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Evaluation of the efficacy of multiple physical, biological and natural antimicrobial interventions for control of pathogenic *Escherichia coli* on beef

Alexandros Ch. Stratakos† and Irene R. Grant*

Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Belfast, UK

(† Current address: Food Microbiology Branch, Agriculture, Food and Environmental Science Division, Agri-Food and Biosciences Institute for Northern Ireland, Belfast, UK)

***Correspondence to:**

Dr Irene R. Grant

Institute for Global Food Security

School of Biological Sciences

Queen's University Belfast

97 Lisburn Road,

Belfast BT9 7BL, UK.

E-mail: i.grant@qub.ac.uk

ABSTRACT

Antimicrobial effects of multiple physical, biological and natural interventions on pathogenic *Escherichia coli* in raw beef were assessed. A cocktail of *E. coli* strains was inoculated onto gamma-irradiated beef and enumerated immediately after each intervention and during storage at 4°C for 7 days. Of the physical interventions, silver-containing antimicrobial packaging and ozone gas treatment did not show significant antimicrobial effects, however cold plasma treatment reduced *E. coli* levels by 0.9 and 1.82 $\log_{10}$ CFU/cm^2 after 2 and 5 min treatments, respectively. A phage cocktail reduced *E. coli* counts by 0.63 and 1.16 $\log_{10}$ CFU/g after 24 h storage at 4 and 12°C, respectively. Of the natural interventions, vinegar and lactic acid (5%) washes for 5 min caused reductions of ~1 $\log_{10}$ CFU/g immediately after treatment, whereas lactoferrin and nisin treatments, separately or in combination, had insignificant antimicrobial effects. Nanoemulsions containing carvacrol or thyme essential oils caused immediate *E. coli* reductions of 1.41 and 1.36 $\log_{10}$ CFU/g, respectively, plus a progressive reduction in viable numbers during storage at 4°C. Our findings suggest that cold plasma, bacteriophages, vinegar, lactic acid, or carvacrol and thyme essential oil nanoemulsions could potentially be of use to the beef industry for controlling pathogenic *E. coli* contamination.

Keywords: *Escherichia coli* O157:H7, decontamination, beef, cold plasma, bacteriophages essential oil nanoemulsions.

1. Introduction

Foodborne illness is a major concern for industry, public authorities and consumers, with the global impact reaching 600 million cases and 420,000 deaths on an annual basis (World Health Organisation, 2015). Over the past few decades, the food producing sector has been experiencing an increase in the demand for meat products. Nevertheless, the meat sector has also been found to be the least trusted by consumers, probably due to the increase in the occurrence of foodborne outbreaks associated with meat (European Commission 2010;

Misra and Jo, 2017). *Escherichia coli* O157 is considered a worldwide health threat and is the serogroup of *E. coli* most commonly associated with illnesses and deaths in humans (Scallan et al., 2011); with clinical manifestations ranging from abdominal pain and diarrhoea to potentially fatal haemolytic-uraemic syndrome (Food Standards Agency, 2014). Although many food products have been implicated in foodborne outbreaks, foods of bovine origin are the most frequently reported as vehicles for human *E. coli* O157 infection (European Food Safety Authority, 2011). Initial *E. coli* O157 contamination of beef products occurs mainly at the de-hiding stage of slaughtering because of bacterial transfer and adherence to the carcasses (Chagnot et al., 2013). Hazard analysis and critical control point systems have been introduced in many countries aiming to reduce or eradicate these pathogens, but even with these systems in place, the absence of *E. coli* O157 from meat cannot be guaranteed and there are still outbreaks of this pathogen that can be traced back to beef and beef products. Due to the potential meat safety concerns, researchers and the industry are continuously investigating different strategies to tackle this issue. The use of antimicrobial interventions on animal tissues with the use of hot water washing and steam pasteurization, organic acids, chlorine dioxide trisodium phosphate and cetylpyridinium chloride has been extensively studied (Mohan and Pohlman, 2016). However, the frequent foodborne disease outbreaks associated with ground beef necessitates further research. Organic acids have been approved for meat decontamination in the United States (USDA Food Safety and Inspection Service, 1996) and in 2013 lactic acid was approved for decontamination of beef carcasses by the European Commission (2013). Thermal treatments have been found to be effective in inactivating pathogenic *E. coli* and other pathogens; however they can also result in unwanted physical and chemical changes. Non-thermal processing technologies have also been investigated as substitutes for thermal processes to reduce microbial contamination while increasing quality and nutrient retention (Wheeler et al., 2014). Food irradiation, specifically electron-beam irradiation, has been found to significantly reduce *E. coli* O157 on beef, without negative effects on the sensory characteristics of the meat (Arthur et al., 2005). However, negative consumer opinion regarding food irradiation hinders its

widespread adoption. Ultraviolet radiation and ozone treatments are also of interest to the meat industry since they do not result in chemical residues or damage (Khadre et al., 2001). High pressure processing (HPP) is another non-thermal technology with high antimicrobial efficacy which has been gaining increasing importance and has been used under commercial conditions in many countries (Patterson, 2005; Hsu et al., 2015). HPP in a range 400–600 MPa has been shown to be effective in controlling most major foodborne pathogenic bacteria (e.g. *E. coli* O157:H7, *Salmonella* spp.) present in meat products such as beef and ground chicken, but is can also cause detrimental changes in meat quality (Chien et al., 2016). Among the non-thermal technologies, the application of cold plasma to improve the microbiological safety and quality of meat and meat products is very new. A few recent studies have demonstrated the potential of cold plasma technology as a novel intervention for ensuring the safety of ready-to-eat beef jerky, chicken and pork (Dirks et al., 2012; Kim et al., 2013; Kim et al., 2014). Essential oils have also been gaining importance as food preservatives, since many studies have found that they possess significant antimicrobial properties against a broad range of foodborne pathogens (Zhang et al., 2016). The antimicrobial efficiency of the essentials oils has been attributed to the high content of phenolic compounds they possess, such as carvacrol, eugenol and thymol, which can also be extracted, isolated and used as food antimicrobials (Burt, 2004). Furthermore, many studies have shown that the concept of combined decontamination treatments (hurdle approach) could be a more efficient strategy for reducing or eliminating pathogens than the application of single interventions (Sofos, 2005).

The aim of this study was to assess and compare the antimicrobial effects of different non-thermal physical (antimicrobial packaging, cold plasma, and ozone), biological (bacteriophages) and natural (vinegar, lactic acid, encapsulated essential oils, lactoferrin and nisin) interventions, as well as combinations of some treatments, against pathogenic *E. coli* present on beef cuts. The effect on *E. coli* was determined immediately after application of each intervention and throughout a 7-day storage period at refrigeration (4^{o}C), and in some cases mild abuse (12^{o}C) temperatures, after vacuum packaging.

2. Materials and methods

2.1. Bacterial strains used and inoculum preparation

A cocktail of four *E. coli* strains was used for inoculation of beef samples. This cocktail consisted of three *E. coli* strains, ATCC BAA 1427, ATCC BAA 1428 and ATCC BAA 1429, designated by the USDA Food Safety and Inspection Service (2015) as surrogate indicator organisms *for E. coli* O157, and a fourth *E. coli* strain, NCTC 12900, which is a shigatoxin negative serotype O157:H7 strain. For each strain a loopful of a fresh Tryptone soya agar plus 0.6% yeast extract (TSAYE, both Oxoid Limited, Basingstoke, UK) slope culture was inoculated into 10 ml of Brain heart infusion broth (BHI, Oxoid) and incubated at 37 °C for 24 h. Subsequently, 100 µl of a 10^{-4} dilution of this broth culture in maximum recovery diluent (Oxoid), was inoculated into another 10 ml BHI broth and incubated at 37 °C for 24 h, until the stationary phase of growth was reached. The final 10 ml cultures were harvested by centrifuging at 3600 x *g* for 30 min, washed twice in phosphate-buffered saline (PBS), and the pellet re-suspended in a final volume of 10 ml PBS to give approximately 10^8–10^9 CFU/ml. To produce the *E. coli* cocktail, equal volumes of suspensions of the four separate strains were combined and mixed well.

2.2. Preparation and inoculation of beef samples

Bulk packs (10 kg) of beef cuts were obtained from a local producer. Before use in challenge studies, rectangular beef cuts (approx. 5 cm x 5 cm) were aseptically weighed as 25 g ± 0.2 g samples into stomacher bags and sterilised by gamma radiation (15 kGy dose) at a nearby ^{60}Co Gamma beam 650 facility, in order to inactivate any naturally occurring *E. coli* cells. Irradiated beef samples were kept frozen at -20°C until required for experiments.

The *E. coli* cocktail was spot inoculated (250 µl) onto the surface of irradiated beef samples, to simulate surface contamination with faeces (McCann et al., 2006). The final inoculum level was approximately 5 $\log_{10}$ CFU/g or CFU/cm^2. Inoculated, unpackaged beef

samples were stored at 4°C for 1 hour before application of the antimicrobial intervention (Poimenidou et al., 2016). After application of each intervention, beef samples were vacuum packed, using a FoodSaver® vacuum sealing system and associated bags (Sunbeam Products, Inc., Boca Raton, USA), before storage at 4°C (and 12°C in certain cases only) for 7 days; samples were tested for viable *E. coli* at day 0, 1, 3, 5 and 7. Samples were stored at 4°C as this is the storage temperature used by the beef industry.

2.3. Antimicrobial interventions

The antimicrobial effects of a range of different physical, biological and natural antimicrobial interventions were studied. The specific concentrations or processing conditions employed for each intervention were selected on the basis of relevant previously published studies and/or preliminary trials.

2.3.1. Physical interventions

To investigate the effect of antimicrobial packaging, a commercial antimicrobial polyethylene terephthalate film incorporating silver nanoparticles (kindly provided by LINPAC Packaging, Featherstone, UK) was used to wrap the meat before vacuum packing using a conventional film. Control samples were vacuum packed using a conventional food grade film.

To investigate the effect of cold plasma treatment, a dielectric barrier discharge cold plasma jet, as described by Alkawareek et al. (2012), was used. Briefly, the plasma source consisted of a quartz dielectric tube with an inner diameter of 4 mm and an outer diameter of 6 mm and it operated at voltage amplitude of 6 kV and a repetition frequency of 20 kHz. The plasma jet configuration was encased in solid acrylic tubing. The plasma jet was produced using a mixture of helium (99.5%) and oxygen (0.5%) at flow rate of 2 standard litres per min. The temperature of the produced plume was 39°C. Beef samples were placed on a Petri dish at a distance of 15 mm from the plasma source during treatment. Based on preliminary trials (results not shown), 2 and 5 min exposure times were used as

168 they showed promising antimicrobial activity without affecting the organoleptic properties
169 of the beef. An untreated control (no exposure to plasma) was also tested. After
170 treatment, beef samples were vacuum packed and stored under refrigeration (4°C).
171 Ozone was applied to beef samples as a gaseous treatment in a hermetically closed
172 transparent cylinder. Ozone was generated using an ozone generator (ESCO, Labozone
173 model, UK). Two ozone concentrations were tested (7.2 and 32 g O_3/m^3) with an exposure
174 time of 5 min in both cases. Ozone concentration was recorded using an ozone gas analyzer
175 (GM-6000-OEM Ozomat, Germany). An untreated control was also tested.
176
177 2.3.2. Biological intervention
178 A commercially available bacteriophage cocktail (EcoShield™, Intralytix, USA) against *E.*
179 *coli* O157 was purchased for this study. The bacteriophage cocktail contained three lytic
180 phages (ECML-4, ECML-117, and ECML-134) belonging to the family *Myoviridae*. Phage
181 cocktail stocks were stored at 4°C in deionised water, according to manufacturer's
182 instructions, and enumerated by the soft agar overlay method (Jamalludeen et al., 2007).
183 The phage cocktail (250 µl) was spread onto the beef surface to achieve a multiplicity of
184 infection (MOI) of 1000; this MOI was shown to yield the most promising results during
185 preliminary trials (results not shown). For untreated controls, 250 µl of PBS was used
186 instead. The phage preparation was added on the same surface of the beef sample that the
187 *E. coli* cells had previously been inoculated on. Samples treated with the bacteriophage
188 cocktail were also stored at 12°C as studies have shown that higher storage temperatures
189 might affect pathogen survival (e.g. Viazis et al., 2011). This mild abuse temperature was
190 included in order to elucidate this potential effect of phage action.
191
192 2.3.3. Natural interventions
193 The natural antimicrobial interventions applied to beef are summarised in Table 1. Beef
194 samples were immersed in a 1 L antimicrobial solution in all cases. Preliminary trials were
195 conducted in order to identify appropriate exposure times and concentrations. Results

showed that 5 min immersion was promising and was followed or not by 30 s rinsing in sterile water. Vinegar (6% vol/vol acetic acid) purchased from a local supermarket was used undiluted. A 5% (vol/vol) lactic acid (Sigma-Aldrich, Dorset, UK) solution in sterile water was also tested. A nisin solution (Sigma-Aldrich, 1000 IU/ml) was also prepared according to Boziaris & Nychas (2006), and bovine lactoferrin (Sigma-Aldrich) at a final concentration of 0.5 mg/ml. The combination of lactic acid (5%) and nisin (1000 IU/ml) or lactoferrin (0.5 mg/ml) and nisin (1000 IU/ml) was also investigated against *E. coli* by sequential immersion in each of the solutions for 5 min. Physically stable nanoemulsions loaded with carvacrol and thyme oil were also prepared using the spontaneous emulsification method (Chang et al., 2013). Specifically, the preparation was as follows: 4 g carvacrol (Sigma-Aldrich) or thyme essential oil (Sigma-Aldrich) were added to 6 g medium chain triglyceride oil (Miglyol 812, IOI Oleo, Germany) and mixed for 5 min (600 rpm). Subsequently, 10 g Tween 80® (Sigma-Aldrich) was added to the oil mixture and mixed for another 5 min. The Tween 80/oil mixture (20 g) was titrated (2 mL/min) into 80 g 5.0 mM sodium citrate buffer (pH 3.5) and was mixed at 600 rpm for 15 min. Subsequently, the emulsion was sterilized by passing through a sterile 0.22 μm syringe filter and stored in sterile 50 mL tubes at 4°C. Droplet size was measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK). A water wash at room temperature (20°C) was also used as an additional control treatment.

2.4. Enumeration of E. coli on beef samples

Vacuum packed beef samples were opened aseptically, and the contents were transferred to a sterile stomacher bag. A 10^{-1} dilution of the sample was prepared in Maximum recovery diluent (MRD, Oxoid). The dilution was homogenised for 1 min and when necessary further 10 fold dilutions were prepared. An aliquot of 1 ml of each of the 10 fold dilutions was pour plated using Rapid 2 agar (Bio-Rad, Watford, UK) and enumerated after incubation at 37 °C for 24 h. Each sample was plated out in duplicate. With regards to the enumeration of surviving *E. coli* O157 cells after bacteriophage treatment, enumeration was performed as above but with the addition of a virucide (Jassim et al., 1998). The inclusion of

224 the virucide (7 parts 10 mM ferrous sulphate/3 parts tea extract) in the stomacher bags when
225 homogenizing beef samples prevented the overestimation of the phage killing effect due to
226 previously unbound phage particles coming into contact with surviving *E. coli* bacteria during
227 the homogenization process.
228
229 *2.5. Statistical analysis*
230 Each individual experiment was replicated on two different occasions, with three
231 replicates included on each occasion. Data for pathogen counts were subjected to analysis
232 of variance (ANOVA) to compare the interactions between treatment and storage time.
233 Differences between effects were assessed by the Tukey test ($P < 0.05$).
234
235 **3. Results and Discussion**
236
237 *3.1. Physical interventions*
238 Different nanocomposite materials have been developed for antimicrobial packaging
239 applications based on the incorporation and/or coating of nanoparticles (NPs) into/onto
240 synthetic polymers. Previous studies performed on food products have demonstrated that
241 incorporation of silver NPs into packaging films significantly extends the shelf-life of
242 chicken meat, fruits and cheese (Costa et al. 2011; Incoronato et al. 2011; Azlin-Hasim et
243 al. 2015). However, there appears to be a lack of studies on beef to date. In this study, a
244 commercially available antimicrobial packaging (polyethylene terephthalate) with
245 incorporated silver NPs was studied. Figure 1A presents the effect of the antimicrobial
246 packaging in comparison to a conventional food grade packaging film (control) during
247 refrigerated storage. The *E. coli* counts for the samples remained stable during storage, as
248 expected, since this pathogenic bacterium will not grow at a temperature below 8°C
249 (Rajkowski and Marmer, 1995). Vacuum packaging using the antimicrobial film resulted in a
250 reduction in *E. coli* of approximately 0.6 $\log_{10}$ CFU/g, after 3 days of storage (Fig. 1A), with
251 the counts remaining stable throughout the remainder of the 7 d storage period.

Sadeghnejad et al. (2014) developed films with silver nanoparticles and found that they were effective in inhibiting *E. coli* growth *in vitro*. Damm et al. (2008) found that PA-6 films with 1.9% (w/w) of nanosilver incorporated into them reduced *E. coli* levels by approx. 0.5 $\log_{10}$ CFU/ml after 24 h at room temperature *in vitro*. Our results show that antimicrobial packaging did not reduce the *E. coli* counts significantly during storage of beef at 4^{o}C. In a previous study, Stratakos et al. (2015) demonstrated that the efficacy of antimicrobial packaging for chicken meat could be improved if applied in combination with additional hurdles. However, no combination treatments were studied during the study being reported here.

Cold plasma consists of reactive species such as reactive oxygen and nitrogen species, UV radiation, energetic ions, and charged particles (Han et al. 2016). The effectiveness of cold plasma decontamination depends on a plethora of factors which include the type of cold plasma generation device, the type of microorganism characteristics, the surface topography and composition of food, water activity, pH, and the diffusion capacity of the plasma reactive species (Min et al. 2016). In this study, the effect of cold atmospheric plasma generated by a helium and oxygen (0.5%) mixture against *E. coli* inoculated onto beef was investigated. Preliminary trials were conducted to investigate suitable cold plasma exposure times (30 sec, 1 min, 2 min, 5 min, 10 min) for beef decontamination (results not presented). Exposure times shorter that 2 min did not have any significant effect on the levels of *E. coli*, so the longer treatment times were used. Exposure to cold plasma for 2 min resulted in an immediate significant reduction ($P < 0.05$) in *E. coli* counts by 0.9 $\log_{10}$ CFU/cm^2, and a further reduction in viable numbers was observed over the course of 7 days storage (Figure 1B); there were significant differences between the *E. coli* counts in beef at day 1 and counts at days 3 ($P < 0.05$), 5 ($P < 0.05$) and 7 ($P < 0.05$) days storage. At the end of the 7 day storage *E. coli* counts had reduced by 2.28 $\log_{10}$ CFU/cm^2 in total. The 5 min cold plasma treatment resulted in a larger initial reduction in *E. coli* (1.82 $\log_{10}$ CFU/cm^2) compared to the 2 min treatment ($P < 0.05$), and also a significant ($P < 0.05$) progressive reduction in *E. coli* counts over the 7 days of storage; overall reduction after 5 min cold plasma treatment was

280 2.48 $\log_{10}$ CFU/cm^2 at the end of storage. Similar results were reported by Ulbin-Figlewicz et
281 al. (2015) using a helium cold plasma source. After a 5 and 10 min treatment a reduction of
282 1.01 and 2.09 $\log_{10}$ CFU/cm^2 was achieved on the levels of *E. coli* on beef. Ziuzina et al.
283 (2012) found that cold plasma treatment at 70 kV for 2 min reduced *E. coli*, *Salmonella*,
284 and *Listeria monocytogenes* on cherry tomatoes by approximately 3, 6 and
285 7 $\log_{10}$ CFU/sample, respectively, whereas a 5 min treatment was needed to reduce these
286 pathogenic bacteria on strawberries by around 4 $\log_{10}$ CFU/sample. One explanation for the
287 progressive reduction in the levels of *E. coli* observed during storage after cold plasma
288 treatment is that the reactive species generated are able to impart their antimicrobial effect
289 for an extended period of time. However, these reactive species are short-lived and react
290 immediately with the microbial cell. Therefore, this phenomenon of extended action could
291 potentially be attributed to the occurrence of cells sub-lethally injured by cold plasma
292 treatment, which during storage at a non-favourable temperature (4^{o}C) (cold storage acts as
293 an additional hurdle against the pathogen), are unable to repair themselves and eventually
294 die. Our results indicate that cold plasma could potentially be used to decrease the risk of
295 pathogenic *E. coli* in beef and that increasing the exposure time would significantly
296 enhance the decontamination effect of the treatment. Although, no visible changes were
297 observed in the beef after the cold plasma treatments, it would be necessary to investigate
298 any potential negative effects on beef quality attributes.

299 Ozone was studied due to its recognised antimicrobial use in the fresh produce
300 industry (Greene et al. 2012). Ozone was applied to *E. coli* inoculated beef samples in
301 gaseous form in an enclosed chamber. Two ozone concentrations were tested (7.2 and 32 g
302 O_3/m^3 or 3400 ppm and 15000 ppm, respectively) for an exposure time of 5 min. Neither of
303 the ozone treatments was able to cause a significant antimicrobial effect against *E. coli*
304 either immediately after treatment or during storage (approx. reductions of 0.2-0.3 $\log_{10}$
305 CFU/g, results not shown). Coll Cárdenas et al. (2011) found that treating beef samples with
306 gaseous ozone only resulted in a decrease of 0.7 $\log_{10}$ CFU/g in *E. coli* levels after 24 h
307 exposure. McMillin and Michel (2000), working with ozonized minced beef inoculated

with *E. coli*, showed that as ozone concentrations increased (500, 3500 and 5000 ppm) larger reductions of *E. coli* were achieved, reaching a maximum reduction of 2.0 $\log_{10}$ CFU/g. The lack of any significant antimicrobial effect of ozone in this study could be attributed to the suppression of ozone efficiency by the presence of organic material on the beef surfaces. Since ozone can oxidize components of bacterial cells, it would also be able to oxidise any lean or adipose tissue present (Castillo et al., 2003). Güzel-Seydim et al. (2004) demonstrated that suspensions of locust bean gum, sodium caseinate and whipping cream actually protected *E. coli* and *Staphylococcus aureus* against ozone treatment effects. The results indicate the inadequacy of gaseous ozone processing, under the conditions tested during this study, to reduce the risk of *E. coli* on beef. The application of ozonated water could possibly be investigated as a potential alternative to gaseous ozone as it has shown promising results with foods of plant origin (Selma et al., 2008; Wani et al., 2015).

3.2. Biological intervention

The effectiveness of a commercially available bacteriophage cocktail (EcoShield™) in controlling the growth of *E. coli* O157 inoculated in raw beef was investigated. Only *E. coli* O157 strain NCTC 12900 was used to inoculate beef samples for the bacteriophage experiments; because the phage cocktail would not infect the other surrogate *E. coli* strains used as the inoculum when studying other interventions. Figure 2 presents the levels of *E. coli* O157 during refrigerated storage (4°C) and at a mild abuse storage temperature (12°C). Enumeration of surviving *E. coli* O157 cells was conducted with the inclusion of a virucide (Jassim et al., 1998; Chibeu et al., 2013) when stomaching the samples in order to inactivate unbound phages. This approach was taken to avoid overestimation of the phage antimicrobial effect due to previously unbound phage particles coming into contact with surviving bacteria during the stomaching process. The MOI is an important parameter influencing the effectiveness of bacteriophage treatment, as higher MOIs increase likelihood of individual bacteria contacting phages. A preliminary trial with beef samples showed that a MOI of 1000 was necessary to reduce counts of the pathogen. Kudva et al. (1999) also

demonstrated that a MOI of at least 1000 for lytic phages was needed to reduce numbers of *E. coli* O157 in broth culture at 4°C.

During the 7 day storage, *E. coli* counts for untreated (control) beef samples stored at 4°C remained stable, whereas for the untreated (control) samples stored at 12°C *E. coli* O157 counts increased from around 5 $\log_{10}$ CFU/cm^2 at the beginning of storage to 7.81 $\log_{10}$ CFU/cm^2 after 7 days. Treatment of beef with the phage cocktail significantly reduced the *E. coli* counts after 24 h storage ($P < 0.05$); a 0.63 $\log_{10}$ CFU/cm^2 reduction was observed for samples stored at 4°C (P4) after 24 h (Fig. 2). A further progressive reduction in *E. coli* counts was also observed during storage reaching an overall 1.53 $\log_{10}$ reduction after 7 days. Notably, after the first 24 h, the counts for the phage-treated beef samples stored at 4°C were always lower ($P < 0.05$) than control samples (C4) (Fig. 2). For samples stored at the mild abuse temperature a more pronounced decrease in the pathogen counts was observed. After 24 h the *E. coli* counts of phage-treated beef samples stored at 12°C (P12) counts were reduced by 1.16 $\log_{10}$; almost twice as much as the decrease observed for phage-treated beef stored at 4°C (P4) (Fig. 2). In this case also, there was a progressive decrease in *E. coli* counts during storage until day 3, after which point regrowth of the pathogen was observed; however, the counts of the pathogen in the phage-treated beef (P12) remained consistently lower (>2.0 $\log_{10}$ CFU/cm^2, $P < 0.05$) compared to the control (C12). Re-growth of *Listeria monocytogenes* on phage-treated roast beef and cooked turkey during storage has also been reported by Chibeu et al. (2013). The re-growth of *E. coli* observed in this study could be attributed to the inability of phage to reach all bacterial targets in the food matrix, resulting in *E. coli* multiplying in hard to reach protected areas (Guenther et al., 2012). Liu et al. (2015) treated beef with individual phages as well as a bacteriophage cocktail and found similar reductions (also using a virucide in their enumeration process) in the *E. coli* 0157 counts after 24 h and 3 days storage at 4°C. They also stored samples at abuse temperatures, however due to the short duration of storage (6 h) and much higher storage temperatures (22 and 37 °C) results are not comparable with this study. The results obtained indicate that refrigeration at 4°C did not prevent infection,

364 although the low temperature probably prolonged the phage latent period and thus time to
365 bacterial lysis (Ly-Chatain et al., 2014). It is noteworthy that inactivation of *E. coli O157*
366 increased with storage temperature. Higher inactivation at higher temperatures was also
367 observed in previous studies on leafy vegetables (Viazis et al., 2011). In conclusion, this
368 study showed that the EcoShield™ phage cocktail could potentially be used as a hurdle to
369 enhance the safety of raw beef in relation to *E. coli* O157.
370
371 **3.3. Natural interventions**
372 Washing with water is the first step in sanitation of many food commodities and is most
373 commonly used to remove microorganisms and soil and other particles. Water washing
374 alone was able to remove approx. 0.26 - 0.40 $\log_{10}$ *E. coli* CFU/g. The wine vinegar used
375 undiluted in the present study contained 6% (vol/vol) acetic acid. Preliminary trials showed
376 that vinegar caused darkening of the meat when no water rinsing was applied after treatment
377 (data not shown). Thus, a rinsing step for 30 sec in sterile water was considered necessary
378 following vinegar washing. Washing with vinegar resulted in a significant reduction in *E. coli*
379 counts (Fig. 3). Immediately after washing a reduction of 0.98 $\log_{10}$ CFU/g was achieved (P
380 < 0.05) compared to the untreated control. Pathogen reductions were significantly higher
381 compared to those caused by water washing alone throughout storage (P < 0.05).
382 Interestingly, even after the water rinsing step, the vinegar treatment resulted in residual
383 antimicrobial activity which was evident from the declining *E. coli* population during storage.
384 The reduction during storage combined with the immediate reduction in viable *E. coli*
385 resulted in an overall reduction in *E. coli* of 1.51 $\log_{10}$ CFU/g after 7 days. A study by Harris
386 et al. (2006) showed a 2.5 $\log_{10}$ reduction for *E. coli* O157:H7 after spraying with 2% acetic
387 acid with the effect remaining over time in refrigerated and frozen storage. Harris et al.
388 (2012) investigated the effect of sterile water and 2% acetic acid on the levels of *E.*
389 *coli* O157:H7 in beef in a simulated commercial processing environment. All treatments
390 reduced the *E. coli* O157:H7 load by around 0.5 $\log_{10}$ after 24 h in ground beef (not beef
391 cuts, as in this study). According to Carpenter et al. (2010), 2% acetic acid did not show a

392 high decontamination capacity compared to water wash but was able to prevent the growth
393 of *E. coli* O157:H7 on beef. Therefore, results show that vinegar washing appears, even
394 including a rinsing step to avoid beef darkening, to be an effective way to reduce the levels
395 of *E. coli* immediately, as well as during subsequent storage, in beef.
396 Lactic acid is an organic acid that has received GRAS (Generally Recognized as Safe)
397 status and can be used for the removal of surface decontamination in beef carcasses (EFSA
398 2011). Preliminary trials were conducted to identify the most appropriate lactic acid
399 concentration for beef application (data not shown). A 5 % lactic acid concentration was
400 selected for subsequent experiments. Beef washing in a lactic acid solution for 5 min with a
401 subsequent water rinsing for 30 s reduced *E. coli* by 1.10 $\log_{10}$ CFU/g ($P < 0.05$), whereas
402 during 7 days of storage the pathogen gradually reduced to give an overall 2.22 $\log_{10}$ CFU/g
403 reduction (Fig. 3) compared to the control. *E. coli* counts for the water wash were
404 significantly higher than the lactic acid treated samples throughout storage. The
405 decontamination efficiency of lactic acid has been found to be influenced by a number of
406 factors, e.g. acid concentration, volume used, nature of treated surface, application method
407 and level of contamination (Youssef et al., 2012). In order to explore if the rinsing step may
408 have had a quenching effect on the antimicrobial activity the lactic acid intervention study
409 was also performed without incorporating a rinsing step (Fig. 3). Lactic acid resulted in a
410 1.19 $\log_{10}$ CFU/g reduction in *E. coli* counts immediately after the 5 min treatment and
411 reached an overall reduction of 2.74 $\log_{10}$ CFU/g at the end of storage compared to the
412 control. The counts of the lactic acid treatment were always significantly lower compared to
413 the water wash ($P < 0.05$). There was a more pronounced progressive reduction of the
414 pathogen counts during the first 3 days of storage after which pathogen levels remained
415 relatively similar (no significant difference between day 3, 5 and 7). When compared to lactic
416 acid plus rinsing (Fig. 3), the absence of a rinsing step led to a greater reduction in pathogen
417 counts, after day 1. The additional reduction achieved was in the range of 0.30 - 0.75 $\log_{10}$
418 CFU/g during storage. Previous studies have shown that lactic acid (1-4%) applied to beef
419 can significantly reduce populations of *E. coli* (Dorsa et al., 1997; Castillo et al.,

2001). Harris et al. (2006) reported a 1.5 $\log_{10}$ CFU/g reduction for *E. coli* O157:H7 could be achieved after spraying with lactic acid (4%). Youssef et al. (2012) also found that treating beef cuts or trimmings with 5% lactic acid could reduce numbers of *E. coli* by 0.5 - 1 $\log_{10}$ CFU/cm^2.

Nisin is a well-known bacteriocin, which also has GRAS status and is commercially used in the food industry to extend shelf-life and enhance food safety (Abdollahzadeh et al. 2014). When the nisin wash (1000 IU/ml) was applied alone, no significant reduction in *E. coli* counts on beef samples was achieved compared with water wash ($P < 0.05$). This was not unexpected since nisin is effective against Gram positive bacteria such as *Brochothrix thermosphacta*, *Clostridium botulinum*, *Staphylococcus aureus*, *Listeria innocua* or *Listeria monocytogenes* (Mustapha et al. 2002), and not necessarily Gram negative bacteria like *E. coli*. However, studies have shown that if nisin is combined with other hurdles, such as essential oils or chelators, it can be effective against *E. coli* (Fang and Tsai 2003; Solomakos et al. 2008). When a nisin wash (1000 IU/ml) was applied subsequent to a 5% lactic acid wash, followed by a 30 s water rinse, no additive or synergistic antimicrobial effects were observed immediately after treatment or during storage ($P>0.05$) (Fig. 3). Statistical analysis showed that the 5% lactic acid treatment and the lactic acid/nisin treatment did not differ significantly at any point during storage, revealing that the combination of lactic acid (5%) and nisin (1000 IU/ml) does not result in enhanced effectiveness against *E. coli*. Mustapha et al. (2002) treated raw beef with lactic acid (2%) alone and in combination with nisin (200 IU/ml) and they also found that nisin does not contribute to any additional antimicrobial effect compared to lactic acid alone against *E. coli*. In the present study higher concentrations of both lactic acid (5%) and nisin were used (1000 IU/ml) but nevertheless it still did not result in any increase in effectiveness against *E. coli*.

Lactoferrin is an iron-binding antimicrobial glycoprotein that is found in milk and other mammalian exocrine secretions (Ye et al., 2000; Steijns and van Hooijdonk, 2000). The effect of lactoferrin (0.5 mg/ml) wash alone or in conjunction with nisin was studied. This concentration of lactoferrin was chosen as it has been previously reported that, under *in*

448 *vitro* conditions, this concentration can impart a bactericidal effect (Murdock et al., 2007).
449 Results showed that there were no significant ($P > 0.05$) difference in reductions of *E. coli*
450 between water washing and lactoferrin wash (data not shown); reductions for water and
451 lactoferrin were 0.26-0.42 and 0.35 - 0.46 $\log_{10}$ CFU/g, respectively, during storage. Our
452 results are in agreement with the study of Del Olmo et al. (2012) who showed that lactoferrin
453 (0.5 mg/ml) was not able to significantly reduce the counts of *E. coli* O157 inoculated onto
454 chicken fillets. Bravo et al. (2014) also found that lactoferrin alone applied to beef carpaccio
455 was not able to reduce the counts of *L. monocytogenes*, *Salmonella* Enteritidis or *E.*
456 *coli* O157. Since a study by Murdock et al. (2007) demonstrated that lactoferrin (0.5 mg/ml)
457 and nisin (250 IU/ ml) acted synergistically against *E. coli* O157 *in vitro*, lactoferrin was also
458 evaluated in combination with a nisin wash (1000 IU/ml) in this study, to explore if any
459 increase in effectiveness occurred for beef decontamination. In this case also there was no
460 increase in the effectiveness, with the lactoferrin/nisin treatment achieving a similar reduction
461 (0.30 - 0.49 $\log_{10}$ CFU/g) to the water wash. The present study shows that the bactericidal
462 effect of lactoferrin declines substantially when used for beef and that use of lactoferrin in
463 conjunction with nisin does not offer any benefit under the applied conditions.

464 Plant essential oils, especially those from thyme, oregano, and clove, have strong
465 antimicrobial effects. However, it has been shown that the effectiveness of essential oils is
466 partially inhibited when applied to foods (Burt, 2004; Baranauskiene et al., 2006). The water
467 insoluble nature of essential oils, as well as interaction with food components, can reduce
468 their antimicrobial effects (Shah et al., 2012; Bhargava et al., 2015). Encapsulation of
469 essential oils in nanoemulsions constitutes an alternative strategy to improve their
470 antimicrobial efficiency in foods, by protecting them from interaction with food constituents
471 and increasing their solubility, thus allowing a higher mass transfer of EO to the cell
472 membranes of foodborne pathogens. The effect of two different EO nanoemulsions plus a
473 water rinsing step on *E. coli* counts on beef are presented in Figure 4. In this case a buffer
474 wash was performed instead of a water wash in order to investigate if the lower pH of the
475 buffer (pH 3.5) used to prepare the EO nanoemulsions had any effect on *E. coli* survival. The

476 reduction in *E. coli* counts achieved after the buffer wash were in the range of 0.30-0.51 $\log_{10}$
477 CFU/g during storage, thus demonstrating no additional effect compared to the water wash
478 employed for the other interventions. After initial preparation, the nanoemulsions were
479 diluted 5-fold to avoid droplet size increase (Landry et al., 2014). After this 5-fold dilution, the
480 nanoemulsions had a concentration of 8000 ppm of either carvacrol or thyme. The carvacrol
481 and thyme EO nanoemulsions prepared by spontaneous emulsification had an average
482 particle size of 100 and 60 nm, respectively, with a polydispersity index of 0.20-0.28,
483 measured by light scattering. The carvacrol nanoemulsion resulted in a significant reduction
484 ($P < 0.05$) of 1.13 $\log_{10}$ CFU/g compared to control immediately after treatment. A similar
485 significant reduction was achieved by the thyme EO wash (1.09 $\log_{10}$ CFU/g) immediately
486 after the treatment. Both nanoemulsions also showed similar overall reductions by the end of
487 storage (1.41 and 1.36 $\log_{10}$ CFU/g for carvacrol and thyme EO, respectively). Statistical
488 analysis showed that the counts of *E. coli* between the two nanoemulsion treatments did not
489 differ significantly at any point during storage, showing that both of them appear to be
490 equally effective. *E. coli* counts during storage showed similar decreases (P>0.05) at each of
491 the time-points during storage for the two EO nanoemulsion treatments.

492 The antimicrobial effect of the nanoemulsion is attributed to the disruption of the cell
493 membrane leading to the release of cell components, such as carbohydrates, proteins, DNA,
494 and potassium ions (Burt, 2004; Moghimi et al., 2016). To the best of our knowledge this is
495 the first study where nanoemulsions were applied to beef to combat *E. coli* contamination.
496 Using oregano oil nanoemulsion (average particle size 148 nm diameter), Bhargava et al.
497 (2015) observed reductions in *E. coli* O157 counts, inoculated on lettuce leaves, of 3.05 and
498 3.35 $\log_{10}$ CFU/g by 500 ppm and 1000 ppm, respectively, 24 h after application. According
499 to Landry et al. (2015), carvacrol nanoemulsion (4000 ppm and 8000 ppm) wash of radish
500 and broccoli seeds reduced *E. coli* O157 counts by 2-3 $\log_{10}$ CFU/g after a 60 min
501 treatment. Nanoemulsified thyme oil has also been demonstrated to result in significant
502 reductions of *E. coli* (after 4 and 8 h) in 2% reduced fat milk and has also been found to be
503 more effective than free thyme oil (Xue et al., 2015). Moghimi et al. (2016) also found that

504 thyme essential oil nanoemulsions (diameter ~ 150 nm) have high antibacterial activity
505 against *E. coli in vitro*. Furthermore, the susceptibility of bacteria to essential oils has been
506 shown to be dependent on the pH of the treatment (Shah et al., 2012). The nanoemulsions
507 used in this study had a low pH (3.5), due to the sodium citrate buffer used to prepare them.
508 The antimicrobial effect they imparted on *E. coli* could be due to combined low pH stress and
509 the antimicrobial effects of carvarcol or thyme EO.

510 Nanoemulsion washes were also applied without the rinsing step. Carvacrol and thyme
511 EO nanoemulsion showed significant reductions ($P < 0.05$) of 0.92 and 0.86 $\log_{10}$ CFU/g
512 after the 5 min treatment and 1.79 and 1.64 $\log_{10}$ CFU/g, respectively, at the end of storage
513 compared to the control. In this case also, no significant differences ($P > 0.05$) were
514 observed in the effectiveness of the two nanoemulsions. The absence of a rinsing step led to
515 a further reduction in the pathogen counts at day 1, after which counts did not differ
516 significantly between storage days (day 1 v day 7). It also led to moderately greater
517 reductions compared to when the rinsing step was included.

518 Although, there was an increase in nanoemulsion effectiveness in the absence of the
519 rinsing step, the reduction obtained in this study was lower compared to other studies in
520 other food matrices. This could possibly be explained by the different surface topography of
521 the beef meat (e.g. surface roughness, crevices) which could allow bacteria to avoid contact
522 with the nanoemulsions. Another explanation for the lower reductions would be the presence
523 of higher amounts of organic load on the meat which could lead to loss of nanoemulsion
524 effectiveness (Landry et al., 2015). Even though a high quantity of the essential oils was
525 loaded into the nanoemulsions, the exposure time adopted was significantly shorter
526 compared to previous studies on the same essential oils. The shorter exposure time would
527 increase the applicability of this intervention and reduce the possibility of changes in the
528 organoleptic characteristics of the meat; however further studies would be needed to prove
529 this. Although less pronounced for nanoemulsions, the increased effectiveness observed for
530 both lactic acid and nanoemulsions indicate the quenching effect that rinsing has on the
531 antimicrobial activity of these two interventions.

4. Conclusion

This study has identified several effective physical, biological and natural control interventions to reduce pathogenic *E. coli* on beef - cold plasma treatment, application of a phage cocktail, and use of vinegar, 5% lactic acid, and carvacrol and thyme oil nanoemulsions - that could potentially find application in the beef industry. Other interventions studied, namely silver-containing packaging, ozone applied in gaseous form, and lactoferrin and nisin, even when applied in combination, did not demonstrate useful reductions in pathogenic *E. coli* numbers. Further research would be needed to assess the impact of the effective *E. coli* control interventions on the quality and sensory properties of beef, as they could potentially impact consumer acceptance. Any potential effect of these interventions on the virulence of surviving pathogenic *E. coli* O157 cells should also be evaluated.

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Table 1

Summary of natural interventions studied for control of pathogenic *E. coli* on raw beef.

Intervention	Concentration	Exposure time (min)	Rinsing[a]
Sterile water	-	5	-
Vinegar[b]	6% acetic acid	5	Yes
Lactic acid	5%	5	Yes
Lactic acid	5%	5	No
Nisin	1000 IU/ml	5	Yes
Lactic acid - Nisin	5% - 1000 IU/ml	5	Yes
Lactoferrin	0.5 mg/ml	5	Yes
Lactoferrin - Nisin	0.5 mg/ml - 1000 IU/ml	5	Yes
Carvacrol EO nanoemulsion	8000 ppm	5	Yes
Carvacrol EO nanoemulsion	8000 ppm	5	No
Thyme EO nanoemulsion	8000 ppm	5	Yes
Thyme EO nanoemulsion	8000 ppm	5	No

[a] rinsing after intervention treatment in sterile water.

[b] Shop bought vinegar used without dilution; label indicated 6% acetic acid.

781 **Figure legends**

782

783 **Fig. 1.** Effect of antimicriobial packaging (A) and cold plasma (2 and 5 min treatment) (B) on
784 levels of *E. coli* on artificially contaminated beef during storage at 4°C. Each point is the
785 average of six replicates. Error bars represent ± standard deviation.

786

787 **Fig. 2.** Effect of the EcoShield™ phage cocktail wash on numbers of *E. coli* on artificially
788 contaminated beef during storage at 4°C and 12°C. (C4: control samples stored at 4°C; P4:
789 phage treated samples stored at 4°C; C12: control samples stored at 12°C; P12: phage
790 treated samples stored at 12°C). Each point is the average of six replicates. Error bars
791 represent ± standard deviation.

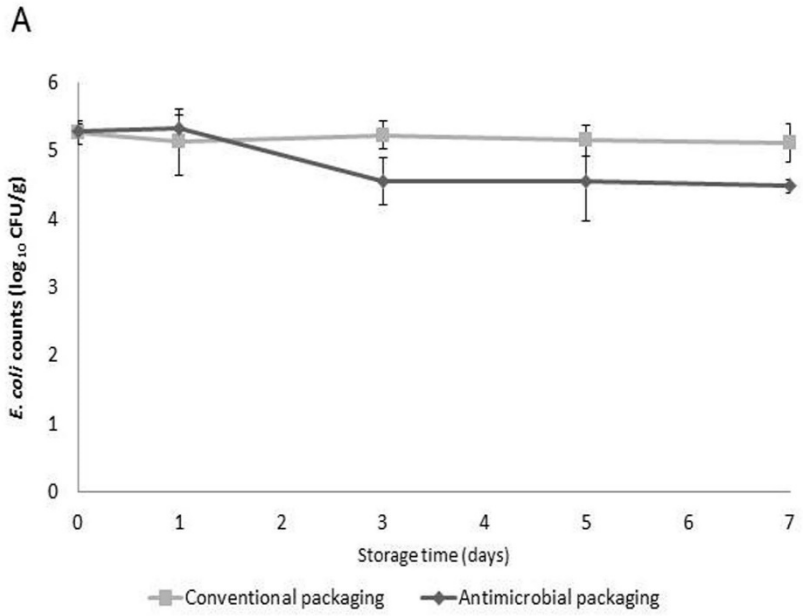
792

793 **Fig. 3.** Effect of vinegar, lactic acid (with and without rinsing) (5%), nisin (1000 IU/ml) alone
794 or in combination with lactic acid on the levels of *E. coli* on artificially contaminated beef
795 during storage at 4°C. Each point is the average of six replicates. Error bars represent ±
796 standard deviation.

797

798 **Fig. 4.** Effect of carvacrol and thyme essential oil (EO) nanoemulsions with and without
799 water rinsing (30 s) on levels of *E. coli* on artificially contaminated beef during storage at
800 4°C. Each point is the average of six replicates. Error bars represent ± standard deviation.

Fig. 1.


A
E. coli counts (log10 CFU/g)
Storage time (days)
Conventional packaging
Antimicrobial packaging

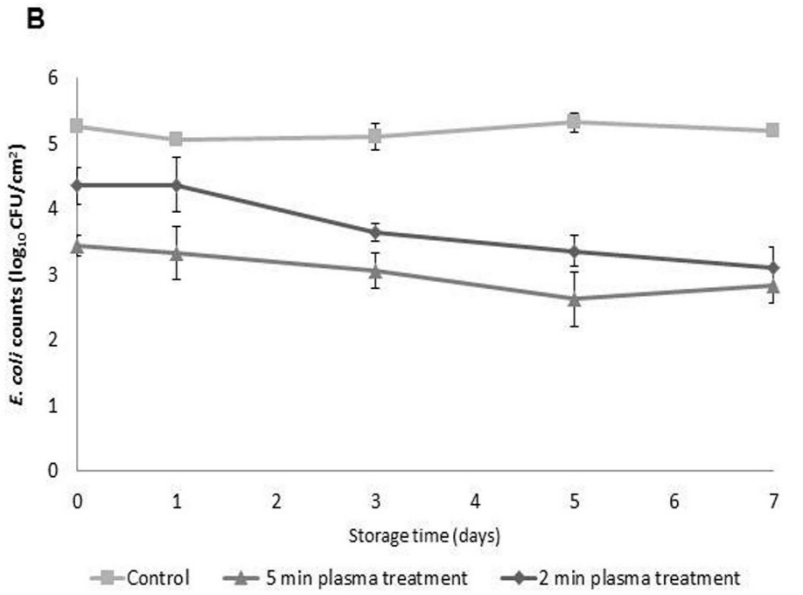

B
E. coli counts (log10 CFU/cm2)
Storage time (days)
Control
5 min plasma treatment
2 min plasma treatment

Fig. 2.

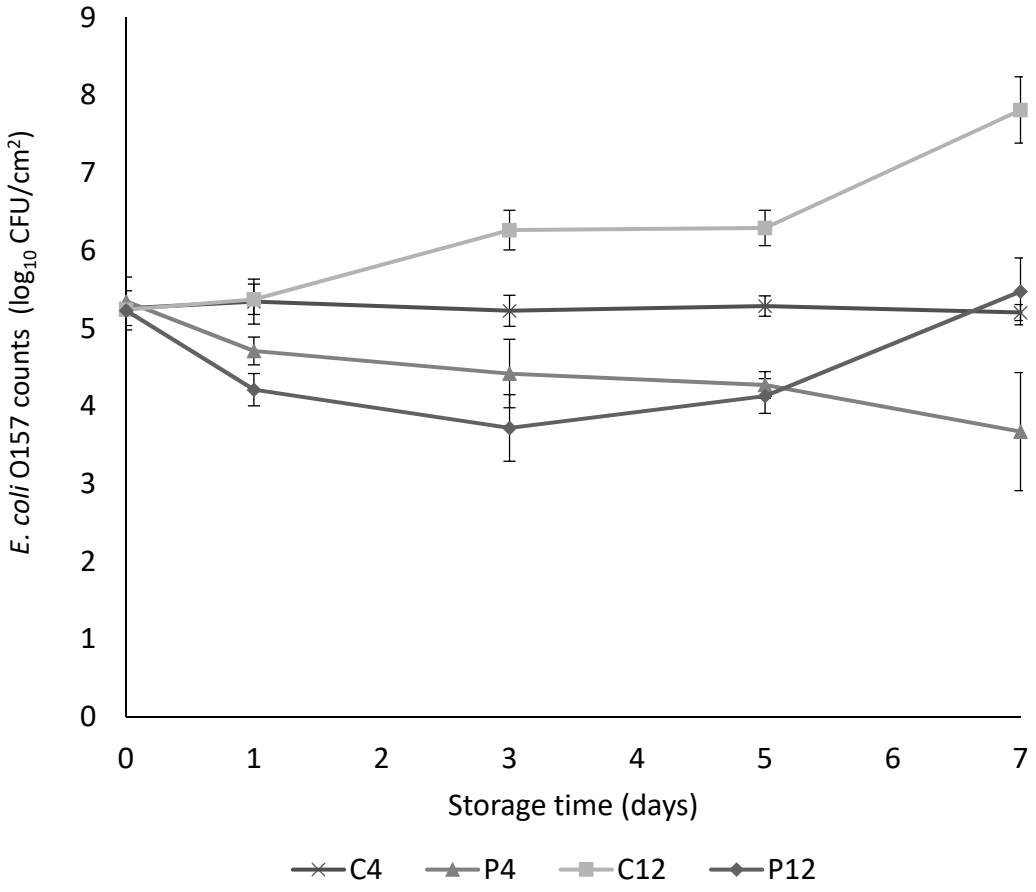
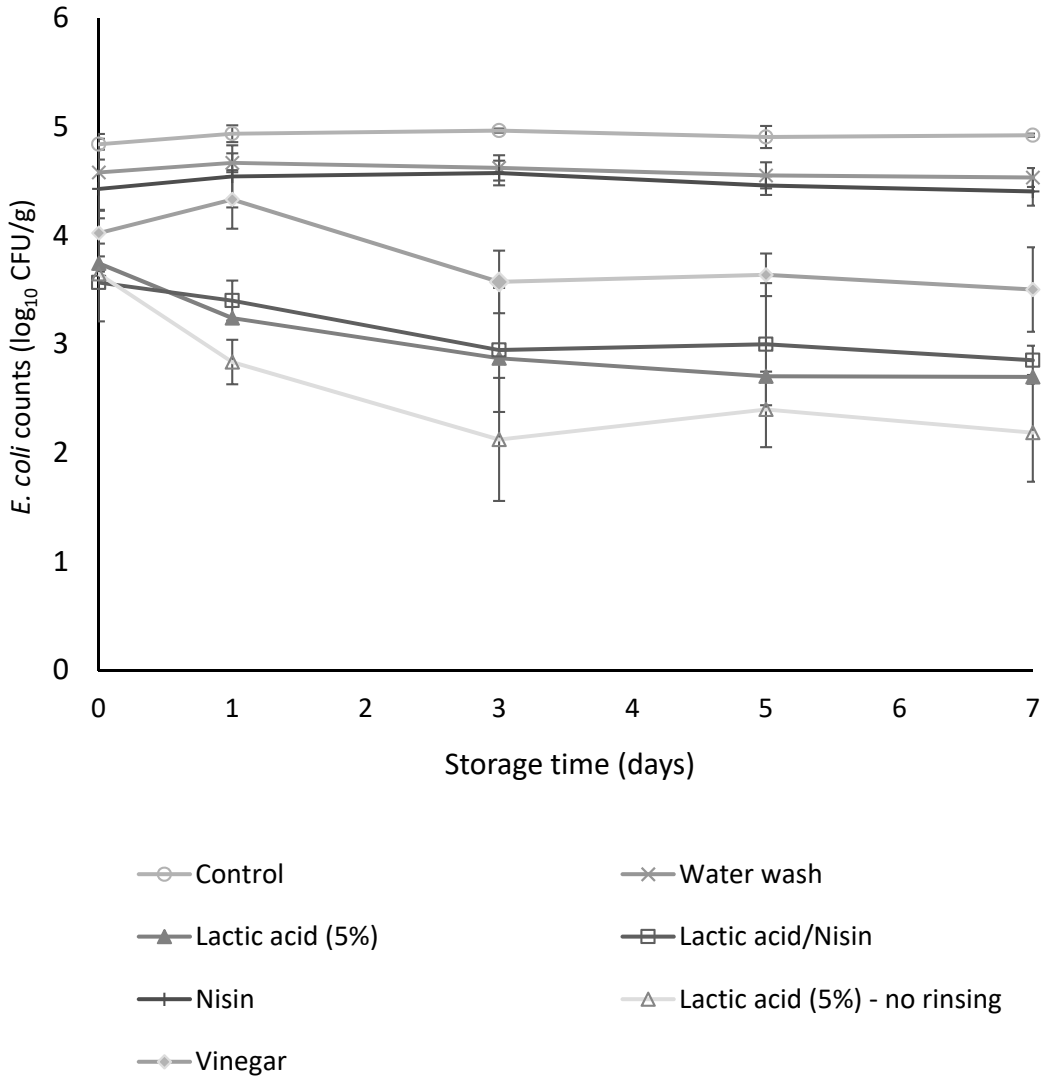
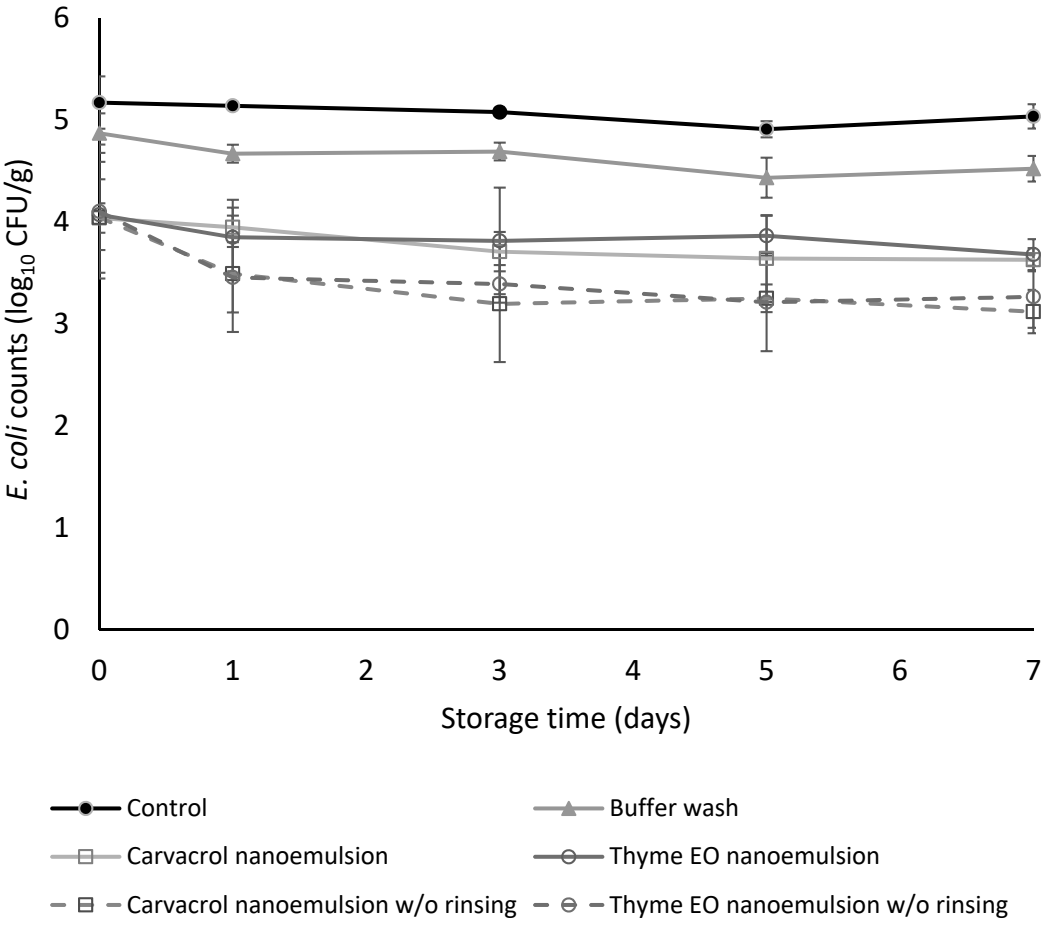

9
8
7
6
5
4
3
2
1
0
E. coli O157 counts (log10 CFU/cm2)
0
1
2
3
4
5
6
7
Storage time (days)
C4
P4
C12
P12

Fig. 3.


6
5
4
3
2
1
0
E. coli counts (log10 CFU/g)
0
1
2
3
4
5
6
7
Storage time (days)
Control
Water wash
Lactic acid (5%)
Lactic acid/Nisin
Nisin
Lactic acid (5%) - no rinsing
Vinegar


6
5
4
3
2
1
0
E. coli counts (log10 CFU/g)
0
1
2
3
4
5
6
7
Storage time (days)
Control
Buffer wash
Carvacrol nanoemulsion
Thyme EO nanoemulsion
Carvacrol nanoemulsion w/o rinsing
Thyme EO nanoemulsion w/o rinsing