risk of LHR-positive pathogen to meat safety.

In conclusion, the present study demonstrated that addition of 3% NaCl increased heat resistance in LHR-negative isogenic *E. coli* strain and EHEC cocktails in ground beef if bacterial cells were well adapted to osmotic stress, resulting in cell reductions for EHEC by less than 5 $\log_{10}$(CFU/g) after the cooking process recommended as a safe handling. These highlight that salt addition in meat may incur the generation of heat resistant EHEC, thus creating an additional risk to meat safety. Nevertheless, the protective effect of NaCl was not observed if bacterial cells were cooled to 4 °C prior to mixing with cold meat and NaCl, indicating that the response of *E. coli* to osmotic shock contributes to this effect. Therefore, chilling the meat prior to salt addition in conjunction with a subsequent chilling

process after salt addition is proposed to mitigate the protective effect of NaCl against heat. Chitosan potentiated the thermal destruction of LHR-positive *E. coli* in ground beef stored at 4 °C for 2 days, indicating that the combination of chitosan with the cooking process recommended currently has the potential to reduce the potential risk of pathogenic *E. coli* to meat safety.

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453

454 **Figure 1.** Protocols used for inoculation of beef patties, addition of salt, and heat treatment
455 to study the effect of NaCl on survival of *E. coli* in ground beef.
456 **Figure 2.** Core temperature profile the of un-inoculated ground beef patties during grilling
457 process. Data were calculated as average of two temperature probes placed 0.5 cm from
458 the geometic centre of the patty and are presented as means ± standard deviation of
459 quintuplicate independent experiments.
460 **Figure 3.** Reduction of heat resistant *E. coli* (Panel A) and heat sensitive *E. coli* (Panel B)
461 before and after grilling the burger to 71 °C core temperature with 0%, 1% and 3% NaCl
462 addition. Samples were prepared following protocol A, and the whole grilled burger was
463 sampled for enumeration. Cell counts were enumerated on LB agar. Heat resistant strains
464 in panel A: *E. coli* AW1.7 (grey dot), *E. coli* AW1.7ΔpHR1(pLHR) (black triangle), and
465 *E. coli* GM14.3 (grey square). Heat sensitive strains in panel B: *E. coli* AW1.7ΔpHR1
466 (white dot), *E. coli* AW1.7ΔpHR1(pRK767) (white triangle), and *E. coli* AW1.3 (white
467 square). Data are presented as means ± standard deviation of triplicate independent
468 experiments. Cell counts that are different from the cell counts in burgers with 0% NaCl
469 group are indicated by an asterisk ($P<0.05$).
470 **Figure 4.** Temperature profile of raw samples treated with Protocol B (Panel A) or Protocol
471 D (Panel B) shown in Figure 1. In Protocol B, after *E. coli* cells that were washed at 23-
472 25 °C, NaCl and raw ground beef were mixed together and stomached for 2 min, one
473 thermal probe was inserted into the geometric centre of raw patty to monitor internal
474 temperature of raw patty for 35 minutes at 23-25 °C. In Protocol D, after raw ground beef
475 was inoculated with *E. coli* cells washed at 4 °C, raw patty was stored at 4 °C and one
476 thermal probe was inserted into the geometric centre of raw patty to monitor internal

477 temperature of raw patty for 720 min prior to NaCl addition (Panel B, from 5 min to 720
478 min). After refrigerated storage, NaCl was mixed with cold patty and stomached for 2 min
479 at 23-25 °C (Panel B, from 720 min to 725 min), and then internal temperature of raw patty
480 was further monitored as above for 35 min at 23-25 °C (Panel B, from 730 min to 760 min).
481 **Figure 5.** Effect of NaCl on survival of EHEC in burgers grilled to a core temperature of
482 71 °C. Samples were prepared and enumerated following protocol A (white bar) or protocol
483 D (grey bar) shown in Fig. 1. In each protocol, core part of grilled burger (10 g) was
484 sampled for enumeration. Cell reduction before and after grilling were determined on LB
485 agar. Data are presented as means ± standard deviation of triplicate independent
486 experiments. Cell counts that are different from the cell counts in burgers with 0% NaCl
487 are indicated by an asterisk ($P<0.05$).
488 **Figure 6.** Reduction of *E. coli* AW1.7ΔpHR1(pLHR) (Panel A) and *E. coli*
489 AW1.7ΔpHR1(pRK767) (Panel B) before and after grilling the burgers with different
490 additives to 71 °C core temperature. Core part of grilled burger (10 g) was sampled for
491 enumeration. Cell counts were enumerated on LB agar (white bar) and VRB agar (grey
492 bar). Data are presented as means ± standard deviation of triplicate independent
493 experiments. Cell reduction that are different from cell reduction in burgers without
494 additives are indicated by an asterisk ($P<0.05$).
495 **Figure 7.** Effect of chitosan on the survival of *E. coli* AW1.7ΔpHR1(pRK767) (non-
496 hatched bar) and *E. coli* AW1.7ΔpHR1(pLHR) (hatched bar) in burgers during 2d of
497 storage at 4 °C. Cell counts were enumerated on LB agar (Panel A) and VRB agar (Panel
498 B). Core part of grilled burger (10 g) was sampled for enumeration. Cell counts of
499 inoculated samples without chitosan and inoculated samples with chitosan were shown as

white bar and grey bar, respectively. Data are presented as means ± standard deviation of triplicate independent experiments. Cell counts that are significantly lower than the cell counts of cooked samples without additives are indicated by an asterisk ($P<0.05$).

Table 1. *E. coli* strains used in this study

Strains	Description	Reference
AW1.7	LHR[a)]-positive wild type isolate from carcass	Dlusskaya et al. (2011)
AW1.7ΔpHR1	LHR-negative, heat sensitive derivative of AW1.7	Pleitner et al. (2012)
AW1.7ΔpHR1(pLHR)	Transgenic LHR-positive derivative of AW1.7	Mercer et al. (2015)
AW1.7ΔpHR1(pRK767)	Transgenic LHR-negative derivative of AW1.7	Mercer et al. (2015)
AW1.3	Wild type isolate from carcass	Aslam et al. (2003)
GM14.3	Wild type isolate from ground meat	Aslam et al. (2003)
O157:H7; C0283	enterohaemorrhagic *E. coli*	Liu et al. (2012)
O145:NM; 03-6430	enterohaemorrhagic *E. coli*	Liu et al. (2012)
O26:H11; 05-6544	enterohaemorrhagic *E. coli*	Liu et al. (2012)
O121:H19; 03-2832	enterohaemorrhagic *E. coli*	Liu et al. (2012)
O145:NM; PARC 449	enteropathogenic *E. coli*	Mercer et al. (2015)

[a)] LHR, locus of heat resistance

Table 2. Effect of different protocols on survival of *E. coli* AW1.7ΔpHR1(pLHR) and *E. coli* AW1.7ΔpHR1(pRK767) in burger with NaCl.

	E. coli AW1.7ΔpHR1(pLHR)	*E. coli* AW1.7ΔpHR1(pRK767)
Treatment	Cell reduction after grilling [$\log_{10}$ (CFU/g)]	
Addition of culture and NaCl together (A)[a)]		
Control	3.4 ± 0.3	> 6.1
3% NaCl	3.7 ± 0.2	5.1±0.4
Addition of washed cell cultures and NaCl together (B)		
Control	3.5 ± 0.3	> 6.1
3% NaCl	3.4 ± 0.4	5.3 ± 0.7
Addition of cell culture and NaCl together, followed by storage at 4°C for 2 d (C)		
Control	ND	> 6.1
3% NaCl	ND	5.8 ± 0.6
Addition of washed cultures, followed by storage at 4°C for 12 h and addition of NaCl (D)		
Control	ND	> 6.1
3% NaCl	ND	> 6.1
Addition of washed cultures, followed by storage at 4°C for 12 h, addition of NaCl and storage at 4°C for 2 d (E)		
Control	3.7 ± 0.3	> 6.1
3% NaCl	3.8 ± 0.2	> 6.1

Detection limit: 1.6 $\log_{10}$ (CFU/g).

"ND" indicates "not determined"

[a)] The protocols used for treatment are described in detail in Fig. 1 and the corresponding Materials and methods section. In each protocol, inoculated samples without NaCl addition were considered as control group, and core part of burger (10 g) was sampled for enumeration after core temperature of burger reached 71°C. Cell reduction before and after grilling were determined on LB agar.

Figure 1.

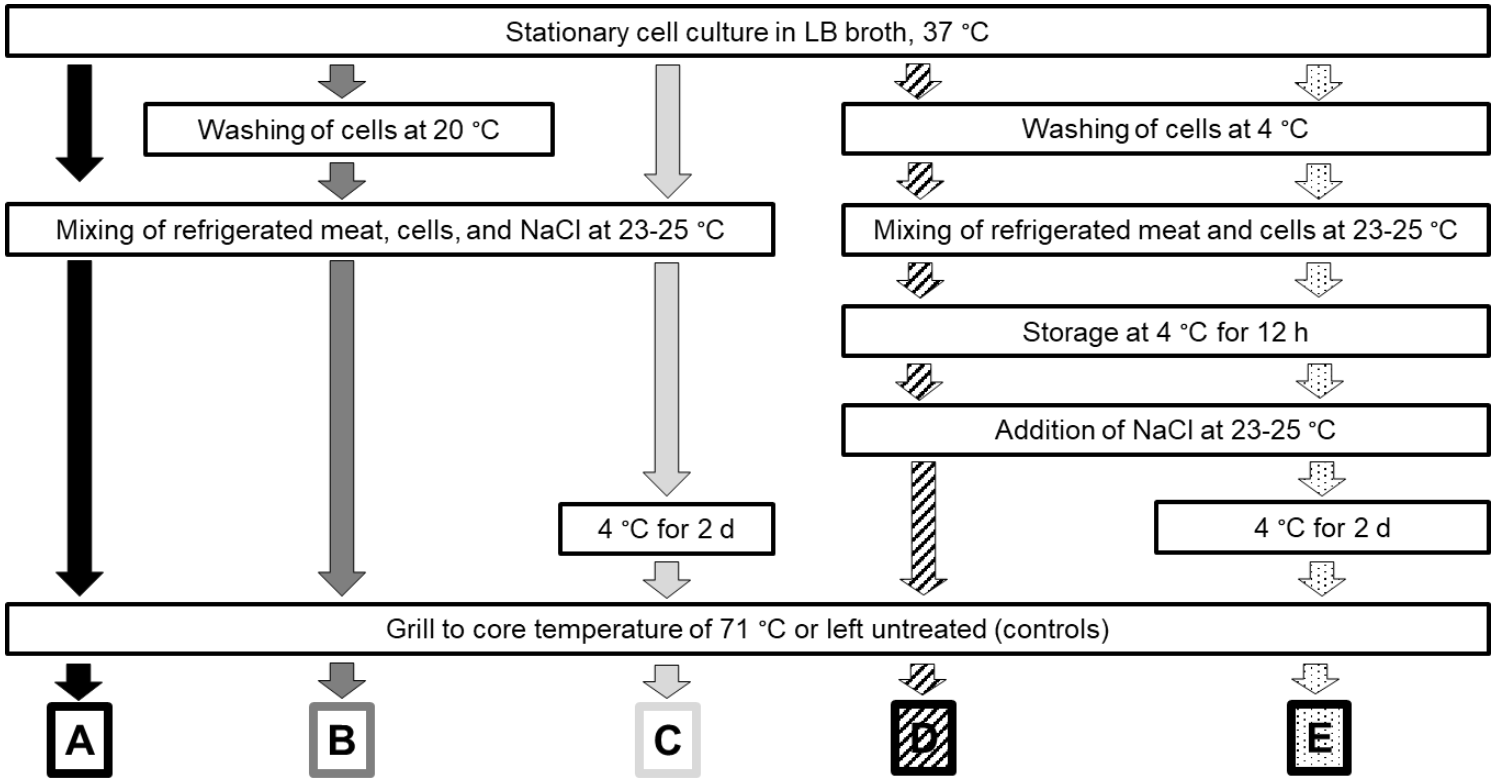

Stationary cell culture in LB broth, 37 °C
Washing of cells at 20 °C
Washing of cells at 4 °C
Mixing of refrigerated meat, cells, and NaCl at 23-25 °C
Mixing of refrigerated meat and cells at 23-25 °C
Storage at 4 °C for 12 h
Addition of NaCl at 23-25 °C
4 °C for 2 d
4 °C for 2 d
Grill to core temperature of 71 °C or left untreated (controls)
A
B
C
D
E

Figure 2

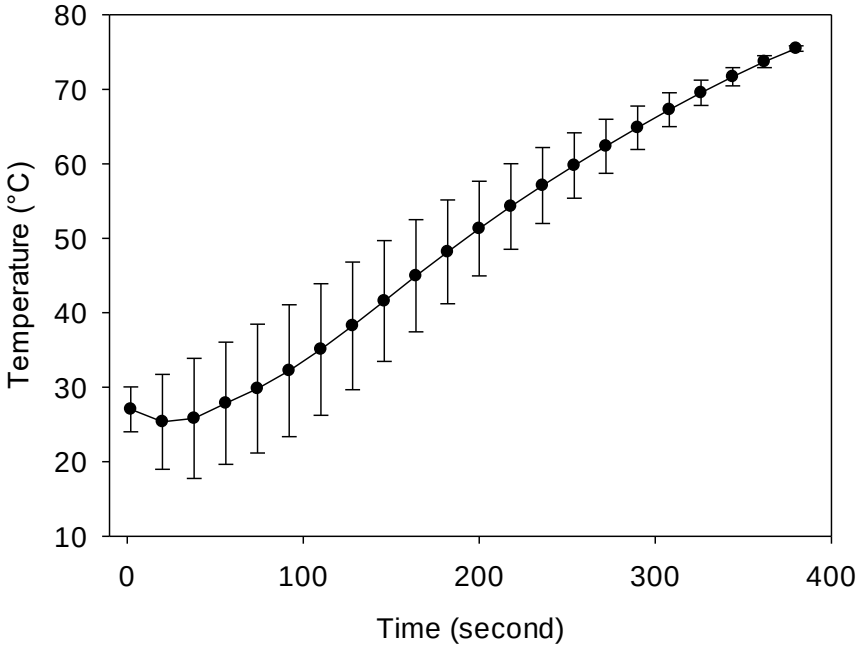

80
70
60
50
40
30
20
10
Temperature (°C)
0
100
200
300
400
Time (second)

Figure 3

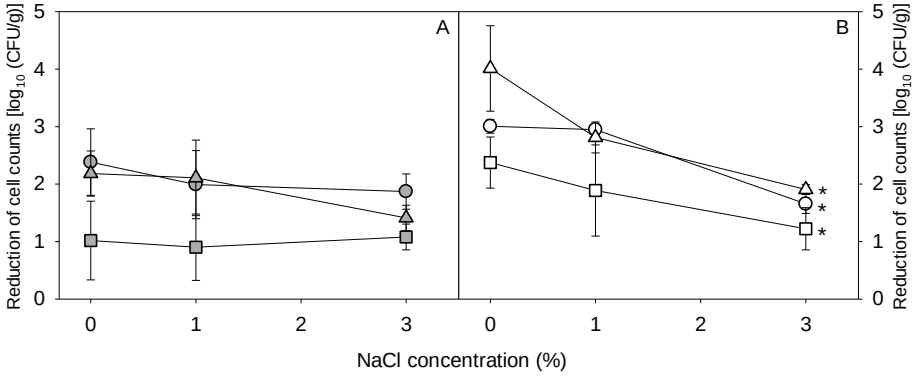

A
B
Reduction of cell counts [log10 (CFU/g)]
Reduction of cell counts [log10 (CFU/g)]
NaCl concentration (%)
0
1
2
3
4
5
*
*
*

Figure 4

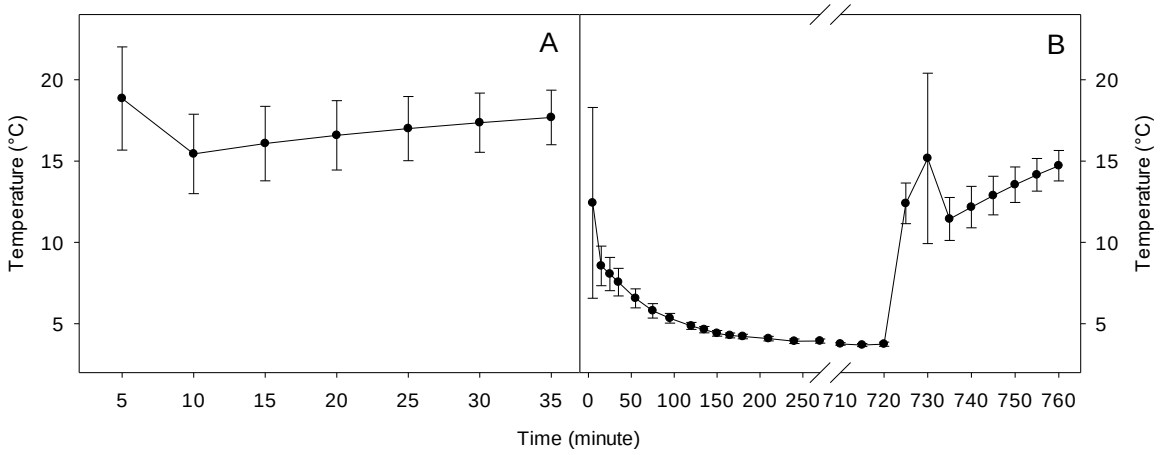

A
B
Temperature (°C)
Temperature (°C)
20
15
10
5
5 10 15 20 25 30 35
0 50 100 150 200 250 710 720 730 740 750 760
Time (minute)

Figure 5

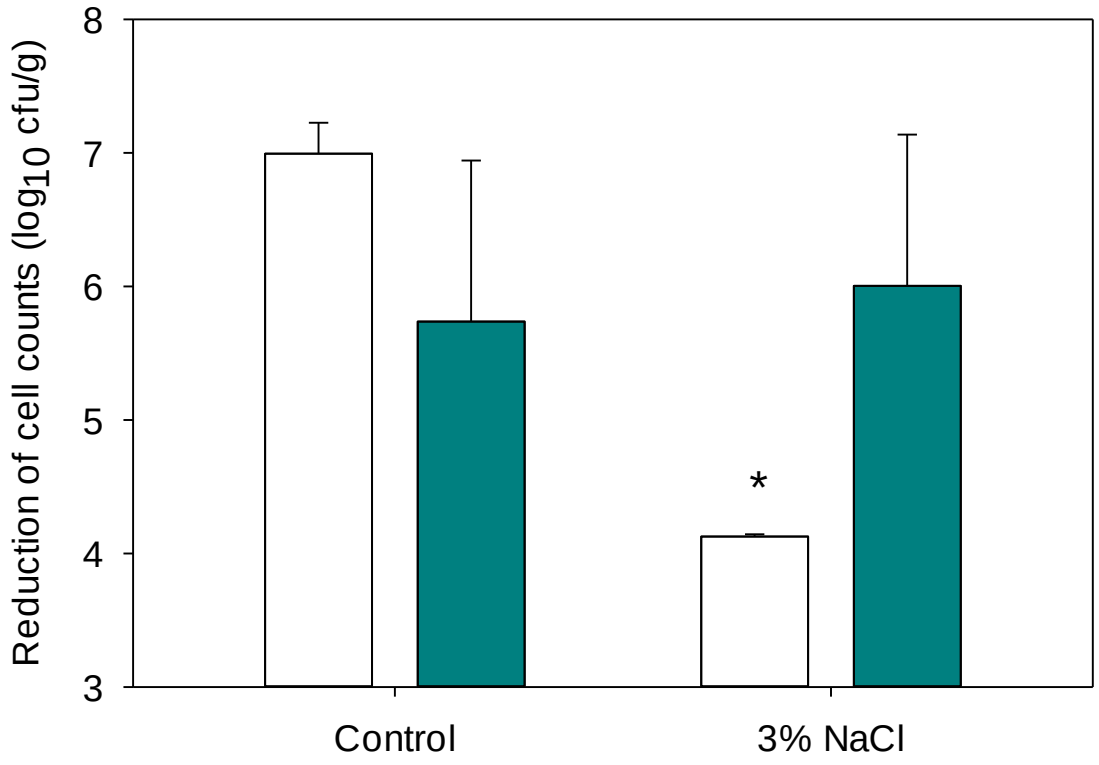


Figure 6

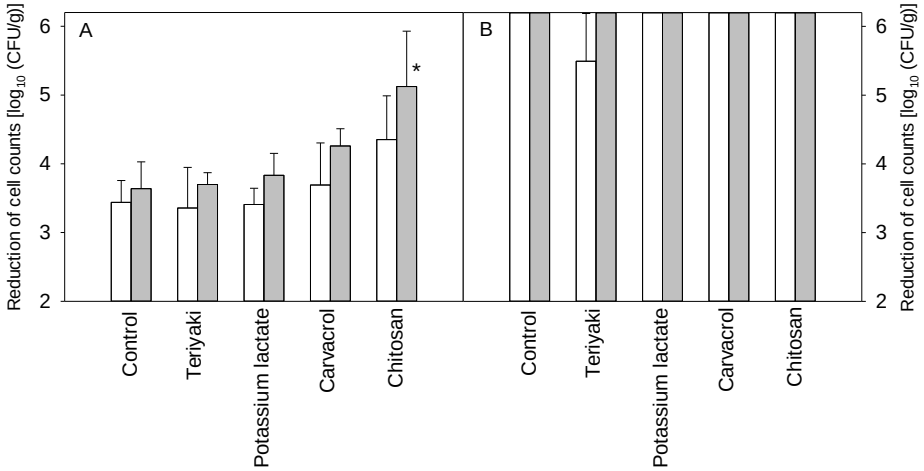
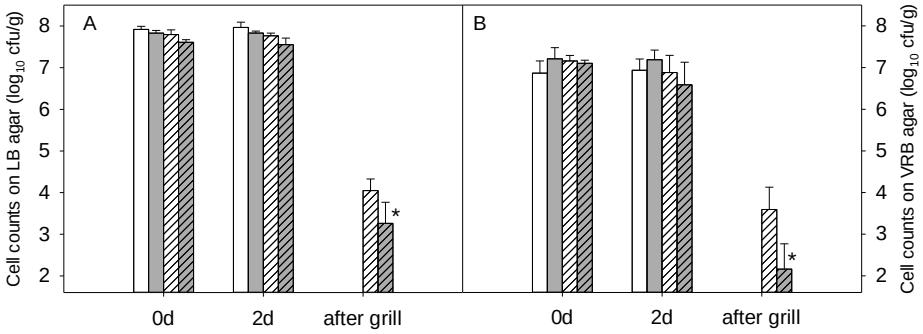

A
B
Reduction of cell counts [log10 (CFU/g)]
Reduction of cell counts [log10 (CFU/g)]
2
3
4
5
6
*
Control
Teriyaki
Potassium lactate
Carvacrol
Chitosan
Control
Teriyaki
Potassium lactate
Carvacrol
Chitosan

Figure 7


A
B
Cell counts on LB agar (log10 cfu/g)
Cell counts on VRB agar (log10 cfu/g)
8
7
6
5
4
3
2
*
*
0d
2d
after grill
0d
2d
after grill