Effect of hydrostatic pressure and antimicrobials on survival of *Listeria monocytogenes* and enterohaemorrhagic *Escherichia coli* in beef

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15 **Abstract**

16 The effects of marinades on *Listeria monocytogenes* and enterohaemorrhagic
17 *Escherichia coli* were investigated in pressure treated beef steaks. Meat was treated
18 with 600 MPa or 450 MPa. Marinades did not enhance pressure inactivation of *E. coli*
19 in beef steaks and marinades prevented pressure-induced sublethal injury in *L.*
20 *monocytogenes*. Membrane-active essential oils carvacrol and thymol, and
21 thiol-reactive allyl-isothiocyanate (AITC) and cinnamaldehyde were selected to
22 investigate potential synergistic activity of clean label antimicrobials with pressure.
23 Carvacrol accelerated pressure inactivation of *E. coli* in beef steaks; however,
24 carvacrol increased pressure resistance of *E. coli* in buffer, and had no effect on
25 survival of *E. coli* in ground beef. Thymol had no effect in either buffer or meat.
26 AITC and cinnamaldehyde exhibited synergistic activity with pressure on *E. coli* in
27 buffer; however, cinnamaldehyde did not affect survival of *E. coli* after pressure
28 treatment of meat. Synergistic inactivation of AITC with pressure was observed only
29 at concentrations that are negatively affect meat quality. AITC and carvacrol may be
30 practically applied for enhancing the bacterial inactivation and extending the shelf life
31 of beef steaks.

32 **Keywords**

33 High pressure, allyl isothiocyanate, cinnamaldehyde, beef, *Listeria*, EHEC, O157

1. Introduction

Meat marination is used to improve the taste and tenderness of meat products by immersing in solutions containing sodium chloride, polyphosphates, sugars and other ingredients (Verbeke et al., 2010; Vlahova-Vangelova & Dragoev, 2014). Effects of marinades on meat microbiota of meat depend on the ingredients. Marination with salt, phosphates and spices alone has little effect on the survival of pathogens on meat but shifts spoilage microbiota towards growth of some psychrotrophic lactic acid bacteria (Björkroth 2005). Extension of the shelf life of marinated meats and the reduction of pathogen levels thus necessitates the combination of marination and antimicrobial ingredients or pressure processing (Wang et al., 2015). Marination mitigates pressure effects on meat quality, discoloration and lipid oxidation (Buckow et al., 2013), when the marinade is formulated with coloring and anti-oxidant ingredients. Marination in combination with treatment at 450 MPa lowered the expressible moisture of beef steaks, and extended their shelf life to 85 days without adverse effect on meat quality. Treatment at 500 or 600 MPa negatively affected meat texture and color with no further increase on shelf life (Wang et al., 2015).

Treatment with 600 MPa was suggested to control risks associated with *E. coli* and *L. monocytogenes* in marinated beef loins (Hugas et al., 2002; Jofré et al., 2009); however, treatment of meat does not eliminate pressure resistant strains of *E. coli* or *L. monocytogenes* (Liu et al., 2012 and 2015; Marcos et al., 2008). Marinating may allow enhancing the pressure inactivation of microorganisms by adding antimicrobial

55 compounds in meat. Among antimicrobial compounds used in meat preservation,
56 essential oils have received increased interest owing to their antimicrobial activity,
57 their synergistic activity with pressure, and because they allow marketing of "clean
58 label" meat products (Feyaerts et al., 2015; Gayán et al., 2012). Synergistic effects of
59 antimicrobial compounds depend on the environment and treatment conditions
60 (Karatzas et al., 2001; Espina et al., 2013, Hofstetter et al., 2013). Pressure induces
61 endogenous oxidative stress in bacteria (Aertsen et al., 2005; Gänzle & Liu, 2015;
62 Malone et al., 2006) which contributes to cell death during and after pressure
63 treatment. Synergistic interaction of antimicrobial compounds with pressure was
64 suggested to depend on the reactivity of the antimicrobial compound with thiols
65 (Feyaerts et al., 2015). The synergistic activity of thiol reactive antimicrobials and
66 pressure, however, has not been described in food applications.

67 Shiga-toxigenic *Escherichia coli* (STEC) are associated with beef (Frenzen et al.,
68 2005; Karch et al., 2005) and causes severe disease with an infectious dose of less
69 than 10 cells (Paton et al., 1996; Tilden et al., 1996). *L. monocytogenes* also occurs in
70 fresh meat and meat products (Frenzen et al., 2005; Sofos, 2008). This study
71 determined the effect of meat marination on the lethality of pressure on pathogenic *E.*
72 *coli* and *L. monocytogenes*, and to assess the combined effect of antimicrobials and
73 pressure. Pressure was applied at a level of 450 MPa, providing optimal quality of
74 marinated beef steaks (Wang et al., 2015), or at 600 MPa, the current upper limit of
75 equipment used in food processing.

2. Methods and Materials

2.1 Bacterial strains and culture conditions.

The *L. monocytogenes* strain cocktail was composed of strains FSL J1-177, FSL C1-056, FSL N3-013, FSL R2-499, FSL N1-227 (Fugett et al. 2006). *E. coli* strains were selected according to Garcia-Hernandez et al. (2015). The cocktail of pathogenic strains was composed the *eae*-positive STEC strains 05-6544 (O26:H11), 03-2832 (O121:H19), 03-6430 (O145:NM) and C0283 (O157:H7), and the *stx*-negative enteropathogenic *E. coli* (EPEC) PARC 449. These strains were selected from more than 100 *E. coli* strains to represent the most pressure resistant strains (Liu et al., 2015). For reasons pertaining to laboratory safety, and to expand the study to a larger number of strains, experiments with pathogenic *E. coli* were complemented with experiments using strain cocktail of non-pathogenic surrogate strains that was composed of *E. coli* AW1.7, AW1.3, GM16.6, DM18.3 and MG1655 and has an comparable resistance to pressure as the cocktail composed of pathogenic strains (Garcia Hernandez et al., 2015; Li et al., 2016). The pressure resistant *E. coli* strain AW 1.7 was also used singly as model organism.

Stock cultures of *Listeria monocytogenes* were streaked onto PALCAM agar (Oxoid, Basingstoke, Hants, England) at 35 °C, and subcultured at 35 °C for 20-24 h with 200 rpm agitation in Tryptone Soy broth (BD, Sparks, MD, USA). *E. coli* were streaked onto Luria-Bertani (LB, Difco, Sparks, MD, USA) agar at 37 °C, and

96 subcultured at 37 °C for 16-18 h with 200 rpm agitation in LB broth. Equal volumes
97 of single cultures were mixed to form the respective strain cocktails.

98 2.2 Meat products, marinades and chemicals.

99 Lean ground beef (15% fat) and beef steaks were provided by a federally inspected
100 meat processing facility. Beef steaks were surface-decontaminated by flaming with
101 ethanol and removal of the denatured surface with a sterile knife. Steaks were then
102 cored perpendicular to the muscle fibres with a sterile corer with diameter 4.8mm to
103 obtain aseptic cuts with a thickness of 2 cm. Ground beef was obtained at the day of
104 processing and stored frozen at -20°C. Cell counts of uninoculated ground beef were
105 determined by plating on LB agar; the cell counts were below the detection limit of
106 200 cfu/g. Two marinades (honey garlic and teriyaki) were provided in powder form
107 by Food Processing Development Centre of Leduc Alberta. The ingredients of the
108 marinade mix are listed in Table 1; marination according to the supplier's suggestions
109 increases the NaCl concentration to 1%. Carvacrol, thymol and allyl isothiocyanate
110 (AITC) were purchased from Fisher Scientific (New Jersey, USA); cinnamaldehyde
111 was purchased from Sigma-Aldrich (St. Louis, USA).

112 2.3 Preparation of marinated meat for pressure treatment.

113 Meat was inoculated with *L. monocytogenes* or *E. coli* by dipping beef steaks into cell
114 suspensions for 15 seconds, or by mixing 1 ml of cell suspensions with 10 g of ground
115 beef thoroughly. The initial population of *L. monocytogenes* or *E. coli* ranged from

116 10^7 to 10^8 cfu/ml. Inoculated meat was mixed with marinade mix (honey garlic or
117 teriyaki) and water at a proportion of 83.3%, 5.7% and 11.0% (w/w), packed into
118 3-cm R3603 tygon tubes (Akron, PA, USA), and heat-sealed. To prevent
119 contamination of the pressure equipment with pathogens, the packaged samples were
120 inserted into 2-ml cryovials (Wheaton, Millville, NJ) filled with 10% bleach

121 2.4 Pressure treatment.

122 Pressure treatments were carried out in a Multivessel Apparatus U111 (Unipress
123 Equipment, Warsaw, Poland) as described (Liu et al., 2012). Beef steaks were treated
124 with 450 MPa for 3 min; ground beef and buffer were treated at 600 MPa and for 3-15
125 min. The time of compression was 45-60 s; the pressure transmission fluid was glycol.
126 The temperature of the pressure vessels was maintained at 20° by a thermostat jacket,
127 and monitored by an internal thermocouple. Temperature changes during compression
128 and decompression were 2°C or less. After pressure treatment, samples were
129 immediately taken for microbial analysis, or removed to refrigerated storage room at
130 4 °C over 16 days. Experiments were performed in triplicate.

131 2.5 Enumeration of *Listeria monocytogenes* and *E. coli* in pressure treated steaks.

132 After treatments at 450 MPa, 20 °C for 3 min, cell counts were enumerated by surface
133 plating onto non-selective agar to allow recovery of injured cells, and selective agar to
134 suppresses the recovery of injured cells. *L. monocytogenes* was enumerated by plating
135 onto Tryptone Soy agar and PALCAM agar with selective supplement; *E. coli* was

136 enumerated by plating onto LB and Violet Red Bile agar (Difco, Sparks, MD US). No
137 contaminating microbiota were observed in uninoculated control steaks, and the
138 colony morphology of samples matched the colony morphology of the inoculum in all
139 samples before treatment, after treatment, and after treatment and storage.

140 2.6 Selection of antimicrobials and determination of concentrations.

141 Compounds with two different mechanisms of antibacterial activity were selected to
142 investigate their combined effect with pressure on inactivation of *E. coli*. Carvacrol
143 and thymol were selected as membrane-active compounds. AITC and
144 cinnamaldehyde selected as thiol reactive compounds. Stock solutions of the four
145 compounds were prepared by mixing with ethanol in a ratio of 1:1 (v/v). The
146 concentrations for their application were determined in 100 mM MES (Fisher, Ottawa,
147 Canada) buffer at pH 5.5. At the ambient temperature, stock solution of each
148 compound was added into the buffer to achieve the following concentrations: 0.01,
149 0.025, 0.04, 0.06, 0.08, 0.10, 0.15, 0.20, 0.25 and 0.30%. Each dilution was inoculated
150 with cells from an overnight culture of *E. coli* AW1.7 to an initial cell count of around
151 10^8 cfu/ml, and incubated at ambient temperature for 4 h. After incubation, cell counts
152 in each sample were obtained by surface plating of appropriate dilutions on LB agar.
153 Sample with inoculation of *E. coli* but without addition of antimicrobials was used as
154 a control. For further applications, the highest concentration of antimicrobial
155 compounds causing inactivation of less than 1 log (cfu/ml) was chosen. These
156 concentrations were as 0.04%, 0.025%, 0.15%, and 0.10% for carvacrol, thymol,

157 AITC, and cinnamaldehyde, respectively.

158 2.7 Effects of antimicrobials with HHP on *E. coli* in buffer and raw ground beef.

159 Experiments were carried out in 100 mM MES (Fisher, Ottawa, Canada) buffer, pH
160 5.5, or with ground beef. Carvacrol, thymol, AITC and cinnamaldehyde stock
161 solutions were added into MES buffer (pH 5.5) or ground beef to a final concentration
162 of 0.04%, 0.025%, 0.15%, and 0.10%, respectively. Samples with ethanol but without
163 essential oils were used as a control. Samples were inoculated with *E. coli* and treated
164 with 600 MPa at 20^{o}C for 3 or 6 min. Cell counts were obtained after surface plating
165 of appropriate dilutions on LB agar. Data are shown as mean ± standard deviation of
166 three independent experiments.

167 2.8 Effects of antimicrobials with HHP on *E. coli* in marinated meat.

168 Experiments were carried out with marinated ground beef and marinated beef steaks.
169 For investigation of essential oils, the marinade of honey garlic was prepared at the
170 ratio of 5.7: 11 by weight (powder: water). Carvacrol, AITC and cinnamaldehyde
171 were dissolved in ethanol and added to the honey garlic marinade. Meat was
172 inoculated with *E. coli* to an initial cell counts of about 10^{7}-10^{8} cfu/ml. Honey garlic
173 marinade supplemented with essential oils was then mixed with meat at a ratio of 16.7:
174 83.3 (marinade: meat) by weight. The resulting final concentrations of carvacrol,
175 AITC and cinnamaldehyde were 0.04 or 0.10, 0.06 or 0.15, and 0.10%, respectively.
176 Marinated meat was treated by high pressure at 450 MPa, 20^{o}C for 3 min. Cell counts

were obtained after surface plating of appropriate dilutions on LB agar. Data are shown as mean ± standard deviation of three independent experiments.

2.9 Statistical analysis

Significant differences between two treatments were determined using Student's *t* test; significant differences between more than two treatments were determined using one way ANOVA with the Holm-Sidak method for pairwise multiple comparison. Significance was assessed at an error probability of 5 % ($P<0.05$).

3. Results

3.1 Effect of marination on survival of *L. monocytogenes* and *E. coli* during pressure treatment of beef steaks.

To determine the effect of meat marination on the lethality of pressure, cell counts of *L. monocytogenes* and *E. coli* cocktails were determined in beef steaks that were marinated with two different marinades. Beef steaks were treated by pressure at 450 MPa and 20°C for 3 min, conditions which significantly extend the shelf life without adverse effect on meat quality (Wang et al., 2015). Pressure treatment reduced cell counts of *L. monocytogenes* on marinated beef steaks by 90% (Figure 1). Marination did not influence the survival of *L. monocytogenes* during pressure treatment but improved survival during storage. Cell counts of un-injured cells obtained on selective agar were lower in control steaks when compared to marinated steaks. During storage, cell counts of *L. monocytogenes* in marinated steaks remained stable. In control steaks,

197 total cell counts tended to decrease while counts of un-injured cells increased,
198 indicating that sublethally injured cells died or recovered during storage (Figure 1).

199 Pressure treatment at 450 MPa reduced cell counts of the cocktail of pathogenic
200 *E. coli* by about 99% (Figure 2). Meat marination did not influence survival of *E. coli*
201 during or after pressure treatment and cell counts remained essentially unchanged
202 over 16 days of refrigerated storage.

203 3.2 Effects of antimicrobials on *E. coli* AW1.7 in buffer.

204 Both *L. monocytogenes* and *E. coli* cocktails showed high resistance to pressure in
205 marinated beef steaks. Subsequent studies explored the use of clean label
206 antimicrobial compounds to enhance the lethal effect of pressure. These experiments
207 were carried out in MES buffer with the pressure resistant model organism *E. coli*
208 AW 1.7. Pressure treatments were carried out at 600 MPa and 20°C to match current
209 industrial practice for pressure treated food, and to allow sensitive detection on
210 synergistic or antagonistic activity (Figure 3). The four antimicrobial compounds
211 were applied at the level of their respective minimum bactericidal concentrations.
212 Both AITC and cinnamaldehyde showed strong synergistic activity with pressure
213 (Figure 3). For example, treatments in presence of AITC or cinnamaldehyde increased
214 the lethality of pressure by about 5 and 3 log(cfu/ml), respectively (Fig. 3). Thymol
215 addition at 0.025% had no effect on survival of *E. coli*. Carvacrol at a concentration of
216 0.025% reduced the cell counts of *E. coli* by around 1 log, however, cell counts after

217 combined application of carvacrol and 600 MPa were not different from those
218 obtained after treatment with 600 MPa without addition of carvacrol (data not shown).
219 Addition of 0.04% carvacrol reduced cell counts of *E. coli* to levels below the
220 detection limit in untreated samples (Figure 3). In pressure treated samples with
221 addition of 0.04% carvacrol, however, *E. coli* was reduced by less than 5 log (cfu/ml).

222 3.3 Effects of antimicrobials on *E. coli* AW1.7 in ground beef.

223 To determine whether the synergistic activity of AITC and cinnamaldehyde is also
224 observed in a meat matrix, these antimicrobials were added to ground beef inoculated
225 with *E. coli* AW1.7. Samples were treated at 600 MPa for 3 or 6 min (Figure 4).
226 AITC (0.15%) showed a synergistic effect on pressure inactivation of *E. coli* at the
227 treatment for 6 min, however, the effect was less pronounced when compared to the
228 effect observed in buffer (Fig. 3 and 4). Addition of 0.1% cinnamaldehyde did not
229 affect the pressure inactivation of *E. coli* in ground beef.

230 Effects of carvacrol and thymol were also investigated in ground beef. The addition of
231 thymol and carvacrol to ground beef at concentrations ranging from 0.04 – 0.1% did
232 not influence survival of *E. coli* when compared to control treatments containing
233 ethanol only (data not shown).

234 3.4 Effects of antimicrobials on survival of *E. coli* AW1.7 in marinated beef steaks
235 and marinated ground beef.

236 The effect of antimicrobial compounds was also evaluated at 450 MPa, i.e. conditions

237 that allow shelf life extension without compromising quality (Wang et al., 2015).
238 Treatment of marinated steaks was compared to an equivalent treatment of marinated
239 ground beef (Figure 5). None of the antimicrobial compounds increased the
240 bactericidal effect of treatment at 450 MPa when supplemented to marinated ground
241 beef (Figure 5). In beef steaks with honey garlic marinade, however, addition of 0.10 %
242 carvacrol or 0.15% AITC enhanced the pressure inactivation of *E. coli*. Reduced
243 concentrations of 0.04% and 0.06% carvacrol and AITC, respectively, did not
244 influence inactivation of *E. coli* at 450 MPa; likewise, 0.10% cinnamaldehyde had no
245 effect on pressure inactivation of *E. coli* in beef steaks (Figure 5).

246 3.5 Effects of antimicrobials on survival of the surrogate cocktail of *E. coli* in
247 marinated beef steaks supplemented with clean label antimicrobials.

248 To validate the combined activity of antimicrobials with a strain cocktail, and to
249 assess their influence on survival of *E. coli* during post-treatment storage, carvacrol
250 and AITC were added to marinade and the survival of a 5 strain surrogate cocktail of
251 *E. coli* in marinated beef steaks was observed after pressure treatment and during
252 post-pressure storage (Figure 6). Survival of the surrogate cocktail of *E. coli* in
253 marinated beef steaks was comparable to the survival of the STEC and EPEC
254 cocktails (compare Fig. 2 and Fig. 6). Carvacrol and AITC reduced cell counts of *E.*
255 *coli* by more than 1 log (cfu/ml) when compared to marinated beef steaks without
256 addition of antimicrobials (Figure 6). The effect of carvacrol was already observed in
257 untreated samples (Figure 6); however, the effect of AITC was observed only after

pressure treatment. Cell counts of the *E. coli* cocktail remained essentially unchanged on pressure-treated marinated beef steaks, and in the corresponding samples with addition of carvacrol (Figure 6). Cell counts of *E. coli* decreased more than 1 log (cfu/ml) reduction during storage of pressure treated beef steaks supplemented with AITC.

4 Discussion

Marination improves the sensory quality of meats; marinades may additionally include antimicrobials to enhance the shelf life and the safety of meat products (Björkroth 2005). Synergistic activity of pressure with antimicrobial compounds added to the marinade potentially eliminates pressure resistant pathogens. Plant essential oils are used as antimicrobial preservatives in meat and meat products (Jayasena & Jo, 2013) and allow "clean label" meat preservation. For example, AITC and mustard powder reduced cell counts of *E. coli* in beef and in fermented sausages (Chacon, et al., 2006; Luciano, et al., 2011; Nadarajah, et al., 2005). The activity of AITC is related to its reactivity with thiols (Luciano & Holley, 2009), while other essential oils destabilize the cytoplasmic membrane (Gharsallaoui, et al., 2015). Synergistic or antagonistic activities of antimicrobial compounds with pressure applications relate to their mode of action (Feyaerts et al., 2015; Hofstetter et al., 2013). This study compared the effect of meat marination and antimicrobial compounds differing in their mode of action. Applications in whole muscle meat were compared to applications in ground beef.

279 Treatment with 450 MPa reduced cell counts of *L. monocytogenes* in meat only by 90
280 to 99%, in keeping with prior reports on the pressure resistance of *L. mon*cy*togenes*
281 (Ates et al., 2016; Balamurugan et al., 2016; Teixeira et al., 2016). Meat marination
282 increased the pressure resistance of *L. monocytogenes* and prevented pressure-induced
283 sub-lethal injury. Salt addition to cooked ham exerted a comparable effect on pressure
284 resistance and pressure-induced sublethal injury of *L. monocytogenes* (Teixeira et al.,
285 2016) and the protective effect of marinades is thus likely attributable to the presence
286 of salt in marinades. An increased osmotic pressure generally enhances the tolerance
287 of microorganisms to pressure (Georget et al., 2015; Molina-Höppner et al., 2004;
288 Van Opstal et al., 2003). In *L. monocytogenes*, baroprotective effects of NaCl are
289 attributed to the accumulation of glycine betaine and carnitine (Smiddy et al., 2004).

290 This study describes combined effect of clean label antimicrobials with pressure on *E.*
291 *coli* in beef. Feyaerts et al. (2015) proposed that thiol reactive antimicrobial
292 compounds act synergistic with pressure because they enhance pressure-induced
293 oxidative stress. We evaluated two membrane-active antimicrobials, carvacrol and
294 thymol (Jayasena, et al., 2013; Sikkema et al., 1994), and two thiol reactive
295 antimicrobials, cinnamaldehyde and AITC. To allow comparison of the different
296 compounds, all four compounds were applied at the level of their minimum
297 bactericidal concentration. Treatments of *E. coli* in buffer confirmed that the thiol
298 reactive antimicrobials AITC and cinnamaldehyde but not the membrane-active
299 carvacrol and thymol show synergistic activity with pressure (Feyaerts et al., 2015;

300 this study). Likewise, the combination of carvacrol and pressure did not exert
301 synergism on inactivation of *L. monocytogenes* in milk (Karatzas et al., 2001).
302 Carvacrol suppressed inactivation of *E. coli* after pressure treatment in buffer
303 (Feyaerts et al., 2015), and reduced inactivation of *Bacillus cereus* spores at a
304 temperature of ≤65°C (Luu-Thi et al. 2015).

305 Synergisms of AITC or cinnamaldehyde with pressure were previously reported in
306 buffer (Feyaerts et al., 2015; Ogawa et al., 2000). Their synergistic antimicrobial
307 effect with pressure likely relate to the effect of these antimicrobials on the bacterial
308 oxidative stress resistance (Feyaerts et al., 2015). AITC reacts with proteins (Luciano
309 & Holley, 2009), cysteine and glutathione (Hanschen et al., 2012; Luciano et al. 2008)
310 and thus disturbs redox homeostasis. AICT also reduces oxidative stress resistance of
311 *E. coli* by inhibition of thiol-containing enzymes such as thioredoxin reductase and
312 glutathione reductases (Carmel-Harel et al., 2000; Luciano & Holley 2009).
313 Cinnamaldehyde also reacts with thiol group of proteins or cysteine (Weibel &
314 Hansen, 1989), and decreases glutathione levels in bacteria (Cocchiara et al., 2005).

315 AITC and cinnamaldehyde are also reactive towards amino groups and thiol groups
316 that are present in the food matrix (Hanschen et al., 2012; LoPachin, et al., 2009;
317 Nakamura et al., 2009). The antioxidant capacity of the food matrix thus provides
318 protection to bacterial cells. Accordingly, thiol-mediated compounds exhibited strong
319 synergistic effects with pressure in buffer, but this activity was diminished or
320 abolished when antimicrobials and pressure were applied to *E. coli* on meat (Figures 4

321 & 5). The amount of AITC that can be applied to foods is limited by its effect on
322 sensory properties. The use of 0.05% AITC in dry fermented sausages resulted in an
323 acceptable level of spiciness while a level of 0.75% or 0.1% AITC resulted in an
324 unacceptable level of spiciness (Chacon et al. 2006).

325 We observed substantial differences in the efficacy of antimicrobials when applied on
326 ground beef, or for marination of whole muscle meat. During marination of whole
327 muscle meat, the antimicrobial compounds are initially concentrated on the surface of
328 the meat and equilibrate only slowly during storage. In contrast, marination of ground
329 beef rapidly distributes antimicrobial compounds throughout the meat matrix. The
330 initial concentration of essential oils on the surface beef steaks is thus substantially
331 higher than in ground beef and carvacrol and AITC accelerated pressure inactivation
332 of *E. coli* in marinated steaks but not in marinated ground beef (Figures 4, 5, and 6).

333 In conclusion, the use of clean label antimicrobial additives to meat marinades can
334 contribute to the elimination of pathogens during pressure processing, or during
335 post-process refrigerated storage. The addition of antimicrobial additives to marinade
336 thus complements the use of marinade to improve the quality and to extend the shelf
337 life pressure-treated meat (Wang et al., 2015). Depending on their mode of action,
338 antimicrobial compounds exert synergistic and antagonistic activities with pressure.
339 The antioxidant capacity of the meat matrix diminishes the activity of thiol-reactive
340 antimicrobials. The application of antimicrobials in marination of whole muscle meat,
341 however, can take advantage of an initial high concentration in the marinade, which is

342 effective against microorganisms on the surface until their concentration has
343 equilibrated throughout the meat matrix.

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References

Aertsen, A., Spiegeleer, P.D., Vanoirbeek, K., Lavilla, M., & Michiels, C.W. (2005). Induction of oxidative stress by high hydrostatic pressure in *Escherichia coli. Applied Microbiology and Biotechnology*, *71*, 2226-2231.

Ates, M.B., Rode1, T.M., Skipnes, D., & Lekang, O-I. (2016). Modeling of *Listeria monocytogenes* inactivation by combined high‐pressure and mild‐temperature treatments in model soup. *European Food Research and Technology, 242*, 279–287.

Balamurugan, S., Ahmed, R., Chibeu, A., Gao, A., Koutchma, T. & Strange, P. (2016). Effect of salt types and concentrations on the high-pressure inactivation of *Listeria monocytogenes* in ground chicken. *International Journal of Food Microbiology,* 218, 51-56.

Björkroth, J. (2005) Microbiological ecology of marinated meat products. *Meat Science*, *70*, 477-480.

Buckow, R., Sikes, A., & Tume, R. (2013). Effect of high pressure on physicochemical properties of meat. *Critical Reviews in Food Science and Nutrition*, *53*, 770-786.

Carmel-Harel, O., & Storz, G. (2000). Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annual Review of Microbiology*, *54*, 439-461.

368 Chacon, P.A., Buffo, R.A., & Holley, R.A. (2006). Inhibitory effects of
369 microencapsulated allyl isothiocyanate (AIT) against *Escherichia coli* O157:H7 in
370 refrigerated, nitrogen packed, finely chopped beef. *International of Food*
371 *Microbiology*, *107*, 231-237.

372 Cocchiara, J., Letizia, C.S., Lalko, J., Lapczynski, A., & Api, A.M. (2005). Fragrance
373 material review on cinnamaldehyde. *Food and Chemical Toxicology*, *43*, 867-923.

374 Espina, L., Garcia-Gonzalo, D., Laglaoui, A., Mackey, B.M., & Pagan, R. (2013).
375 Synergistic combinations of high hydrostatic pressure and essential oils or their
376 constituents and their use in preservation of fruit juices. *International of Food*
377 *Microbiology*, *161*, 23–30.

378 Feyaerts, J., Rogiers, G., Corthouts, J., & Michiels, C. (2015). Thiol-reactive natural
379 antimicrobials and high pressure treatment synergistically enhance bacterial
380 inactivation. *Innovative Food Science and Emerging Technology*, *27*, 26-34.

381 Frenzen, P.D., Drake, A., Angulo, F.J., and the Emerging Infections Program Foodnet
382 Working Group. (2005). Ecomomic cost of illness due to *Escherichia coli* O157
383 infections in the United States. *Journal of Food Protection*, *68*, 2623-2630.

384 Fugett, E., Fortes, E., Nnoka, C. & Wiedmann, M. (2006). International Life Sciences
385 Institute North America *Listeria monocytogenes* strain collection: development of
386 standard *Listeria monocytogenes* strain sets for research and validation studies. *Journal*
387 *of Food Protection*, *69*, 2929–2938.

388 Gänzle, M.G., & Liu, Y. (2015). Mechanisms of pressure-mediated cell death and
389 injury in *Escherichia coli*: from fundamentals to food applications. Frontiers in
390 Microbiology *6*, 599.

391 Garcia-Hernandez, R., McMullen, L., & Gänzle, M.G., (2015). Development and
392 validation of a surrogate strain cocktail to evaluate bactericidal effects of pressure on
393 verotoxigenic *Escherichia coli. International of Food Microbiology*, *205*, 16-22.

394 Gayán, E., Antonio Torres, J., & Paredes-Sabja, D. (2012). Hurdle approach to
395 increase the microbial inactivation by high pressure processing: effect of essential oils.
396 *Food Engineering Reviews*, *4*, 141-148.

397 Georget, E., Sevenich, R., Reineke, K., Mathys, A., Heinz, V., Callanan, M., Rauh, C.,
398 & Knorr, D., (2015). Inactivation of microorganisms by high isostatic pressure
399 processing in complex matrices: A review. *Innovative Food Science and Emerging*
400 *Technology*, *27*, 1-14.

401 Gharsallaoui, A., Oulahal, N., Joly, C., & Degraeve, P. (2015). Nisin as a Food
402 Preservative: Part 1: Physicochemical Properties, Antimicrobial Activity, and Main
403 Uses. *Critical review of Food Science and Nutrition*,
404 *DOI*:10.1080/10408398.2013.763765.

405 Hanschen, F.S., Bruggemann, N., Brodehl, A., Mewis, I., Schreiner, M., Rohn, S. &
406 Kroh, L.W. (2012). Characterization of products from the reaction of
407 glucosinolate-derived isothiocyanates with cysteine and lysine derivatives formed in

408 either model systems or broccoli sprouts. *Journal of Agricultural and Food Chemistry*,
409 *60*, 7735–7745.

410 Hofstetter, S., Gebhardt, D., Ho, L., Gänzle, M., & McMullen, L.M. (2013). Effects of
411 nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and
412 high pressure. *Food Microbiology 34,* 46-51.

413 Hugas, M., Garriga, M., & Monfort, J. (2002). New mild technologies in meat
414 processing: high pressure as a model technology. *Meat Science*, *62*, 359-371.

415 Jayasena, D.D., & Jo, C. (2013). Essential oils as potential antimicrobial agents in
416 meat and meat products: A review. *Trends in Food Science and Technology*, *34*,
417 96-108.

418 Jofré, A., Aymerich, T., Grèbol, N., & Garriga, M. (2009). Efficiency of high
419 hydrostatic pressure at 600 MPa against food-borne microorganisms by challenge
420 tests on convenience meat products. *LWT-Food Science and Technology*, *42*, 924-928.

421 Karatzas, A.K., Kets, E.P.W., Smid, E.J., & Bennik, M.H.J. (2001). The combined
422 action of carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A.
423 *Jounal of Applied Microbiology. 90*, 463–469.

424 Karch, H., Tarr, P.I., & Bielaszewska, M. (2005) Enterohaemorrhagic *Escherichia coli*
425 in human medicine. *International Journal of Medical Microbiology*, *295*, 405-418.

426 Li, H., Garcia-Hernandez, R., Driedger, D., McMullen, L.M., & Gänzle, M.G. (2016)
427 Effect of the food matrix and food constituents on pressure resistance of Escherichia
428 coli. *Food Microbiology, 57*, 96-102.

429 Liu, Y., Betti, M., & Gänzle, M.G. (2012). High pressure inactivation of *Escherichia*
430 *coli*, *Campylobacter jejuni*, and spoilage microbiota on poultry meat. *Journal of Food*
431 *Protection*, *75*, 497-503.

432 Liu, Y., Gill, A., McMullen, L., & Gänzle, M.G. (2015). Variation in heat and pressure
433 resistance of verotoxigenic and nontoxigenic *Escherichia coli. Journal of Food*
434 *Protection*, *78*, 111-120.

435 LoPachin, R.M., Gavin, T., Petersen, D.R., & Barber, D.S. (2009). Molecular
436 mechanisms of 4-hydroxy-2-nonenal and acrolein toxicity: Nucleophilic targets and
437 adduct formation. *Chemical Research in Toxicology*, *22*, 1499–1508.

438 Luciano, F.B., Hosseinian, F.S., Beta, T., & Holley, R.A. (2008). Effect of free-SH
439 containing compounds on allyl isothiocyanate antimicrobial activity against
440 *Escherichia coli* O157:H7. *Journal of Food Science*, *73*, 214–220.

441 Luciano, F.B., & Holley, R.A. (2009). Enzymatic inhibition by allyl isothiocyanate
442 and factors affecting its antimicrobial action against *Escherichia coli* O157:H7.
443 *International Journal of Food Microbiology*, *131*, 240–245.

444 Luciano, F.B., Belland, J., & Holley, R.A. (2011). Microbial and chemical origins of
445 the bactericidal activity of thermally treated yellow mustard powder toward

446 *Escherichia coli* O157:H7 during dry sausage ripening. *International Journal of Food*
447 *Microbiology*, *145*, 69-76.

448 Luu-Thi, H., Corthouts, J., Passaris, I., Grauwet, T., Aertsen, A., Hendrickx, M., &
449 Michiels, C.W. (2015). Carvacrol suppresses high pressure high temperature
450 inactivation of *Bacillus cereus* spores. *International Journal of Food Microbiology*,
451 *197*, 45-52.

452 Malone, A.S., Chung, Y.K., & Yousef, A.E. (2006). Genes of *Escherichia coli*
453 O157:H7 that are involved in high-pressure resistance. *Applied and Environmental*
454 *Microbiology, 72*, 2661-2671.

455 Marcos, B., Aymerich, T., Monfort, J.M., & Garriga, M. (2008). High-pressure
456 processing and antimicrobial biodegradable packaging to control *Listeria*
457 *monocytogenes* during storage of cooked ham. *Food Microbiology 25,* 177-182.

458 Molina-Höppner, A., Doster,W., Vogel, R.F., & Gänzle, M.G. (2004). Protective effect
459 of sucrose and sodium chloride for *Lactococcus lactis* during sublethal and lethal
460 highpressure treatments. *Applied and Environmental Microbiology*, *70*, 2013–2020.

461 Nadarajah, D., Han, J.H., & Holley, R. A. (2005). Inactivation of *Escherichia coli*
462 O157:H7 in packaged ground beef by allyl isothiocyanate. *International Journal of*
463 *Food Microbiology*, *99*, 269-279.

464 Nakamura, T., Kawai, Y., Kitamoto, N., Osawa, T., & Kato, Y. (2009). Covalent
465 modification of lysine residues by allyl isothiocyanate in physiological conditions:

466 Plausible transformation of isothiocyanate from thiol to amine. *Chemical Research in*
467 *Toxicology*, *22*, 536-542.

468 Ogawa, T., Nakatani, A., Matsuzaki, H., Isobe, S., & Isshiki, K. (2000). Combined
469 effects of hydrostatic pressure, temperature, and the addition of allyl isothiocyanate
470 on inactivation of *Escherichia coli*. *Journal of Food Protection*, *63*, 884-888.

471 Paton, A.W., Ratcliff, R.M., Doyle, R.M., Seymour-Murray, J., Davos, D., Lanser,
472 J.A., & Paton, J.C. (1996). Molecular microbiological investigation of an outbreak of
473 hemolytic-uremic syndrome caused by dry fermented sausage contaminated with
474 Shiga-like toxin-producing *Escherichia coli*. *Journal of Clinical Microbiology*, *34*,
475 1622-1627.

476 Sikkema, J., Debont, J.A.M., & Poolman, B. (1994). Interactions of cyclic
477 hydrocarbons with biological membranes. The *Journal of Biological Chemistry*, *269*,
478 8022-8028.

479 Smiddy, M., Sleator, R.D., Patterson, M.F., Hill, C. & Kelly, A.L. (2004). Role for
480 compatible solutes glycine betaine and L-carnitine in listerial barotolerance. *Applied*
481 *and Environmental Microbiology, 70,* 7555-7557.

482 Sofos, J. (2008). Challenges to meat safety in the 21st century. *Meat Science*, *78*, 3-13.

483 Tilden, J., Young, W., McNamara, A.M., Custer, C., Boesel, B., Lambert-Fair, M.A.,
484 Majkowski, J., Vugia, D., Werner, S.B., Hollingsworth, J., & Morris, J.G. (1996). A

485 new route of transmission for *Escherichia coli*: infection from dry fermented salami.
486 *American Journal of Public Health*, *86*, 1142-1145.

487 Teixeira, J.S., Maier, M.B., Miller, P., Gänzle, M.G., McMullen, L.M. (2016). The
488 effect of growth temperature, process temperature, and sodium chloride on the high
489 pressure inactivation of *Listeria monocytogenes* on ham. *European Food Research*
490 *and Technology, in press.*

491 Van Opstal, I., Vanmuysen, S.C., & Michiels, C.W. (2003). High sucrose
492 concentration protects *E. coli* against high pressure inactivation but not against high
493 pressure sensitization to the lactoperoxidase system. *International Journal of Food*
494 *Microbiology*, *88*, 1-9.

495 Verbeke, W., Pérez-Cueto, F., de Barcellos, M., Krystallis, A., & Grunert, K. (2010).
496 European citizen and consumer attitudes and preferences regarding beef and pork.
497 *Meat Science*, *84*, 284-292.

498 Vlahova-Vangelova, D., & Dragoev, S. (2014). Marination: effect on meat safety and
499 human health. A review. *Bulgarian Journal of Agricultural Science*, *20*, 503-509.

500 Wang, H., Yao, J., Erin, K., & Gänzle, M. (2015). Effect of pressure on quality and
501 shelf life of marinated beef semitendinosus steaks. *Meat Science*, *99*, 148.

502 Weibel, H., & Hansen, J. (1989). Interaction of cinnamaldehyde (a sensitizer in
503 fragrance) with protein. *Contact Dermatitis*, *20*, 161-166.

504 **Figure legends**

505 **Figure 1.** The effect of meat marination on survival of a 5 strain *Listeria*
506 *monocytogenes* cocktail in beef steaks after pressure treatment. Samples were treated
507 at 450 MPa and 20°C for 3 min, and stored at 4°C for 16 days after treatment. Prior to
508 treatment, steaks were marinated with honey garlic (■) or teriyaki (◆) marinades.
509 Un-marinated steaks were used as control (●). Cell counts were obtained on
510 Tryptone Soy agar (closed symbols) or PALCAM agar (open symbols). Data are
511 shown as mean ± standard deviation of three independent experiments.

512 **Figure 2.** The effect of meat marination on survival of a cocktail of 5 pathogenic
513 strains of *E. coli* on beef steaks after pressure treatment. Samples were treated at 450
514 MPa, 20°C for 3 min, and stored at 4°C for 16 days after treatment. Prior to treatment,
515 steaks were marinated with honey garlic (■) or teriyaki (◆) marinades.
516 Un-marinated steaks were used as control (●). Cell counts were obtained on
517 Luria-Bertani agar (closed symbols) or Violet Red Bile agar (open symbols). Data are
518 shown as mean ± standard deviation of three independent experiments.

519 **Figure 3.** Effects of carvacrol (CAR), thymol (THY), allyl isothiocyanate (AITC),
520 and cinnamaldehyde (CIN) on the pressure resistance of *E. coli* AW 1.7 in MES
521 buffer (pH 5.5). The four compounds were dissolved in ethanol (1:1 v/v), and added
522 into MES buffer to a final concentration of 0.025-0.15% before inoculation. Samples
523 with only ethanol (EtOH) but without essential oils were used as controls. Samples
524 were treated by at 600 MPa and 20°C for 3 (gray bars) or 6 (black bars) min. Cell

525 counts of untreated controls are shown as white bars. Surviving cells were enumerated
526 on Luria-Bertani agar. Data are shown as mean ± standard deviation of three
527 independent experiments.

528 **Figure 4.** Effects of cinnamaldehyde and allyl isothiocyanate (AITC) on cell counts
529 of *E. coli* AW 1.7 in ground beef after pressure treatments. Before inoculation, the
530 essential oils were diluted with ethanol (1:1 v/v), and added into ground beef to a final
531 concentration of 0.10% and 0.15%, respectively. Samples with addition of ethanol
532 (EtOH) served as control. Samples were treated at 600 MPa and 20°C for 3 min (gray
533 bars) or 6 min (black bars). Untreated samples are shown as white bars. Surviving
534 cells were enumerated on Luria-Bertani agar. Data are shown as mean ± standard
535 deviation of three independent experiments. The asterisk indicates significance
536 different between AITC and ethanol control (p<0.05).

537 **Figure 5.** Effect of antimicrobial compounds on pressure resistance of *E. coli* AW1.7
538 in marinated beef steaks (black bars) and marinated ground beef (gray bars). Samples
539 were treated at 450 MPa 20°C for 3 min. Prior to pressure treatment, meat was
540 marinated with honey garlic (HG) marinade supplemented with carvacrol (CAR),
541 allyl isothiocyanate (AITC) or cinnamaldehyde (CIN) at a final concentration of
542 0.04/0.1%, 0.06/0.15% or 0.10%. UT (untreated) represents marinated meat without
543 pressure treatment. Marinated meat with no antimicrobial supplement (HG) was also
544 used as control. Cell counts were enumerated on Luria-Bertani agar. Different letters
545 above the bars indicate significant differences (p<0.05) to the marinated and pressure

546 treated control without addition of antimicrobial compounds. Data are shown as mean
547 ± standard deviation of three independent experiments.

548 **Figure 6.** Effect of essential oils on survival of a 5-strain surrogate cocktail of *E. coli*
549 in marinated beef steaks after pressure treatment and refrigerated storage. Samples
550 were treated at 450 MPa and 20°C for 3 min, and stored at 4°C after treatment. A
551 storage time of -0.5 days represents untreated controls; a storage time of 0 days
552 represents cell counts taken immediately after pressure treatment. Prior to pressure
553 treatment, steaks were marinated with marinades of honey garlic (HG) supplemented
554 with 0.1% carvacrol (CAR, ●) or 0.15% allyl isothiocyanate (AITC, ▼). Marinated
555 steaks without antimicrobial supplement were used as control (○). Cell counts were
556 enumerated on Luria-Bertani agar. Different letters beside the symbols indicate
557 significant differences between the three samples for a corresponding given time
558 (p<0.05). Data are shown as mean ± standard deviation of three independent
559 experiments.
560

561 **Table 1**. Ingredients of honey garlic and teriyaki marinade.
562

Marinade	Ingredients
Honey Garlic	Sugar, salt, fructose, honey powder (honey, wheat starch, soy flour, soy lecithin), granulated garlic, sodium phosphates, soy sauce powder (soy sauce from wheat and soybeans, corn maltodextrin), garlic powder, caramel, calcium silicate, spices, monounsaturated vegetable oil, artificial flavor.
Teriyaki	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, sulphites.

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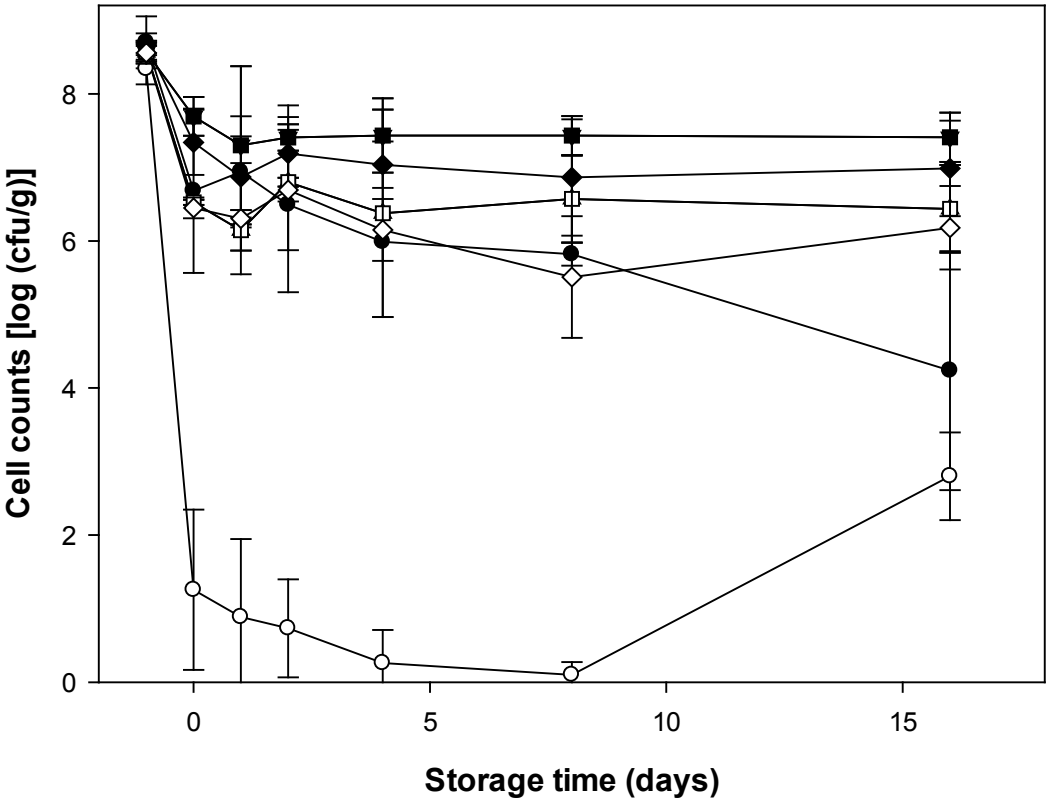

565

566 **Figure 1.** The effect of meat marination on survival of a 5 strain *Listeria monocytogenes*
567 cocktail in beef steaks after pressure treatment. Samples were treated at 450 MPa and 20°C
568 for 3 min, and stored at 4°C for 16 days after treatment. Prior to treatment, steaks were
569 marinated with honey garlic (■) or teriyaki (◆) marinades. Un-marinated steaks were used
570 as control (●). Cell counts were obtained on Tryptone Soy agar (closed symbols) or
571 PALCAM agar (open symbols), respectively. Data are shown as mean ± standard deviation of
572 three independent experiments.

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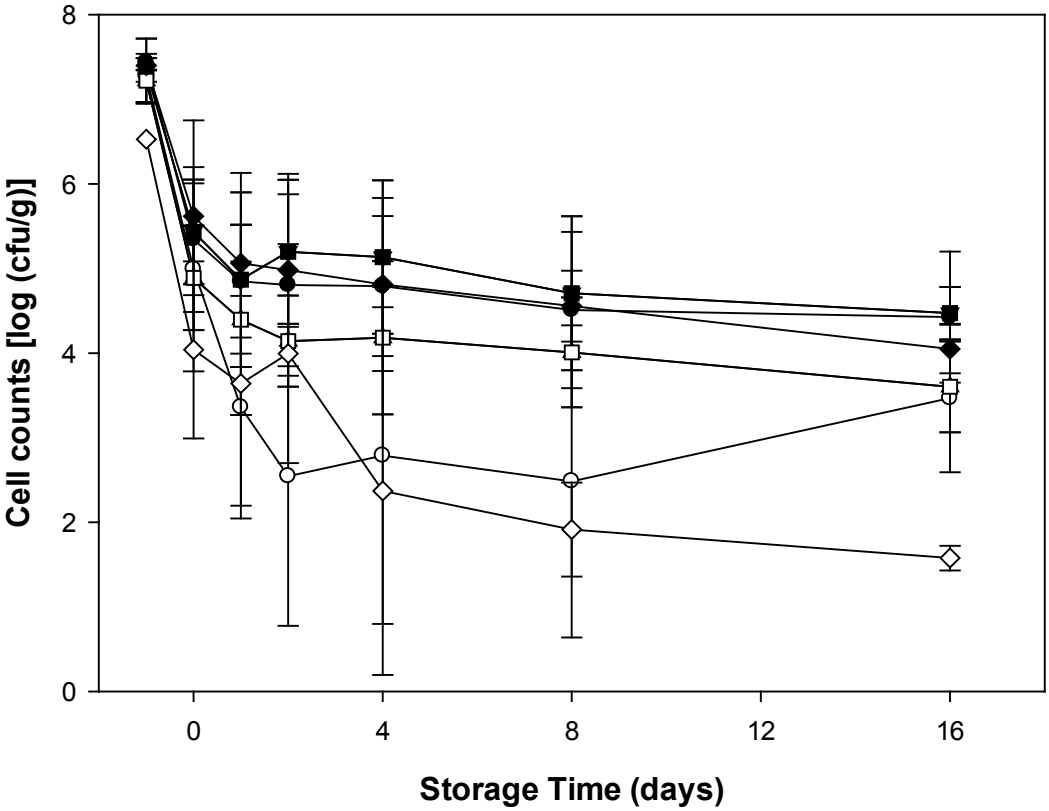

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576 **Figure 2.** The effect of meat marination on survival of a cocktail of 5 pathogenic strains of *E.*
577 *coli* on beef steaks after pressure treatment. Samples were treated at 450 MPa, 20°C for 3 min,
578 and stored at 4°C for 16 days after treatment. Prior to treatment, steaks were marinated with
579 honey garlic (■) or teriyaki (◆) marinades. Un-marinated steaks were used as control (●).
580 Cell counts were obtained on Luria-Bertani agar (closed symbols) or Violet Red Bile agar
581 (open symbols). Data are shown as mean ± standard deviation of three independent
582 experiments.

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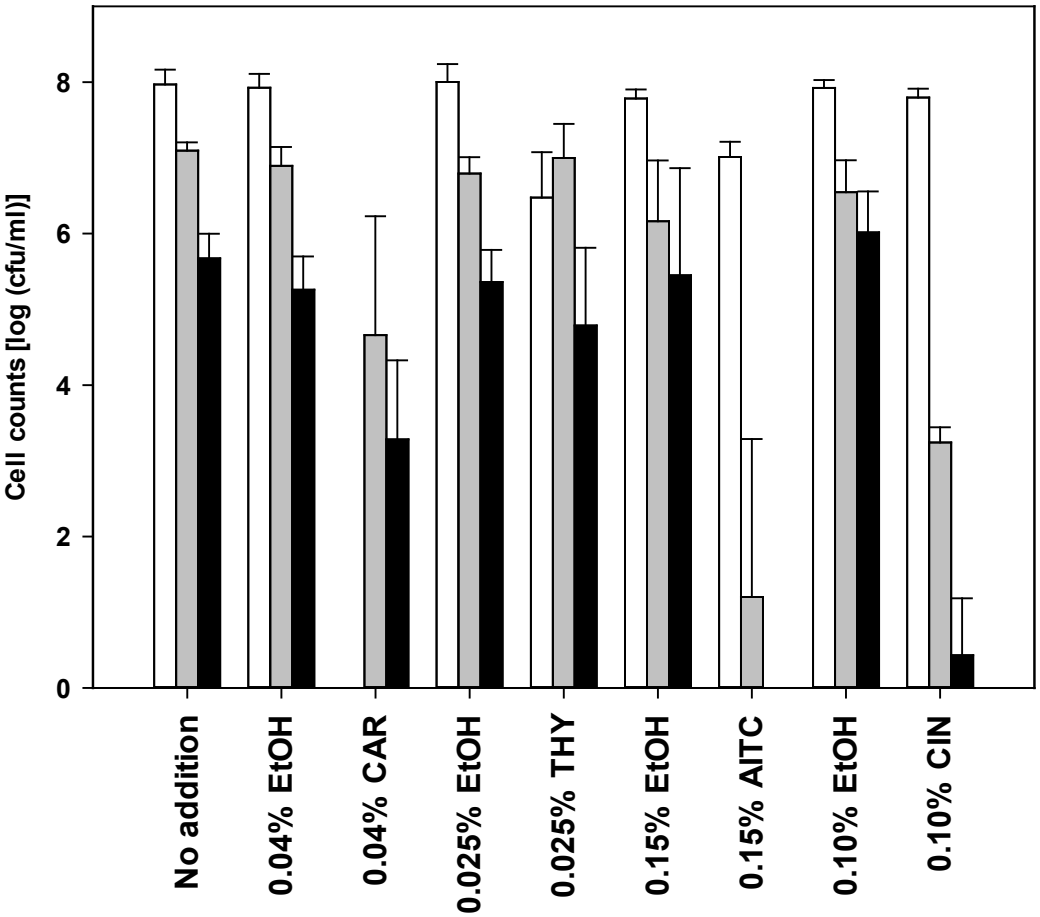

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585 **Figure 3.** Effects of carvacrol (CAR), thymol (THY), allyl isothiocyanate (AITC), and
586 cinnamaldehyde (CIN) on the pressure resistance of *E. coli* AW 1.7 in MES buffer (pH 5.5).
587 The four compounds were dissolved in ethanol (1:1 v/v), and added into MES buffer to a
588 final concentration of 0.025-0.15% before inoculation. Samples with only ethanol (EtOH) but
589 without essential oils were used as controls. Samples were treated by at 600 MPa and 20°C
590 for 3 (gray bars) or 6 (black bars) min. Cell counts of untreated controls are shown as white
591 bars. Surviving cells were enumerated on Luria-Bertani agar. Data are shown as mean ±
592 standard deviation of three independent experiments.

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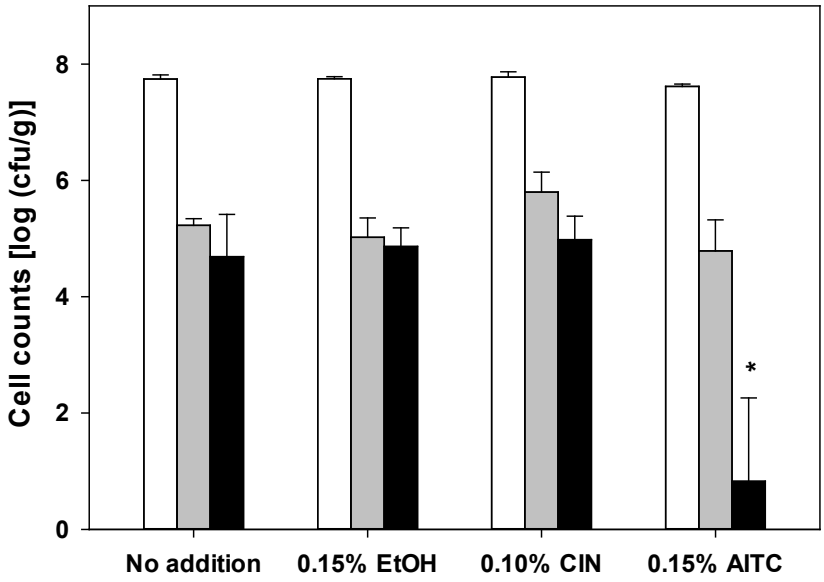

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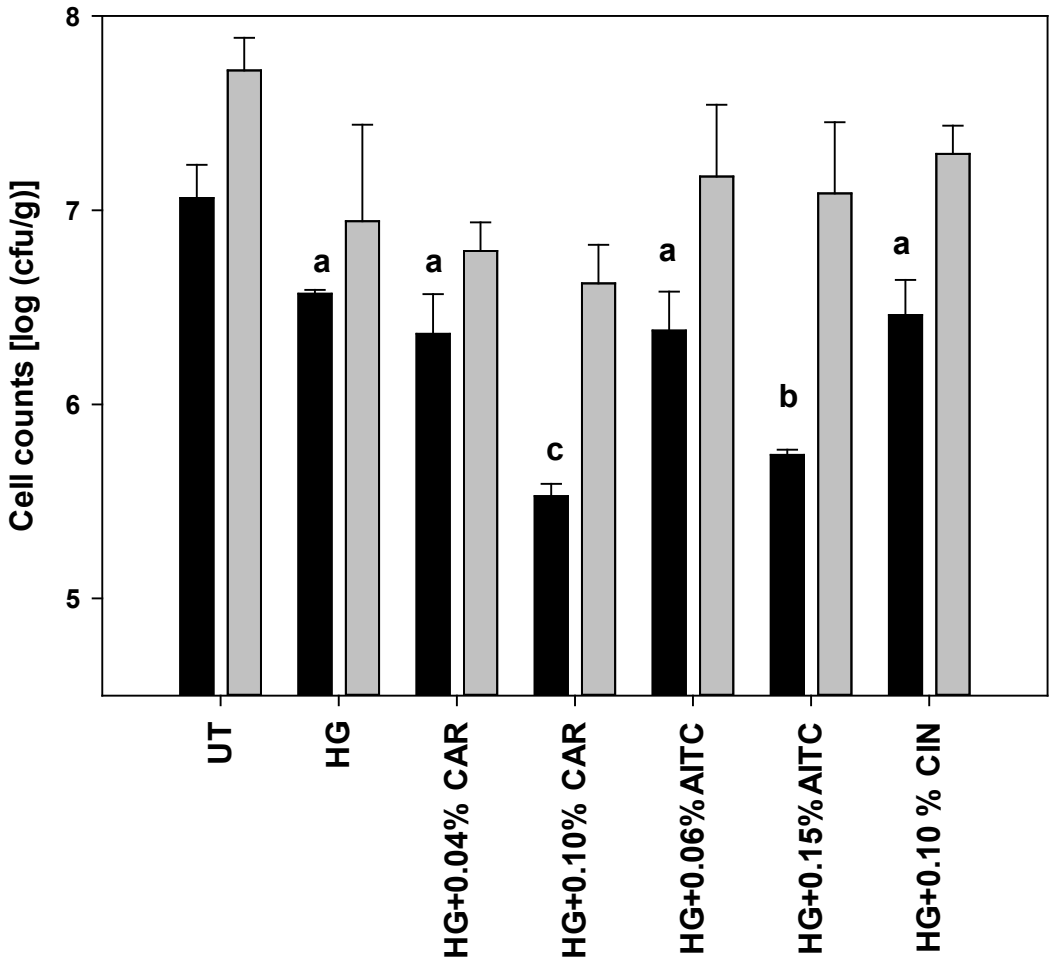

607

608 **Figure 5.** Effect of antimicrobial compounds on pressure resistance of *E. coli* AW1.7 in
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610 treated at 450 MPa 20°C for 3 min. Prior to pressure treatment, meat was marinated with
611 honey garlic (HG) marinade supplemented with carvacrol (CAR), allyl isothiocyanate (AITC)
612 or cinnamaldehyde (CIN) at a final concentration of 0.04/0.1%, 0.06/0.15% or 0.10%. UT
613 (untreated) represents marinated meat without pressure treatment. Marinated meat with no
614 antimicrobial supplement (HG) was also used as control. Cell counts were enumerated on
615 Luria-Bertani agar. Different letters above the bars indicate significant differences (p<0.05) to
616 the marinated and pressure treated control without addition of antimicrobial compounds. Data
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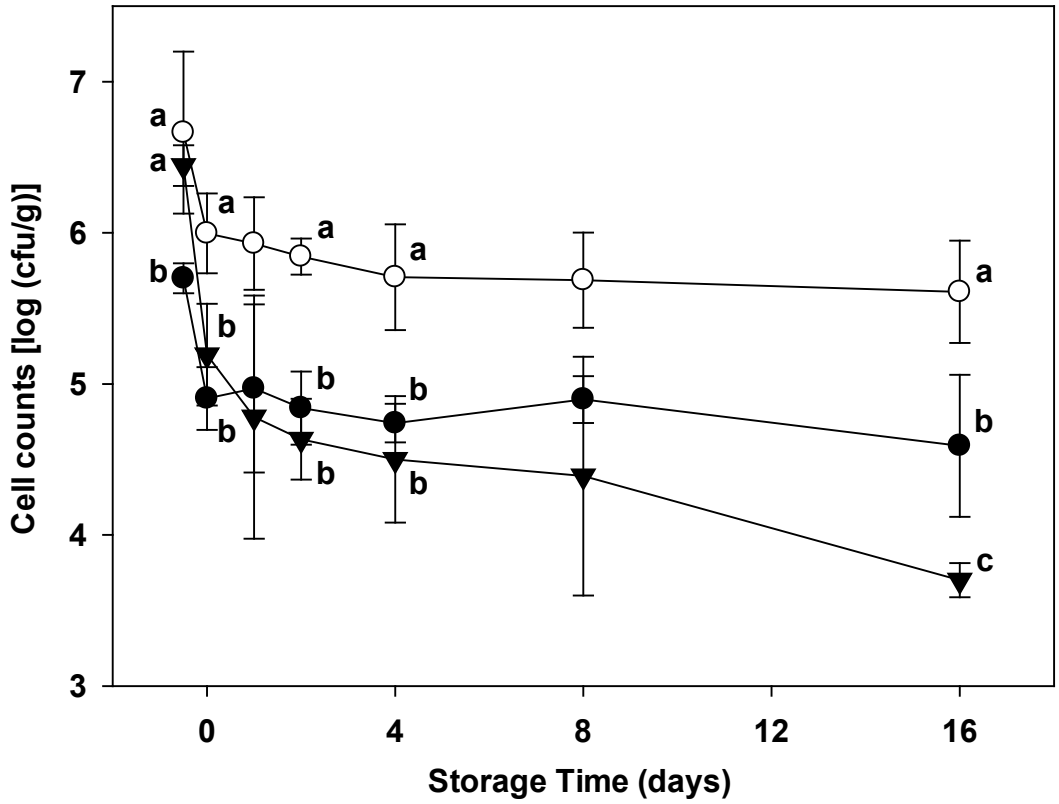

Figure 6. Effect of essential oils on survival of a 5-strain surrogate cocktail of *E. coli* in marinated beef steaks after pressure treatment and refrigerated storage. Samples were treated at 450 MPa and 20°C for 3 min, and stored at 4°C after treatment. A storage time of -0.5 days represents untreated controls; a storage time of 0 days represents cell counts taken immediately after pressure treatment. Prior to pressure treatment, steaks were marinated with marinades of honey garlic (HG) supplemented with 0.1% carvacrol (CAR, ●) or 0.15% allyl isothiocyanate (AITC, ▼). Marinated steaks without antimicrobial supplement were used as control (○). Cell counts were enumerated on Luria-Bertani agar. Data are shown as mean ± standard deviation of three independent experiments. Data obtained at the same storage time are significantly different ($p<0.05$) if they do not share a common superscript.