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

Ziyi Hu^a, Alina Rohde^a, Lynn McMullen^a, Michael Gänzle^{a,b,*}^a University of Alberta, Dept. of Agricultural, Food and Nutritional Science, Edmonton, Canada^b Hubei University of Technology, College of Bioengineering and Food Science, Wuhan, PR China

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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 $> 3 \log_{10}$ (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.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) can cause substantial morbidity and mortality even after ingestion of < 10 cells (Croxen et al., 2013; Paton et al., 1996; Tilden Jr 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 et al., 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 $D_{60^\circ\text{C}}$ values of < 1 min, LHR-positive *E. coli* have $D_{60^\circ\text{C}}$ values of 15 to > 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 $< 2 \log_{10}$ (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

* Corresponding author at: University of Alberta, Dept. of Agricultural, Food and Nutritional Science, 4-10 Ag/For Centre, Edmonton T6G 2P5, AB, Canada.

E-mail address: mgaenzle@ualberta.ca (M. Gänzle).

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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 uninoculated 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 $3.5 \pm 0.9 \log_{10}$ (CFU/g) and $2.3 \pm 0.3 \log_{10}$ (CFU/g), respectively.

Chitosan with high molecular weight (210 kDa) was supplied by Yuhan Ocean Biochemistry Co. Ltd. (Taufzhou, 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 $5311 \times g$ 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

The effects of other additives on the heat resistance of *E. coli* in ground beef were assessed with the protocol A (Fig. 1). Teriyaki powder was mixed with sterile water at the ratio of 5.7:11 by weight to create marinade solution; this marinade solution was then mixed with 10 mL of stationary culture and 200 g ground beef. The composition of the final raw burger was 83.3% meat, 11% water, and 5.7% teriyaki powder by weight. The effect of carvacrol and potassium lactate was investigated by inoculation of 200 g ground beef with 10 mL of stationary culture. Carvacrol was dissolved in ethanol at the ratio of 1:1 by volume, dispersed in teriyaki marinade, and the carvacrol / teriyaki marinade then mixed with inoculated ground beef at a ratio of 16.7:83.3 (marinade solution: meat). The concentration of additives was chosen on the basis of their current commercial use (potassium lactate, marinade) or their in vitro MIC against *Escherichia coli* (chitosan, carvacrol). The resulting final concentration of carvacrol was 0.10%. Potassium L-lactate solution was mixed with inoculated ground beef at a ratio of 3:100 (v/w). Chitosan solution (1%) was mixed with

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 <i>E. coli</i> | Liu et al. (2012) |
| O145:NM; 03-6430 | Enterohaemorrhagic <i>E. coli</i> | Liu et al. (2012) |
| O26:H11; 05-6544 | Enterohaemorrhagic <i>E. coli</i> | Liu et al. (2012) |
| O121:H19; 03-2832 | Enterohaemorrhagic <i>E. coli</i> | Liu et al. (2012) |
| O145:NM; PARC 449 | Enteropathogenic <i>E. coli</i> | Mercer et al. (2015) |

^a LHR, locus of heat resistance.

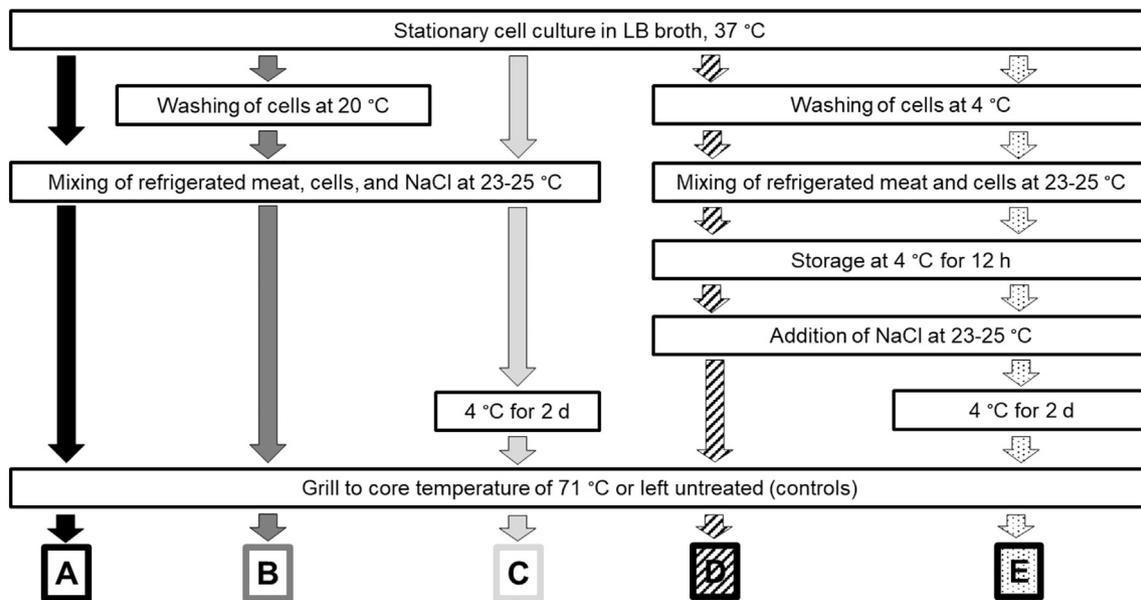


Fig. 1. Protocols used for inoculation of beef patties, addition of salt, and heat treatment to study the effect of NaCl on survival of *E. coli* in ground beef.

200 g ground beef, followed by mixing with 10 mL of stationary culture. Mixing of ground beef, cells, and additives was achieved by stomaching for 2 min; after 35 min at 23–25 °C, beef patties were grilled as described below.

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

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

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

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

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

Treatment lethality was calculated as follows:

$$\text{reduction of cell counts} = \log(N_0) - \log(N_t)$$

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

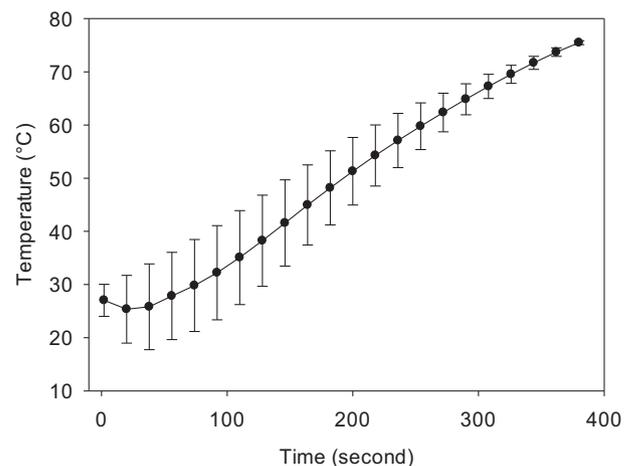


Fig. 2. Core temperature profile of the un-inoculated ground beef patties during grilling process. Data were calculated as average of two temperature probes placed 0.5 cm from the geometric centre of the patty and are presented as means \pm standard deviation of quintuplicate independent experiments.

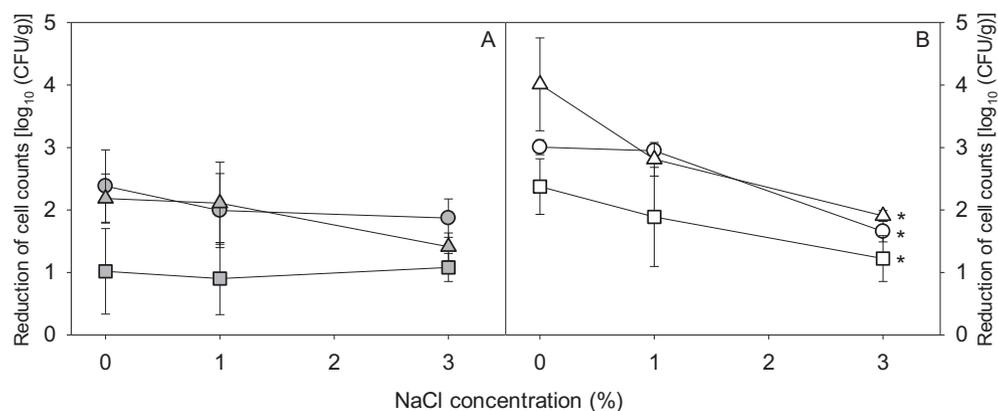


Fig. 3. Reduction of heat resistant *E. coli* (Panel A) and heat sensitive *E. coli* (Panel B) before and after grilling the burger to 71 °C core temperature with 0%, 1% and 3% NaCl addition. Samples were prepared following protocol A, and the whole grilled burger was sampled for enumeration. Cell counts were enumerated on LB agar. Heat resistant strains in panel A: *E. coli* AW1.7 (grey dot), *E. coli* AW1.7ΔpHR1(pLHR) (black triangle), and *E. coli* GM14.3 (grey square). Heat sensitive strains in panel B: *E. coli* AW1.7ΔpHR1 (white dot), *E. coli* AW1.7ΔpHR1(pRK767) (white triangle), and *E. coli* AW1.3 (white square). Data are presented as means ± standard deviation of triplicate independent experiments. Cell counts that are different from the cell counts in burgers with 0% NaCl group are indicated by an asterisk ($P < 0.05$).

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. 3A), 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. 3B).

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 log₁₀ (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 > 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 > 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 log₁₀(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 of *E. coli* AW1.7ΔpHR1(pLHR) remained at about 4 log₁₀(cfu/g) after cooking to 71 °C; the addition of chitosan reduced cell counts of this strain by 0.5 to 1 log₁₀(CFU/g) (Fig. 7).

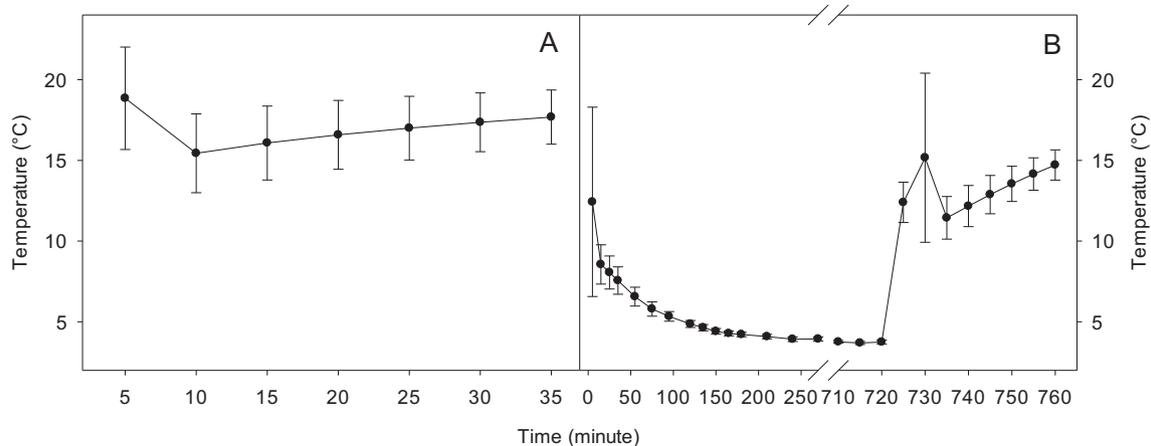


Fig. 4. Temperature profile of raw samples treated with Protocol B (Panel A) or Protocol D (Panel B) shown in Fig. 1. In Protocol B, after *E. coli* cells that were washed at 23–25 °C, NaCl and raw ground beef were mixed together and stomached for 2 min, one thermal probe was inserted into the geometric centre of raw patty to monitor internal temperature of raw patty for 35 min at 23–25 °C. In Protocol D, after raw ground beef was inoculated with *E. coli* cells washed at 4 °C, raw patty was stored at 4 °C and one thermal probe was inserted into the geometric centre of raw patty to monitor internal temperature of raw patty for 720 min prior to NaCl addition (Panel B, from 5 min to 720 min). After refrigerated storage, NaCl was mixed with cold patty and stomached for 2 min at 23–25 °C (Panel B, from 720 min to 725 min), and then internal temperature of raw patty was further monitored as above for 35 min at 23–25 °C (Panel B, from 730 min to 760 min).

Table 2

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

| | <i>E. coli</i> AW1.7ΔpHR1(pLHR) | <i>E. coli</i> 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.

4. Discussion

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

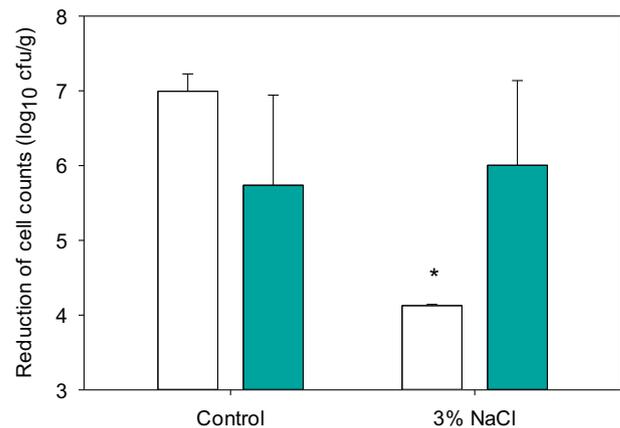


Fig. 5. Effect of NaCl on survival of EHEC in burgers grilled to a core temperature of 71 °C. Samples were prepared and enumerated following protocol A (white bar) or protocol D (grey bar) shown in Fig. 1. In each protocol, core part of grilled burger (10 g) was sampled for enumeration. Cell reduction before and after grilling were determined on LB agar. Data are presented as means ± standard deviation of triplicate independent experiments. Cell counts that are different from the cell counts in burgers with 0% NaCl are indicated by an asterisk ($P < 0.05$).

in meat cooked according to the recommended cooking guidelines.

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

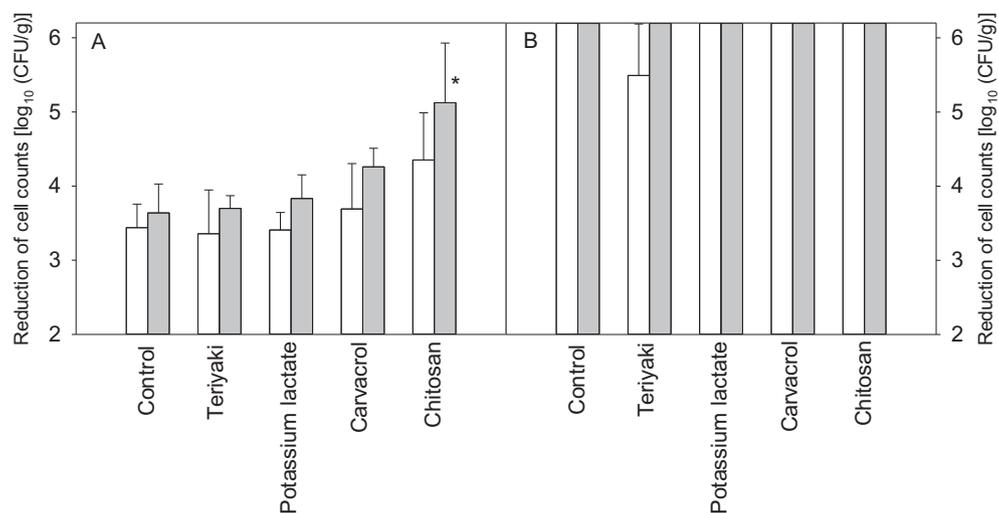


Fig. 6. Reduction of *E. coli* AW1.7ΔpHR1(pLHR) (Panel A) and *E. coli* AW1.7ΔpHR1(pRK767) (Panel B) before and after grilling the burgers with different additives to 71 °C core temperature. Core part of grilled burger (10 g) was sampled for enumeration. Cell counts were enumerated on LB agar (white bar) and VRB agar (grey bar). Data are presented as means ± standard deviation of triplicate independent experiments. Cell reduction that are different from cell reduction in burgers without additives are indicated by an asterisk ($P < 0.05$).

NaCl was observed only if bacterial cells adapted to the osmotic up-shock 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 flavor (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 log₁₀ (CFU/g), and the combination of rutin or resveratrol with chitosan was more effective, enhancing thermal destruction of EHEC by 5 log₁₀(CFU/g) (Surenran 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 < 5 log₁₀(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

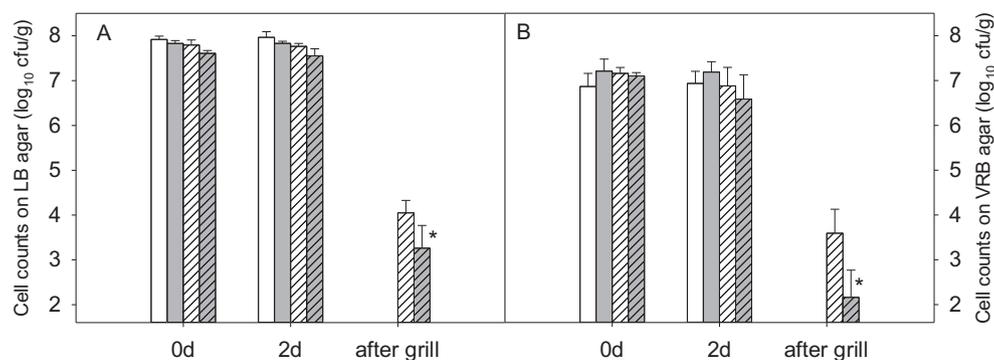


Fig. 7. Effect of chitosan on the survival of *E. coli* AW1.7ΔpHR1(pRK767) (non-hatched bar) and *E. coli* AW1.7ΔpHR1(pLHR) (hatched bar) in burgers during 2d of storage at 4 °C. Cell counts were enumerated on LB agar (Panel A) and VRB agar (Panel B). Core part of grilled burger (10 g) was sampled for enumeration. Cell counts of 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 ($P < 0.05$).

potential risk of pathogenic *E. coli* to meat safety.

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References

- Aslam, M., Nattress, F., Greer, G., Yost, C., Gill, C., McMullen, L.M., 2003. Origin of contamination and genetic diversity of *Escherichia coli* in beef cattle. *Appl. Environ. Microbiol.* 69, 2794–2799.
- Aslam, M., Greer, G., Nattress, F., Gill, C., McMullen, L.M., 2004. Genotypic analysis of *Escherichia coli* recovered from product and equipment at a beef-packing plant. *J. Appl. Microbiol.* 97, 78–86.
- Boll, E.J., Marti, R., Hasman, H., Overballe-Petersen, S., Stegger, M., Ng, K., Knöchel, S., Krogfelt, K.A., Hummerjohann, J., Struve, C., 2017. Turn up the heat—food and clinical *Escherichia coli* isolates feature two transferrable loci of heat resistance. *Front. Microbiol.* 8, 579.
- Canadian Food Inspection Agency (CFIA), 2015. Annex O: policy on the control of *E. coli* O157:H7/NM contamination in raw beef products. Available at: <http://www.inspection.gc.ca/food/meat-and-poultry-products/manual-of-procedures/chapter-4/annex-o/eng/1370616273137/1370616333827>.
- Cao-Hoang, L., Dumont, F., Marechal, P.A., Gervais, P., 2010. Inactivation of *Escherichia coli* and *Lactobacillus plantarum* in relation to membrane permeabilization due to rapid chilling followed by cold storage. *Arch. Microbiol.* 192, 299–305.
- Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M., Finlay, B.B., 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clin. Microbiol. Rev.* 26, 822–880.
- Dlusskaya, E., McMullen, L.M., Gänzle, M.G., 2011. Characterization of an extremely heat-resistant *Escherichia coli* obtained from a beef processing facility. *J. Appl. Microbiol.* 110, 840–849.
- FDA, 2011. Agency response letter GRAS notice no. GRN 000397. Available at: <https://wayback.archive-it.org/7993/20170606195036/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm287638.htm>.
- FDA, 2018. Substances added to food (formerly EAFUS) and Carvacrol. Available at: <https://www.accessdata.fda.gov/scripts/fdcc/?set=FoodSubstances&id=CARVACROL&sort=Sortterm&order=ASC&startrow=1&type=basic&search=carvacrol>.
- Ferens, W.A., Hovde, C.J., 2011. *Escherichia coli* O157: H7: animal reservoir and sources of human infection. *Foodborne Pathog. Dis.* 8, 465–487.
- Gill, C., 2009. Effects on the microbiological condition of product of decontaminating treatments routinely applied to carcasses at beef packing plants. *J. Food Prot.* 72, 1790–1801.
- Health Canada, 2015. Available at: <http://www.hc-sc.gc.ca/fn-an/legislation/guide-ld/guide-cook-cuiss-meat-viand-eng.php>.
- Helander, I., Nurmiaho-Lassila, E., Ahvenainen, R., Rhoades, J., Roller, S., 2001. Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. *Int. J. Food Microbiol.* 71, 235–244.
- Herberhold, H., Royer, C.A., Winter, R., 2004. Effects of chaotropic and kosmotropic cosolvents on the pressure-induced unfolding and denaturation of proteins: an FT-IR study on staphylococcal nuclease. *Biochemistry* 43, 3336–3345.
- Hu, Z.Y., Gänzle, M.G., 2019. Challenges and opportunities related to the use of chitosan as food preservative. *J. Appl. Microbiol.* 126, 1318–1331.
- Huang, L., Juneja, V.K., 2003. Thermal inactivation of *Escherichia coli* O157: H7 in ground beef supplemented with sodium lactate. *J. Food Prot.* 66, 664–667.
- Jayasena, D.D., Jo, C., 2013. Essential oils as potential antimicrobial agents in meat and meat products: a review. *Trends Food Sci. Technol.* 34, 96–108.
- Jin, T., Zhang, H., Boyd, G., Tang, J., 2008. Thermal resistance of *Salmonella enteritidis* and *Escherichia coli* K12 in liquid egg determined by thermal-death-time disks. *J. Food Eng.* 84, 608–614.
- Jones, P.G., Inouye, M., 1994. The cold-shock response—a hot topic. *Mol. Microbiol.* 11, 811–818.
- Juneja, V.K., Friedman, M., 2008. Carvacrol and cinnamaldehyde facilitate thermal destruction of *Escherichia coli* O157: H7 in raw ground beef. *J. Food Prot.* 71, 1604–1611.
- Juneja, V.K., Cadavez, V., Gonzales-Barron, U., Mukhopadhyay, S., 2015. Modelling the effect of pH, sodium chloride and sodium pyrophosphate on the thermal resistance of *Escherichia coli* O157:H7 in ground beef. *Food Res. Int.* 69, 289–304.
- Katsui, N., Tsuchido, T., Takano, M., Shibasaki, I., 1981. Effect of preincubation temperature on the heat resistance of *Escherichia coli* having different fatty acid compositions. *J. Gen. Microbiol.* 122, 357.
- Liu, H., Du, Y., Wang, X., Sun, L., 2004. Chitosan kills bacteria through cell membrane damage. *Int. J. Food Microbiol.* 95, 147–155.
- Liu, Y., Gill, A., Betti, M., McMullen, L.M., Gänzle, M.G., 2012. Determination of heat resistance of 101 strains of verotoxigenic *Escherichia coli*. In: Proceedings of the 58th International Congress of Meat Science and Technology, Montreal, Canada, August 12–17, pp. 1–4 SAFMICROP-35.
- Liu, Y., Gill, A., McMullen, L.M., Gänzle, M.G., 2015. Variation in heat and pressure resistance of verotoxigenic and nontoxigenic *Escherichia coli*. *J. Food Prot.* 78, 111–120.
- Low, J.C., McKendrick, L.J., McKechnie, C., Fenlon, D., Naylor, S.W., Currie, C., Smith, D.G., Allison, L., Gally, D.L., 2005. Rectal carriage of enterohemorrhagic *Escherichia coli* O157 in slaughtered cattle. *Appl. Environ. Microbiol.* 71, 93–97.
- Ma, A., Chui, L., 2017. Identification of heat resistant *Escherichia coli* by qPCR for the locus of heat resistance. *J. Microbiol. Methods* 133, 87–89.
- Mellegård, H., Strand, S., Christensen, B., Granum, P., Hardy, S., 2011. Antibacterial activity of chemically defined chitosans: influence of molecular weight, degree of acetylation and test organism. *Int. J. Food Microbiol.* 148, 48–54.
- Mercer, R.G., Zheng, J., Garcia-Hernandez, R., Ruan, L., Gänzle, M.G., McMullen, L.M., 2015. Genetic determinants of heat resistance in *Escherichia coli*. *Front. Microbiol.* 6, 932.
- Mercer, R.G., Walker, B.D., Yang, X., McMullen, L.M., Gänzle, M.G., 2017. The locus of heat resistance (LHR) mediates heat resistance in *Salmonella enterica*, *Escherichia coli* and *Enterobacter cloacae*. *Food Microbiol.* 64, 96–103.
- Mukherjee, A., Yoon, Y., Belk, K.E., Scanga, J.A., Smith, G.C., Sofos, J.N., 2008. Thermal inactivation of *Escherichia coli* O157: H7 in beef treated with marination and tenderization ingredients. *J. Food Prot.* 71, 1349–1356.
- Parsegian, V.A., Rand, R.P., Rau, D.C., 2000. Osmotic stress, crowding, preferential hydration, and binding: a comparison of perspectives. *Proc. Natl. Acad. Sci. U. S. A.* 97, 3987–3992.
- Paton, A.W., Ratcliff, R.M., Doyle, R.M., Seymour-Murray, J., Davos, D., Lanser, J.A., Paton, J.C., 1996. Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. *J. Clin. Microbiol.* 34, 1622–1627.
- Pleitner, A., Zhai, Y., Winter, R., Ruan, L., McMullen, L.M., Gänzle, M.G., 2012. Compatible solutes contribute to heat resistance and ribosome stability in *Escherichia coli* AW1.7. *Biochim. Biophys. Acta* 1824, 1351–1357.
- Pothast, K., Hamm, R., 1976. The biochemistry of DFD meat. *Fleischwirtschaft* 56, 978–982.
- Rhee, M., Lee, S., Hillers, V.N., McCurdy, S.M., Kang, D., 2003. Evaluation of consumer-style cooking methods for reduction of *Escherichia coli* O157: H7 in ground beef. *J. Food Prot.* 66, 1030–1034.
- Ruan, K., Xu, C., Li, T., Li, J., Lange, R., Balny, C., 2003. The thermodynamic analysis of protein stabilization by sucrose and glycerol against pressure-induced unfolding. *Eur. J. Biochem.* 270, 1654–1661.
- Sleator, R.D., Hill, C., 2010. Compatible solutes: A listeria passe-partout? *Gut Microbes* 1, 77–79.
- Sofos, J.N., Geornaras, I., 2010. Overview of current meat hygiene and safety risks and summary of recent studies on biofilms, and control of *Escherichia coli* O157: H7 in nonintact, and *Listeria monocytogenes* in ready-to-eat, meat products. *Meat Sci.* 86, 2–14.
- Surendran Nair, M., Lau, P., Belskie, K., Fancher, S., Chen, C., Karumathil, D.P., Yin, H., Liu, Y., Ma, F., Upadhyaya, I., 2016. Potentiating the heat inactivation of *Escherichia coli* O157: H7 in ground beef patties by natural antimicrobials. *Front. Microbiol.* 7, 15.
- Tolaimate, A., Desbrieres, J., Rhazi, M., Alagui, A., Vincendon, M., Vottero, P., 2000. On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer.* 41, 2463–2469.
- Tilden Jr, J., Young, W., McNamara, A.M., Custer, C., Boesel, B., Lambert-Fair, M.A., Majkowski, J., Vugia, D., Werner, S.B., Hollingsworth, J., Morris Jr, J.G., 1996. A new route of transmission for *Escherichia coli*: infection from dry fermented salami. *Am. J. Public Health* 86, 1142–1145.
- Timasheff, S.N., 2002. Protein-solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components. *Proc. Natl. Acad. Sci. U. S. A.* 99, 9721–9726.
- Tsai, G., Su, W., 1999. Antibacterial activity of shrimp chitosan against *Escherichia coli*. *J. Food Prot.* 62, 239–243.
- Verbeke, W., Pérez-Cueto, F.J., de Barcellos, M.D., Krystallin, A., Grunert, K.G., 2010. European citizen and consumer attitudes and preferences regarding beef and pork. *Meat Sci.* 84, 284–292.
- Vlahova-Vangelova, D., Dragoev, S., 2014. Marination: effect on meat safety and human health. A review. *Bulg. J. Agric. Sci.* 20, 503–509.
- WHO, 2018. Enterohaemorrhagic *Escherichia coli* (EHEC). Available at: <http://www.who.int/news-room/fact-sheets/detail/e-coli>.