

1
2
3
4 **Evaluation of the efficacy of multiple physical, biological and natural**
5 **antimicrobial interventions for control of pathogenic *Escherichia coli* on beef**
6

7 Alexandros Ch. Stratakos[†] and Irene R. Grant*

8
9 Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast,
10 Belfast, UK
11

12 († Current address: Food Microbiology Branch, Agriculture, Food and Environmental Science
13 Division, Agri-Food and Biosciences Institute for Northern Ireland, Belfast, UK)
14

15 ***Correspondence to:**

16 Dr Irene R. Grant
17 Institute for Global Food Security
18 School of Biological Sciences
19 Queen's University Belfast
20 97 Lisburn Road,
21 Belfast BT9 7BL, UK.

22 E-mail: i.grant@qub.ac.uk
23
24
25
26
27

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 log₁₀ CFU/cm² after 2 and 5 min treatments, respectively. A phage cocktail reduced *E. coli* counts by 0.63 and 1.16 log₁₀ 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 log₁₀ 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 log₁₀ 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 it 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 essential 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°C), and in some cases mild abuse (12°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 10⁸–10⁹ 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 ⁶⁰Co 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 log₁₀ CFU/g or CFU/cm². 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 they showed promising antimicrobial activity without affecting the organoleptic properties of the beef. An untreated

control (no exposure to plasma) was also tested. After treatment, beef samples were vacuum packed and stored under refrigeration (4°C).

Ozone was applied to beef samples as a gaseous treatment in a hermetically closed transparent cylinder. Ozone was generated using an ozone generator (ESCO, Labozone model, UK). Two ozone concentrations were tested (7.2 and 32 g O₃/m³) with an exposure time of 5 min in both cases. Ozone concentration was recorded using an ozone gas analyzer (GM-6000-OEM Ozomat, Germany). An untreated control was also tested.

2.3.2. Biological intervention

A commercially available bacteriophage cocktail (EcoShield™, Intralytix, USA) against *E. coli* O157 was purchased for this study. The bacteriophage cocktail contained three lytic phages (ECML-4, ECML-117, and ECML-134) belonging to the family *Myoviridae*. Phage cocktail stocks were stored at 4°C in deionised water, according to manufacturer's instructions, and enumerated by the soft agar overlay method (Jamalludeen et al., 2007). The phage cocktail (250 µl) was spread onto the beef surface to achieve a multiplicity of infection (MOI) of 1000; this MOI was shown to yield the most promising results during preliminary trials (results not shown). For untreated controls, 250 µl of PBS was used instead. The phage preparation was added on the same surface of the beef sample that the *E. coli* cells had previously been inoculated on. Samples treated with the bacteriophage cocktail were also stored at 12°C as studies have shown that higher storage temperatures might affect pathogen survival (e.g. Viazis et al., 2011). This mild abuse temperature was included in order to elucidate this potential effect of phage action.

2.3.3. Natural interventions

The natural antimicrobial interventions applied to beef are summarised in Table 1. Beef samples were immersed in a 1 L antimicrobial solution in all cases. Preliminary trials were 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 poured 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 the virucide (7 parts 10 mM ferrous sulphate/3 parts tea extract) in the stomacher bags when homogenizing beef

samples prevented the overestimation of the phage killing effect due to previously unbound phage particles coming into contact with surviving *E. coli* bacteria during the homogenization process.

2.5. Statistical analysis

Each individual experiment was replicated on two different occasions, with three replicates included on each occasion. Data for pathogen counts were subjected to analysis of variance (ANOVA) to compare the interactions between treatment and storage time. Differences between effects were assessed by the Tukey test ($P < 0.05$).

3. Results and Discussion

3.1. Physical interventions

Different nanocomposite materials have been developed for antimicrobial packaging applications based on the incorporation and/or coating of nanoparticles (NPs) into/onto synthetic polymers. Previous studies performed on food products have demonstrated that incorporation of silver NPs into packaging films significantly extends the shelf-life of chicken meat, fruits and cheese (Costa et al. 2011; Incoronato et al. 2011; Azlin-Hasim et al. 2015). However, there appears to be a lack of studies on beef to date. In this study, a commercially available antimicrobial packaging (polyethylene terephthalate) with incorporated silver NPs was studied. Figure 1A presents the effect of the antimicrobial packaging in comparison to a conventional food grade packaging film (control) during refrigerated storage. The *E. coli* counts for the samples remained stable during storage, as expected, since this pathogenic bacterium will not grow at a temperature below 8°C (Rajkowski and Marmer, 1995). Vacuum packaging using the antimicrobial film resulted in a reduction in *E. coli* of approximately 0.6 log₁₀ CFU/g, after 3 days of storage (Fig. 1A), with 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 log₁₀ 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°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 than 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 log₁₀ CFU/cm², 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 log₁₀ CFU/cm² in total. The 5 min cold plasma treatment resulted in a larger initial reduction in *E. coli* (1.82 log₁₀ CFU/cm²) 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 2.48 log₁₀ CFU/cm² at the end of storage. Similar results were reported by Ulbin-Figlewicz et al. (2015) using a helium cold plasma source. After a 5 and 10 min treatment a reduction of 1.01 and 2.09 log₁₀

CFU/cm² was achieved on the levels of *E. coli* on beef. Ziuzina et al. (2012) found that cold plasma treatment at 70 kV for 2 min reduced *E. coli*, *Salmonella*, and *Listeria monocytogenes* on cherry tomatoes by approximately 3, 6 and 7 log₁₀ CFU/sample, respectively, whereas a 5 min treatment was needed to reduce these pathogenic bacteria on strawberries by around 4 log₁₀ CFU/sample. One explanation for the progressive reduction in the levels of *E. coli* observed during storage after cold plasma treatment is that the reactive species generated are able to impart their antimicrobial effect for an extended period of time. However, these reactive species are short-lived and react immediately with the microbial cell. Therefore, this phenomenon of extended action could potentially be attributed to the occurrence of cells sub-lethally injured by cold plasma treatment, which during storage at a non-favourable temperature (4°C) (cold storage acts as an additional hurdle against the pathogen), are unable to repair themselves and eventually die. Our results indicate that cold plasma could potentially be used to decrease the risk of pathogenic *E. coli* in beef and that increasing the exposure time would significantly enhance the decontamination effect of the treatment. Although, no visible changes were observed in the beef after the cold plasma treatments, it would be necessary to investigate any potential negative effects on beef quality attributes.

Ozone was studied due to its recognised antimicrobial use in the fresh produce industry (Greene et al. 2012). Ozone was applied to *E. coli* inoculated beef samples in gaseous form in an enclosed chamber. Two ozone concentrations were tested (7.2 and 32 g O₃/m³ or 3400 ppm and 15000 ppm, respectively) for an exposure time of 5 min. Neither of the ozone treatments was able to cause a significant antimicrobial effect against *E. coli* either immediately after treatment or during storage (approx. reductions of 0.2-0.3 log₁₀ CFU/g, results not shown). Coll Cárdenas et al. (2011) found that treating beef samples with gaseous ozone only resulted in a decrease of 0.7 log₁₀ CFU/g in *E. coli* levels after 24 h 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 log₁₀ 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 log₁₀ CFU/cm² at the beginning of storage to 7.81 log₁₀ CFU/cm² 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 log₁₀ CFU/cm² 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 log₁₀ 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 log₁₀; 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 log₁₀ CFU/cm², $P < 0.05$) 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* O157 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, although the low temperature probably prolonged the phage latent period and thus time to bacterial lysis (Ly-Chatain et al., 2014). It is noteworthy that inactivation of *E. coli* O157 increased with storage temperature. Higher inactivation at higher temperatures was

also observed in previous studies on leafy vegetables (Viazis et al., 2011). In conclusion, this study showed that the EcoShield™ phage cocktail could potentially be used as a hurdle to enhance the safety of raw beef in relation to *E. coli* O157.

3.3. Natural interventions

Washing with water is the first step in sanitation of many food commodities and is most commonly used to remove microorganisms and soil and other particles. Water washing alone was able to remove approx. 0.26 - 0.40 log₁₀ *E. coli* CFU/g. The wine vinegar used undiluted in the present study contained 6% (vol/vol) acetic acid. Preliminary trials showed that vinegar caused darkening of the meat when no water rinsing was applied after treatment (data not shown). Thus, a rinsing step for 30 sec in sterile water was considered necessary following vinegar washing. Washing with vinegar resulted in a significant reduction in *E. coli* counts (Fig. 3). Immediately after washing a reduction of 0.98 log₁₀ CFU/g was achieved ($P < 0.05$) compared to the untreated control. Pathogen reductions were significantly higher compared to those caused by water washing alone throughout storage ($P < 0.05$). Interestingly, even after the water rinsing step, the vinegar treatment resulted in residual antimicrobial activity which was evident from the declining *E. coli* population during storage. The reduction during storage combined with the immediate reduction in viable *E. coli* resulted in an overall reduction in *E. coli* of 1.51 log₁₀ CFU/g after 7 days. A study by Harris et al. (2006) showed a 2.5 log₁₀ reduction for *E. coli* O157:H7 after spraying with 2% acetic acid with the effect remaining over time in refrigerated and frozen storage. Harris et al. (2012) investigated the effect of sterile water and 2% acetic acid on the levels of *E. coli* O157:H7 in beef in a simulated commercial processing environment. All treatments reduced the *E. coli* O157:H7 load by around 0.5 log₁₀ after 24 h in ground beef (not beef cuts, as in this study). According to Carpenter et al. (2010), 2% acetic acid did not show a high decontamination capacity compared to water wash but was able to prevent the growth of *E. coli* O157:H7 on beef. Therefore, results show that vinegar washing appears, even including a rinsing step to avoid beef darkening, to be an

effective way to reduce the levels of *E. coli* immediately, as well as during subsequent storage, in beef.

Lactic acid is an organic acid that has received GRAS (Generally Recognized as Safe) status and can be used for the removal of surface decontamination in beef carcasses (EFSA 2011). Preliminary trials were conducted to identify the most appropriate lactic acid concentration for beef application (data not shown). A 5 % lactic acid concentration was selected for subsequent experiments. Beef washing in a lactic acid solution for 5 min with a subsequent water rinsing for 30 s reduced *E. coli* by 1.10 log₁₀ CFU/g ($P < 0.05$), whereas during 7 days of storage the pathogen gradually reduced to give an overall 2.22 log₁₀ CFU/g reduction (Fig. 3) compared to the control. *E. coli* counts for the water wash were significantly higher than the lactic acid treated samples throughout storage. The decontamination efficiency of lactic acid has been found to be influenced by a number of factors, e.g. acid concentration, volume used, nature of treated surface, application method and level of contamination (Youssef et al., 2012). In order to explore if the rinsing step may have had a quenching effect on the antimicrobial activity the lactic acid intervention study was also performed without incorporating a rinsing step (Fig. 3). Lactic acid resulted in a 1.19 log₁₀ CFU/g reduction in *E. coli* counts immediately after the 5 min treatment and reached an overall reduction of 2.74 log₁₀ CFU/g at the end of storage compared to the control. The counts of the lactic acid treatment were always significantly lower compared to the water wash ($P < 0.05$). There was a more pronounced progressive reduction of the pathogen counts during the first 3 days of storage after which pathogen levels remained relatively similar (no significant difference between day 3, 5 and 7). When compared to lactic acid plus rinsing (Fig. 3), the absence of a rinsing step led to a greater reduction in pathogen counts, after day 1. The additional reduction achieved was in the range of 0.30 - 0.75 log₁₀ CFU/g during storage. Previous studies have shown that lactic acid (1-4%) applied to beef can significantly reduce populations of *E. coli* (Dorsa et al., 1997; Castillo et al., 2001). Harris et al. (2006) reported a 1.5 log₁₀ 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 log₁₀ CFU/cm².

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 vitro* conditions, this concentration can impart a bactericidal effect (Murdock et al., 2007). Results showed that there were no significant ($P > 0.05$) difference in reductions of *E. coli*

between water washing and lactoferrin wash (data not shown); reductions for water and lactoferrin were 0.26-0.42 and 0.35 - 0.46 log₁₀ CFU/g, respectively, during storage. Our results are in agreement with the study of Del Olmo et al. (2012) who showed that lactoferrin (0.5 mg/ml) was not able to significantly reduce the counts of *E. coli* O157 inoculated onto chicken fillets. Bravo et al. (2014) also found that lactoferrin alone applied to beef carpaccio was not able to reduce the counts of *L. monocytogenes*, *Salmonella* Enteritidis or *E. coli* O157. Since a study by Murdock et al. (2007) demonstrated that lactoferrin (0.5 mg/ml) and nisin (250 IU/ ml) acted synergistically against *E. coli* O157 *in vitro*, lactoferrin was also evaluated in combination with a nisin wash (1000 IU/ml) in this study, to explore if any increase in effectiveness occurred for beef decontamination. In this case also there was no increase in the effectiveness, with the lactoferrin/nisin treatment achieving a similar reduction (0.30 - 0.49 log₁₀ CFU/g) to the water wash. The present study shows that the bactericidal effect of lactoferrin declines substantially when used for beef and that use of lactoferrin in conjunction with nisin does not offer any benefit under the applied conditions.

Plant essential oils, especially those from thyme, oregano, and clove, have strong antimicrobial effects. However, it has been shown that the effectiveness of essential oils is partially inhibited when applied to foods (Burt, 2004; Baranauskiene et al., 2006). The water insoluble nature of essential oils, as well as interaction with food components, can reduce their antimicrobial effects (Shah et al., 2012; Bhargava et al., 2015). Encapsulation of essential oils in nanoemulsions constitutes an alternative strategy to improve their antimicrobial efficiency in foods, by protecting them from interaction with food constituents and increasing their solubility, thus allowing a higher mass transfer of EO to the cell membranes of foodborne pathogens. The effect of two different EO nanoemulsions plus a water rinsing step on *E. coli* counts on beef are presented in Figure 4. In this case a buffer wash was performed instead of a water wash in order to investigate if the lower pH of the buffer (pH 3.5) used to prepare the EO nanoemulsions had any effect on *E. coli* survival. The reduction in *E. coli* counts achieved after the buffer wash were in the range of 0.30-0.51 log₁₀ CFU/g during storage, thus demonstrating no additional effect compared to the water wash employed for the other

interventions. After initial preparation, the nanoemulsions were diluted 5-fold to avoid droplet size increase (Landry et al., 2014). After this 5-fold dilution, the nanoemulsions had a concentration of 8000 ppm of either carvacrol or thyme. The carvacrol and thyme EO nanoemulsions prepared by spontaneous emulsification had an average particle size of 100 and 60 nm, respectively, with a polydispersity index of 0.20-0.28, measured by light scattering. The carvacrol nanoemulsion resulted in a significant reduction ($P < 0.05$) of 1.13 log₁₀ CFU/g compared to control immediately after treatment. A similar significant reduction was achieved by the thyme EO wash (1.09 log₁₀ CFU/g) immediately after the treatment. Both nanoemulsions also showed similar overall reductions by the end of storage (1.41 and 1.36 log₁₀ CFU/g for carvacrol and thyme EO, respectively). Statistical analysis showed that the counts of *E. coli* between the two nanoemulsion treatments did not differ significantly at any point during storage, showing that both of them appear to be equally effective. *E. coli* counts during storage showed similar decreases ($P > 0.05$) at each of the time-points during storage for the two EO nanoemulsion treatments.

The antimicrobial effect of the nanoemulsion is attributed to the disruption of the cell membrane leading to the release of cell components, such as carbohydrates, proteins, DNA, and potassium ions (Burt, 2004; Moghimi et al., 2016). To the best of our knowledge this is the first study where nanoemulsions were applied to beef to combat *E. coli* contamination. Using oregano oil nanoemulsion (average particle size 148 nm diameter), Bhargava et al. (2015) observed reductions in *E. coli* O157 counts, inoculated on lettuce leaves, of 3.05 and 3.35 log₁₀ CFU/g by 500 ppm and 1000 ppm, respectively, 24 h after application. According to Landry et al. (2015), carvacrol nanoemulsion (4000 ppm and 8000 ppm) wash of radish and broccoli seeds reduced *E. coli* O157 counts by 2-3 log₁₀ CFU/g after a 60 min treatment. Nanoemulsified thyme oil has also been demonstrated to result in significant reductions of *E. coli* (after 4 and 8 h) in 2% reduced fat milk and has also been found to be more effective than free thyme oil (Xue et al., 2015). Moghimi et al. (2016) also found that thyme essential oil nanoemulsions (diameter ~ 150 nm) have high antibacterial activity against *E. coli in vitro*. Furthermore, the susceptibility of bacteria to essential oils has been shown to

be dependent on the pH of the treatment (Shah et al., 2012). The nanoemulsions used in this study had a low pH (3.5), due to the sodium citrate buffer used to prepare them. The antimicrobial effect they imparted on *E. coli* could be due to combined low pH stress and the antimicrobial effects of carvacrol or thyme EO.

Nanoemulsion washes were also applied without the rinsing step. Carvacrol and thyme EO nanoemulsion showed significant reductions ($P < 0.05$) of 0.92 and 0.86 log₁₀ CFU/g after the 5 min treatment and 1.79 and 1.64 log₁₀ CFU/g, respectively, at the end of storage compared to the control. In this case also, no significant differences ($P > 0.05$) were observed in the effectiveness of the two nanoemulsions. The absence of a rinsing step led to a further reduction in the pathogen counts at day 1, after which counts did not differ significantly between storage days (day 1 v day 7). It also led to moderately greater reductions compared to when the rinsing step was included.

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

Acknowledgements

This study was funded by Rangeland Foods, Lough Egish, Castleblayney, County Monaghan, Republic of Ireland. We would like to thank Dr Eugene Carmichael for technical assistance with the dynamic light scattering measurements.

References

- Abdollahzadeh, E., Rezaei, M., Hosseini, H., 2014. Antibacterial activity of plant essential oils and extracts: The role of thyme essential oil, nisin, and their combination to control *Listeria monocytogenes* inoculated in minced fish meat. *Food Cont.* 35, 177-183.
- Alkawareek, M.Y., Algwari, Q.T., Lavery, G., Gorman, S.P., Graham, W.G., O'Connell, D. and Gilmore, B.F., 2012. Eradication of *Pseudomonas aeruginosa* biofilms by atmospheric pressure non-thermal plasma. *PLoS One* 7, e44289.
- Arthur, T.M., Wheeler, T.L., Shackelford, S.D., Bosilevac, J.M., Nou, X., Koohmaraie, M., 2005. Effects of low-dose, low-penetration electron beam irradiation of chilled beef carcass surface cuts on *Escherichia coli* O157: H7 and meat quality. *J. Food Prot.* 68, 666-672.

557 Azlin-Hasim, S., Cruz-Romero, M. C., Morris, M. A., Cummins, E., Kerry, J.P. 2015. Effects
 558 of a combination of antimicrobial silver low density polyethylene nanocomposite films and
 559 modified atmosphere packaging on the shelf life of chicken breast fillets. *Food Pack. Shelf*
 560 *Life*, 26–35.

561 Baranauskiene, R., Venskutonis, P.R., Dewettinck, K., Verhe, R., 2006. Properties of oregano
 562 (*Origanum vulgare* L.), citronella (*Cymbopogon nardus* G.) and marjoram (*Majorana*
 563 *hortensis* L.) flavors encapsulated into milk protein-based matrices. *Food Res. Int.* 39, 413-
 564 425.

565 Bhargava, K., Conti, D. S., da Rocha, S. R., Zhang, Y. 2015. Application of an oregano oil
 566 nanoemulsion to the control of foodborne bacteria on fresh lettuce. *Food Microbiol.* 47, 69-
 567 73.

568 Boziaris, I.S., Nychas, G.J., 2006. Effect of nisin on growth boundaries of *Listeria*
 569 *monocytogenes* Scott A, at various temperatures, pH and water activities. *Food*
 570 *Microbiol.* 23, 779-784.

571 Bravo, D., de Alba, M., Medina, M., 2014. Combined treatments of high-pressure with the
 572 lactoperoxidase system or lactoferrin on the inactivation of *Listeria monocytogenes*,
 573 *Salmonella Enteritidis* and *Escherichia coli* O157:H7 in beef carpaccio. *Food Microbiol.* 41,
 574 27-32.

575 Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods—
 576 a review. *Int. J. Food Microbiol.* 94, 223-253.

577 Chagnot, C., Agus, A., Renier, S., Peyrin, F., Talon, R., Astruc, T., Desvaux, M., 2013. *In vitro*
 578 colonization of the muscle extracellular matrix components by *Escherichia coli* O157:H7:
 579 the influence of growth medium, temperature and pH on initial adhesion and induction of
 580 biofilm formation by collagens I and III. *PLoS One* 8(3): e59386.

581 Chang, Y., McLandsborough, L., McClements, D.J., 2013. Physicochemical properties and
 582 antimicrobial efficacy of carvacrol nanoemulsions formed by spontaneous emulsification.
 583 *J. Agric. Food Chem.* 61, 8906–8913.

584 Chibeu, A., Agius, L., Gao, A., Sabour, P.M., Kropinski, A.M., Balamurugan, S., 2013.
585 Efficacy of a bacteriophage LISTEX™ P100 combined with chemical antimicrobials in
586 reducing *Listeria monocytogenes* in cooked turkey and roast beef. *Int. J.*
587 *Food Microbiol.* 167, 208-214.

588 Chien, S.-Y., Sheen, S., Sommers, C.H., Sheen, L.-Y., 2016. Modeling the inactivation of
589 intestinal pathogenic *Escherichia coli* O157:H7 and uropathogenic *E. coli* in ground
590 chicken by high pressure processing and thymol. *Front Microbiol.* 7, 920.

591 Coll Cárdenas, F.C., Andrés, S., Giannuzzi, L., Zaritzky, N., 2011. Antimicrobial action and
592 effects on beef quality attributes of a gaseous ozone treatment at refrigeration
593 temperatures. *Food Cont.* 22, 1442-1447.

594 Costa, C., Conte, A., Buonocore, G.G., Del Nobile, M.A., 2011. Antimicrobial silver-
595 montmorillonite nanoparticles to prolong the shelf life of fresh fruit salad. *Int. J. Food*
596 *Microbiol.* 148, 164–7.

597 Damm, C., Munstedt, H., Rosch, A., 2008. The antimicrobial efficacy of polyamide 6/silver-
598 nano- and microcomposites. *Mat. Chem. Phys.* 108, 61–6.

599 Del Olmo, A., Calzada, J., Nuñez, M., 2012. Effect of lactoferrin and its derivatives, high
600 hydrostatic pressure, and their combinations, on *Escherichia coli* O157:H7 and
601 *Pseudomonas fluorescens* in chicken filets. *Innov. Food Sci. Emerg. Technol.* 13, 51-56.

602 Dirks, B.P., Dobrynin, D., Fridman, G., Mukhin, Y., Fridman, A., Quinlan, J.J., 2012. Treatment
603 of raw poultry with nonthermal dielectric barrier discharge plasma to reduce *Campylobacter*
604 *jejuni* and *Salmonella enterica*. *J. Food Prot.* 75, 22-28.

605 Dorsa, W.J., Cutter, C.N., Siragusa, G.R., 1997. Effects of acetic acid, lactic acid and trisodium
606 phosphate on the microflora of refrigerated beef carcass surface tissue inoculated with
607 *Escherichia coli* O157: H7, *Listeria innocua*, and *Clostridium sporogenes*. *J. Food Prot.* 60,
608 619-624.

609 European Commission, 2010. The Consumer Markets Scoreboard. Making Markets Work for
610 Consumers (4th edition). Luxembourg: Directorate-General for Health & Consumers.

http://ec.europa.eu/consumers/consumer_evidence/consumer_scoreboards/4_edition/docs/cms_4_en.pdf. Accessed 15 December 2017.

European Food Safety Authority (2011). Scientific opinion on the evaluation of the safety and efficacy of lactic acid for the removal of microbial surface contamination of beef carcasses, cuts and trimmings. *EFSA J.* 9 (7), 2317.

European Commission, 2013. Regulation (EC) No 101/2013 of the European Parliament and of the Council of 4 February 2013 Concerning the use of lactic acid to reduce microbiological surface contamination on bovine carcasses. *Off. J. Eur. Union* 9, 2011–2013.

Fang, T.J., Tsai, H.C., 2003. Growth patterns of *Escherichia coli* O157: H7 in ground beef treated with nisin, chelators, organic acids and their combinations immobilized in calcium alginate gels. *Food Microbiol.* 20, 243-253.

Food Standards Agency, 2014. *E. coli* O157 control of cross contamination guidance for food business operators and local authorities. Available at: <http://www.food.gov.uk/sites/default/files/ecoli-cross-contamination-guidance.pdf> Accessed 17 April 2017.

Greene A.K., Guzel-Seydim Z.B., Seydim A.C., 2012. Chemical and physical properties of ozone, in: O'Donnell C., Tiwari B. K., Cullen P. J., Rice R. G., (Eds.). *Ozone in Food Processing*. Blackwell Publishing, Ltd., Oxford, pp. 19–32.

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., Loessner, M.J., 2012. Biocontrol of *Salmonella typhimurium* in RTE foods with the virulent bacteriophage FO1-E2. *Int. J. Food Microbiol.* 154, 66–72

Han, L., Patil, S., Boehm, D., Milosavljević, V., Cullen, P. J., Bourke, P., 2016. Mechanisms of inactivation by high-voltage atmospheric cold plasma differ for *Escherichia coli* and *Staphylococcus aureus*. *Appl. Environ. Microbiol.* 82, 450-458.

Harris, D., Brashears, M.M., Garmyn, A.J., Brooks, J.C., Miller, M.F., 2012. Microbiological and organoleptic characteristics of beef trim and ground beef treated with acetic acid, lactic

638 acid, acidified sodium chlorite, or sterile water in a simulated commercial processing
639 environment to reduce *Escherichia coli* O157: H7 and *Salmonella*. *Meat Sci.* 90, 783-788.

640 Hsu, H., Sheen, S., Sites, J., Cassidy, J., Scullen, B., Sommers, C., 2015. Effect of high
641 pressure processing on the survival of Shiga Toxin-producing *Escherichia coli* (Big Six vs.
642 O157: H7) in ground beef. *Food Microbiol.* 48, 1-7.

643 Incoronato, A.L., Conte, A., Buonocore, G.G., Del Nobile, M.A. 2011. Agar hydrogel with
644 silver nanoparticles to prolong the shelf life of Fior di Latte cheese. *J. Dairy Sci.* 94, 1697–
645 704.

646 Jamalludeen, N., Jonson, R.P., Friendship, R., Kropinski, A.M., Lingohr, E.J., Gyles, C.L.
647 (2007). Isolation and characterization of nine bacteriophages that lyse O149
648 enterotoxigenic *Escherichia coli*. *Vet. Microbiol.* 124, 47–57.

649 Jassim, S.A.A., Denyer, S.P., Stewart, G.S.A.B., 1998. Antiviral or antifungal composition
650 comprising an extract of pomegranate rind or other plants and method of use. U.S Patent
651 5840308.

652 Khadre, M.A., Yousef, A.E., Kim, J.G., 2001. Microbiological aspects of ozone applications in
653 food: a review. *J. Food Sci.* 66, 1242-1252.

654 Kim, J.S., Lee, E.J., Choi, E.H., Kim, Y.J., 2014. Inactivation of *Staphylococcus aureus* on the
655 beef jerky by radio-frequency atmospheric pressure plasma discharge treatment. *Innov.*
656 *Food Sci. Emerg. Technol.* 22, 124-130.

657 Kim, H.-J. Yong, H.I., Park, S., Choe, W., Jo, C., 2013. Effects of dielectric barrier discharge
658 plasma on pathogen inactivation and the physicochemical and sensory characteristics of
659 pork loin. *Curr. Appl. Phys.* 13, 1420-1425.

660 Kudva, I.T., Jelacic, S., Tarr, P.I., Youderian, P., Hovde, C.J., 1999. Biocontrol of
661 *Escherichia coli* O157 with O157-specific bacteriophages. *Appl. Environ. Microbiol.* 65,
662 3767-3773.

663 Landry, K.S., Chang, Y., McClements, D.J., McLandsborough, L. 2014. Effectiveness of a
664 novel spontaneous carvacrol nanoemulsion against *Salmonella enterica* Enteritidis and

665 *Escherichia coli* O157: H7 on contaminated mung bean and alfalfa seeds. *Int. J. Food*
666 *Microbiol.* 187, 15-21.

667 Landry, K.S., Micheli, S., McClements, D.J., McLandsborough, L. 2015. Effectiveness of a
668 spontaneous carvacrol nanoemulsion against *Salmonella enterica* Enteritidis and
669 *Escherichia coli* O157:H7 on contaminated broccoli and radish seeds. *Food Microbiol.* 51,
670 10-17.

671 Liu, H., Niu, Y.D., Meng, R., Wang, J., Li, J., Johnson, R.P., McAllister, T.A., Stanford, K.,
672 2015. Control of *Escherichia coli* O157 on beef at 37, 22 and 4°C by T5-, T1-, T4-and O1-
673 like bacteriophages. *Food Microbiol.* 51, 69-73.

674 Ly-Chatain, M.H., 2014. The factors affecting effectiveness of treatment in phages
675 therapy. *Front. Microbiol.* 5, 51.

676 McCann, M.S., McGovern, A.C., McDowell, D.A., Blair, I.S., Sheridan, J.J., 2006. Surface
677 decontamination of beef inoculated with *Salmonella* Typhimurium DT104 or *Escherichia coli* O157:
678 H7 using dry air in a novel heat treatment apparatus. *J. appl. Microbiol.* 101, 1177-1187.

679 McMillin, K., Michel, M., 2000. Reduction of *E. coli* in ground beef with gaseous ozone.
680 Louisiana Agriculture 43, 35.

681 Min, S.C., Roh, S.H., Niemira, B.A., Sites, J.E., Boyd, G., Lacombe, A. 2016. Dielectric barrier
682 discharge atmospheric cold plasma inhibits *Escherichia coli* O157: H7, *Salmonella*, *Listeria*
683 *monocytogenes*, and Tulane virus in Romaine lettuce. *Int. J. Food Microbiol.* 237, 114-
684 120.

685 Misra, N.N., Jo, C., 2017. Applications of cold plasma technology for microbiological safety in
686 meat industry. *Trends Food Sci. Technol.* 64, 74-86.

687 Moghimi, R., Ghaderi, L., Rafati, H., Aliahmadi, A., McClements, D.J. 2016. Superior
688 antibacterial activity of nanoemulsion of *Thymus daenensis* essential oil against *E.*
689 *coli*. *Food Chem.* 194, 410-415.

690 Mohan, A., Pohlman, F.W., 2016. Role of organic acids and peroxyacetic acid as antimicrobial
691 intervention for controlling *Escherichia coli* O157:H7 on beef trimmings. *LWT - Food Sci.*
692 *Technol.* 65, 868-873.

693 Murdock, C.A., Cleveland, J., Matthews, K.R., Chikindas, M.L. 2007. The synergistic effect of
 694 nisin and lactoferrin on the inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:
 695 H7. *Lett. appl. Microbiol.* 44, 255-261.

696 Mustapha, A., Ariyapitipun, T., Clarke, A.D., 2002. Survival of *Escherichia coli* O157:H7 on
 697 vacuum-packaged raw beef treated with polylactic acid, lactic acid and Nisin. *J. Food*
 698 *Sci.* 67, 262-267.

699 Patterson M.F., 2005. Microbiology of pressure treated foods. *J. Appl. Microbiol.* 98, 1400–
 700 1409.

701 Rajkowski, K.T., Marmer, B.S., 1995. Growth of *Escherichia coli*
 702 O157:H7 at fluctuating incubation temperatures. *J. Food Prot.* 58, 1307–
 703 1313.

704 Poimenidou, S.V., Bikouli, V.C., Gardeli, C., Mitsi, C., Tarantilis, P.A., Nychas, G.J.
 705 Skandamis, P.N., 2016. Effect of single or combined chemical and natural antimicrobial
 706 interventions on *Escherichia coli* O157: H7, total microbiota and color of packaged spinach
 707 and lettuce. *Int. J. Food Microbiol.* 220, 6-18.

708 Selma, M.V., Ibáñez, A.M., Allende, A., Cantwell, M., Suslow, T., 2008. Effect of gaseous
 709 ozone and hot water on microbial and sensory quality of cantaloupe and potential
 710 transference of *Escherichia coli* O157:H7 during cutting. *Food Microbiol.* 25, 162-168.

711 Shah, B., Davidson, P. M., & Zhong, Q., 2012. Nanocapsular dispersion of thymol for
 712 enhanced dispersibility and increased antimicrobial effectiveness against *Escherichia coli*
 713 O157:H7 and *Listeria monocytogenes* in model food systems. *Appl. Environ. Microbiol.* 78,
 714 8448-8453.

715 Sofos J.N., 2005. Improving the Safety of Fresh Meat. Woodhead Publishing in Food Science
 716 and Technology, CRC Press, New York.

717 Solomakos, N., Govaris, A., Koidis, P., Botsoglou, N., 2008. The antimicrobial effect of thyme
 718 essential oil, nisin and their combination against *Escherichia coli* O157:H7 in minced beef
 719 during refrigerated storage. *Meat Sci.* 80, 159-166.

720 Steijns, J.M., Van Hooijdonk, A.C.M., 2000. Occurrence, structure, biochemical properties
721 and technological characteristics of lactoferrin. *Brit. J. Nutr.* 84, 511-517.

722 Stratakis, A.C., Delgado-Pando, G., Linton, M., Patterson, M.F. and Koidis, A., 2015.
723 Synergism between high-pressure processing and active packaging against *Listeria*
724 *monocytogenes* in ready-to-eat chicken breast. *Innov. Food Sci. Emerg. Technol.* 27, 41-
725 47.

726 Ulbin-Figlewicz N., Jarmoluk, A., Marycz, K., 2015. Antimicrobial activity of low-pressure
727 plasma treatment against selected foodborne bacteria and meat microbiota. *Ann.*
728 *Microbiol.* 65, 1537-1546.

729 USDA Food Safety and Inspection Service (USDA-FSIS), 1996. Pathogen Reduction; Hazard
730 Analysis and Critical Control Point (HACCP) Systems. 61 Federal Register.
731 <https://www.gpo.gov/fdsys/pkg/FR-1996-07-25/pdf/96-17837.pdf>. Accessed 15 December
732 2017

733 USDA Food Safety and Inspection Service, 2015. Use of non-pathogenic *Escherichia coli* (*E.*
734 *coli*) cultures as surrogate indicator organisms in validation studies.
735 [https://askfsis.custhelp.com/app/answers/detail/a_id/1392/~use-of-non-pathogenic-](https://askfsis.custhelp.com/app/answers/detail/a_id/1392/~use-of-non-pathogenic-escherichia-coli-%28e.-coli%29-cultures-as-surrogate)
736 [escherichia-coli-%28e.-coli%29-cultures-as-surrogate](https://askfsis.custhelp.com/app/answers/detail/a_id/1392/~use-of-non-pathogenic-escherichia-coli-%28e.-coli%29-cultures-as-surrogate). Accessed 15 December 2017.

737 Viazis, S., Akhtar, M., Feirtag, J., Diez-Gonzalez, F., 2011. Reduction of *Escherichia coli*
738 O157: H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and
739 trans-cinnamaldehyde. *Food Microbiol.* 28, 149-157.

740 Wani, S., Maker, J.K., Thompson, J.R., Barnes, J., Singleton, I., 2015. Effect of ozone
741 treatment on inactivation of *Escherichia coli* and *Listeria* sp. on spinach. *Agriculture* 5, 155-
742 169.

743 Wheeler, T.L., Kalchayanand, N., Bosilevac, J.M., 2014. Pre-and post-harvest interventions
744 to reduce pathogen contamination in the US beef industry. *Meat Sci.* 98, 372-382.

- World Health Organisation, 2015. WHO's first ever global estimates of foodborne diseases find children under 5 account for almost one third of deaths. <http://www.who.int/mediacentre/news/releases/2015/foodborne-disease-estimates/en/>. Accessed 15 December 2017.
- Xue, J., Davidson, P.M., Zhong, Q., 2015. Antimicrobial activity of thyme oil co-nanoemulsified with sodium caseinate and lecithin. *Int. J. Food Microbiol.* 210, 1-8.
- Ye, X.Y., Wang, H.X., Liu, F., Ng, T.B., 2000. Ribonuclease, cell-free translation-inhibitory and superoxide radical scavenging activities of the iron-binding protein lactoferrin from bovine milk. *Int. J. Biochem. C. Biol.* 32, 235–241.
- Youssef, M.K., Yang, X., Badoni, M., Gill, C.O., 2012. Effects of spray volume, type of surface tissue and inoculum level on the survival of *Escherichia coli* on beef sprayed with 5% lactic acid. *Food Cont.* 25, 717-722.
- Ziuzina, D., Patil, S., Cullen, P.J., Keener, K.M., Bourke, P. 2012. Atmospheric cold plasma inactivation of *Escherichia coli* in liquid media inside a sealed package. *J. Appl. Microbiol.* 114, 778–787.

Table 1Summary 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.

Figure legends

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

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

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

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

Fig. 1.

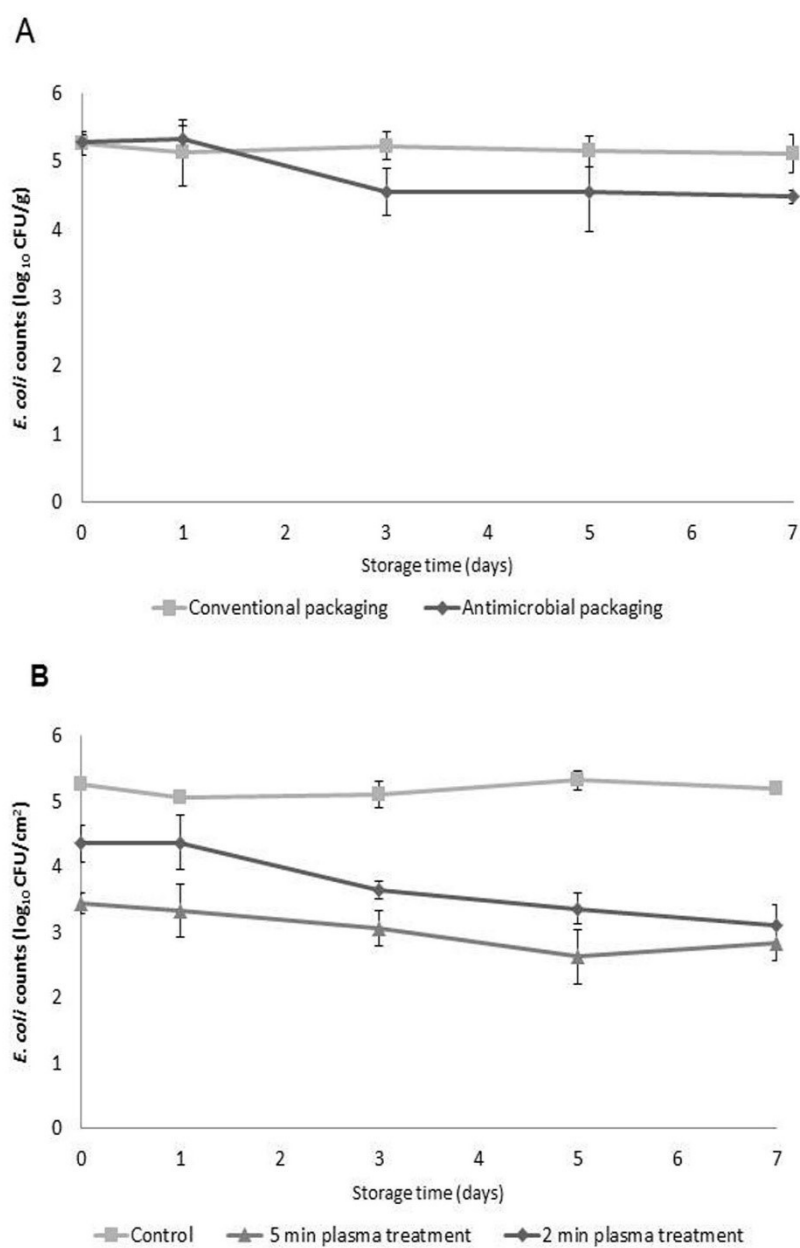


Fig. 2.

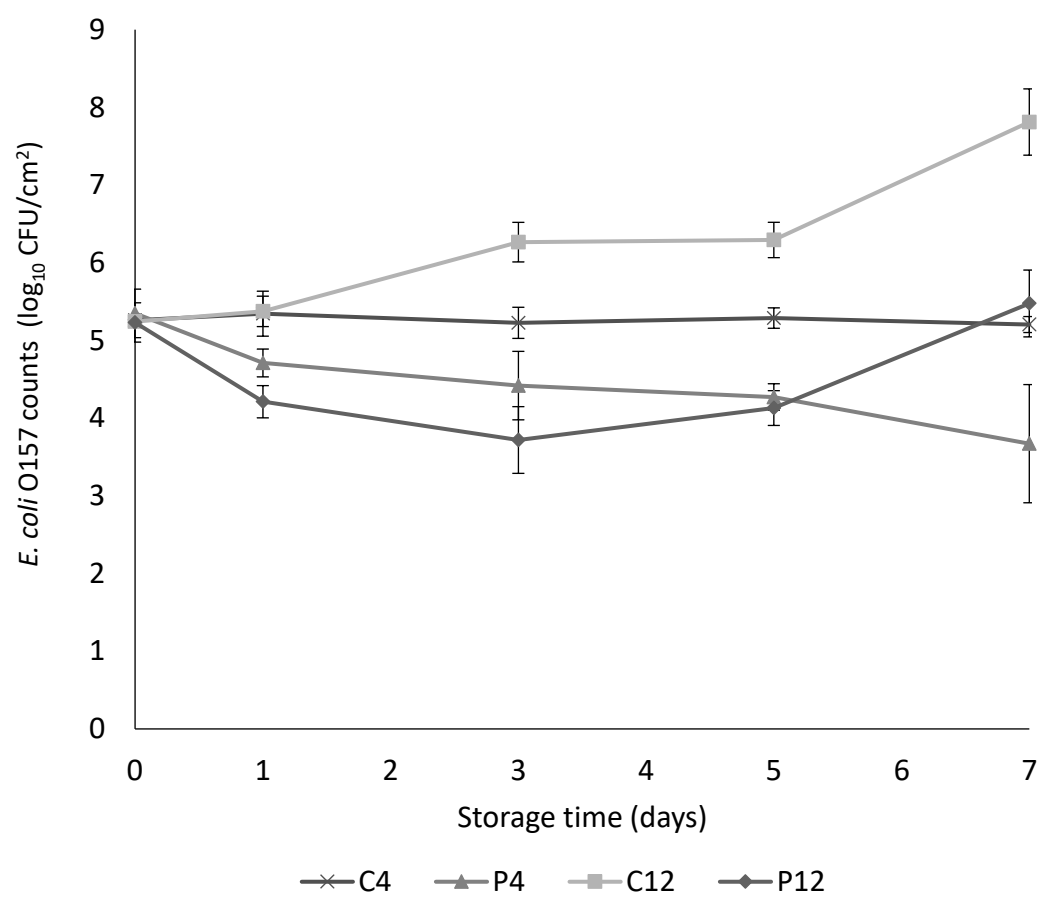
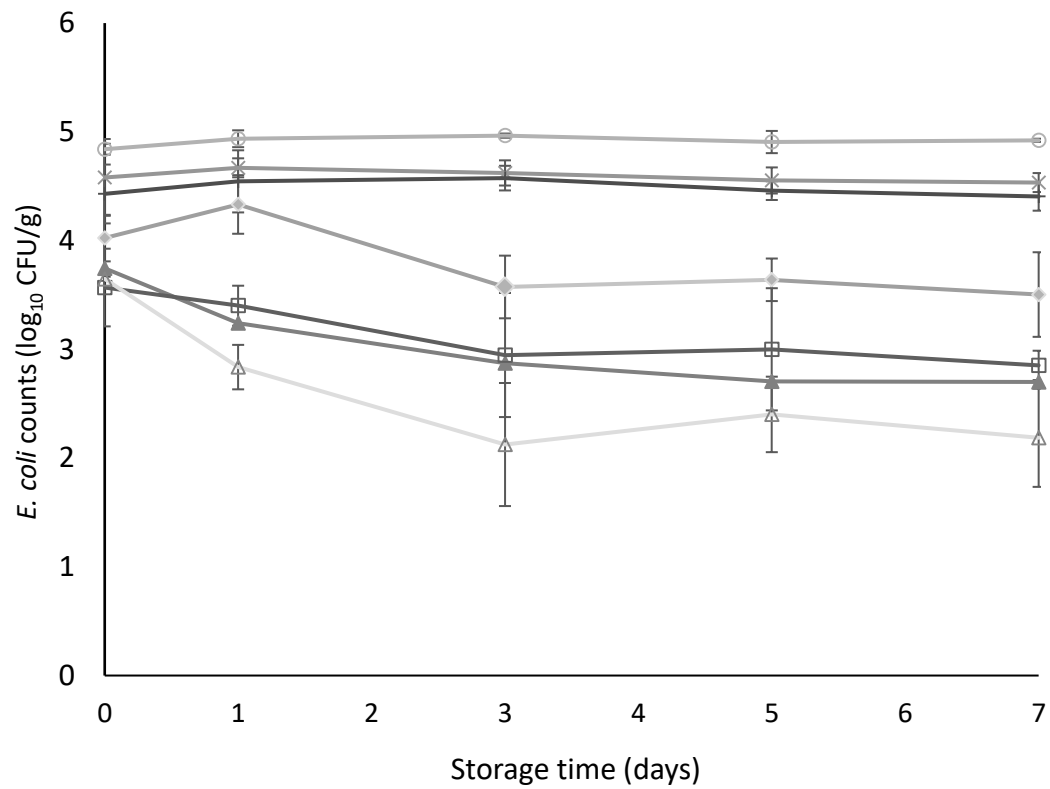


Fig. 3.



○ Control

▲ Lactic acid (5%)

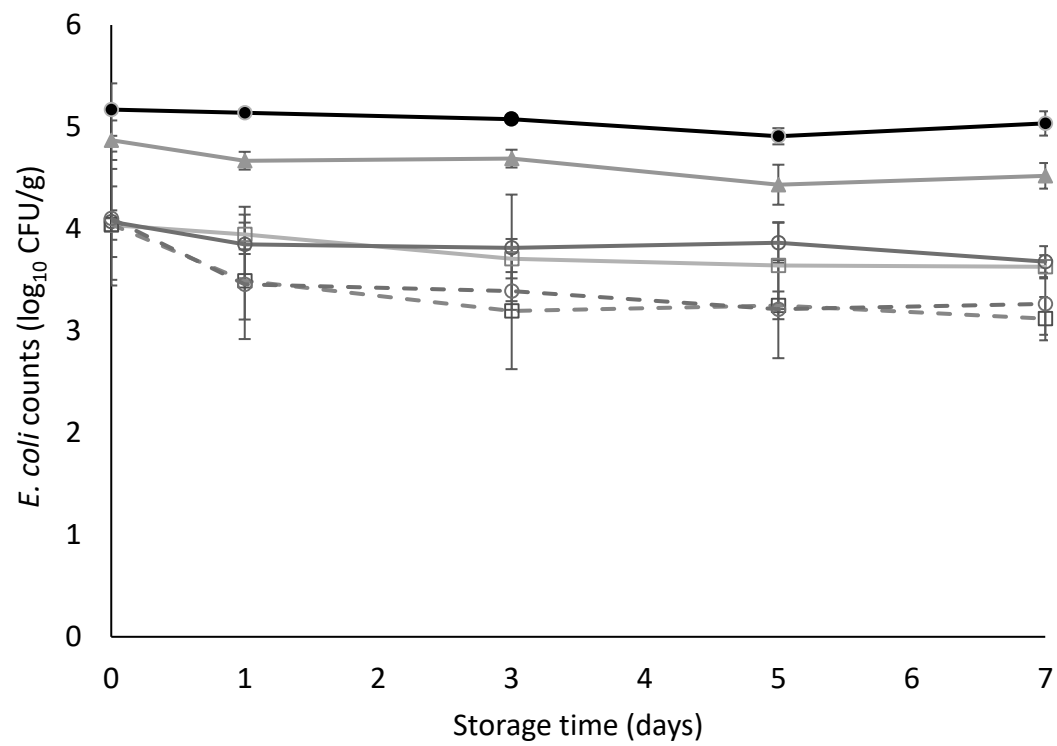
— Nisin

◆ Vinegar

× Water wash

□ Lactic acid/Nisin

△ Lactic acid (5%) - no rinsing



● Control
 ▲ Buffer wash
 □ Carvacrol nanoemulsion
 ○ Thyme EO nanoemulsion
 □ Carvacrol nanoemulsion w/o rinsing
 ○ Thyme EO nanoemulsion w/o rinsing