# Survival of Shiga toxin-producing *Escherichia coli* and Stx bacteriophages in moisture enhanced beef

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# Abstract

Moisture enhancement of meat through injection is a technology to improve the sensory properties and the weight of meat. However, the technology may increase the risk of food borne infections. Shiga toxin-producing *Escherichia coli* (STEC) or bacteriophages carrying cytotoxin genes (Shiga toxin genes, *stx*), which is normally only present on the surface of intact beef, may be transferred to the inner parts of the muscle during the injection process. Pathogens and bacteriophages surviving the storage period may not be eliminated in the cooking process since many consumers prefer undercooked beef. Measures to increase the microbial food safety of moisture enhanced beef may include sterilization or washing of the outer surface of the meat before injection, avoiding recycling of marinade and addition of antimicrobial agents to the marinade. This paper reviews the literature regarding microbial safety of moisture enhanced beef with special emphasis on STEC and Stx bacteriophages. Also, results from a European Union research project, ProSafeBeef (Food-CT-16 2006-36241) is presented.

# Keywords:

Moisture enhanced; marination; beef; Shiga toxin: STEC; bacteriophage

# 1. Introduction

Injection of meat with a marinade or solution is commonly used in the food industry to enhance taste, juiciness, and tenderness of meat products and to increase the weight of the meat (Grobbel, 2009, Wicklund, et al., 2005). Common components of marinades are salt, sugar, phosphate, acids and spices. The marinades may contain lactate, acetate and other antimicrobial components. Meat injected with solutions is often referred to as enhanced or marinated meat.

The microbial safety of raw beef often relies on a proper heat treatment at the end user stage. A whole piece of beef is usually sterile inside with bacteria only present on the surface. Therefore, proper heat treatment of the meat surface by cooking, frying or grilling is sufficient to kill pathogenic bacteria and the beef is microbiologically safe to eat even if it is not cooked thoroughly. Beef is therefore often eaten rare done and regarded as safe. However, the injection process may transfer bacteria including pathogens from the surface to the interior. Many consumers may not be aware of this and prepare non-intact meat as intact meat. Indeed, an outbreak of *Escherichia coli* O157:H7 linked to injected beef was explained by preferences for rare done beef and undercooking (Laine, et al., 2005).

Moisture enhancement of poultry and pork is common in Europe, but in contrary to the US, beef is rarely subjected to this process and therefore the technology, safety and consumer attitudes were important research topics in the European Union project ProSafeBeef (Food-CT-16 2006-36241). This paper will discuss the safety aspects of moisture enhanced beef with emphasis on risk of Shiga toxin-producing *E. coli* (STEC) and *E. coli* Stx bacteriophages and present some results from the European project. The safety of non-intact products in general has been extensively studied and reviewed earlier, for example by the group of John E. Sofos at the Colorado State University (Sofos, 2009; Sofos & Geornaras, 2010).

# 2. Risks associated with enhanced beef

The primary food safety concern involving production of enhanced beef is the introduction or translocation of pathogens into the internal tissues of the meat through injection of marinades or brines. The translocated bacteria can originate from a contaminated meat surface, the solution injected, or from the injection processing equipment. Several human pathogens including *Salmonella, Campylobacter*, *Listeria monocytogenes* and *E. coli* may be present on the surface of the raw materials or the production environment and contaminate the interior of enhanced beef products. Among these pathogens, *E. coli* O157:H7 has specifically been associated with beef production. This pathogen is highly relevant for microbial risk assessment of enhanced beef as it has a low infectious dose and can cause severe illness. A risk assessment of non-intact beef conducted by The US Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) in 2002, concluded that the risk of *E. coli* O157:H7 illness from such products was very low as the pathogen does not survive cooking temperatures (USDA-FSIS, 2002). Since then, non-intact beef, including moisture enhanced beef, have been implicated in several outbreaks and products recalls (Laine, et al., 2005; USDA-FSIS, 2007, 2009). This has clarified the public health risks of *E. coli* O157:H7 not only in ground beef but also in enhanced beef products.

*E. coli* O157:H7 is not the only serotype of *E. coli* that can cause severe infection. In recent years the emergence of non-O157 STEC has gained significant worry and a testing program for the so-called Big six non-O157 STEC serogroups (O26, O103, O45, O111, O121 and O145), has been suggested for implementation in the US meat industry producing non-intact raw beef. A recent study indicates that antimicrobial interventions are as effective against non-O157 STEC as *E. coli* O157:H7. However, no previous studies have included non-O157 serogroups in survival studies on enhanced beef. Other bacteria including pathogens like *Salmonella*, *Campylobacte*r and *L. monocytogenes* may also be translocated through the injection process. However, understanding risks associated with STEC and enhanced beef will also support increased control of these additional potential risk factors.

The risk analyses on intact and enhanced beef have mainly focused on the presence of pathogenic bacteria and not on virulence attributes. Shiga toxin bacteriophages (Stx phages) carry specific *stx* genes and constitute a major virulence attribute in STEC. Studies have shown that phages can survive better than their bacterial hosts (Imamovic & Muniesa, 2011; Schmidt, 2001). Furthermore, Stx phages can infect commensal *E. coli* and convert non-pathogenic strains into Shiga toxin-producing strains (Gamage, Strasser, Chalk, & Weiss, 2003; Herold, Karch, & Schmidt, 2004; Martinez-Castillo et al. 2013). Surviving Stx phages can thus represent a threat to food safety, even in the absence of viable STEC. Another food safety aspect is that factors relevant for the marination process may trigger induction of lytic cycle for bacteriophages (Los, Los, Wegrzyn, & Wegrzyn, 2009). There are studies showing that concentrations of salt used in meat processing give prophage induction, and enhance Stx production (Harris, et al., 2012; Shkilnyj & Koudelka, 2007). Several STEC outbreaks have been linked to beef, and introduction of non-intact beef products into new markets may impact food safety for the consumers. Possible contamination and survival of STEC and Stx phages in these products should therefore not be neglected.

# 3. Microbial contamination during the enhancement process

During the enhancement process, marinade is needle injected into the interior of the muscle matrix and used marinade is often recycled through the whole production day. However, this process does not only transfer marinade into the meat, but also microorganisms present on the meat surface, from contaminated marinade or from the injection equipment.

## 3.1 Beef as a vector for contamination

STEC is in general not frequently present on raw meat cuts and the level of microorganisms on positive samples is expected to be low. Data provided to the European surveillance program on zoonotic agents showed that the mean incidence of STEC and *E. coli* O157 on fresh bovine meat was 2.3 and 0.7%, respectively (9 285 samples)(European Centre for Disease Prevention and Control and European Food Safety Authority, 2011). The concentration of STEC on the meat surfaces was not reported. In an investigation on retail beef products in Egypt, *E. coli* O157:H7 was found in one out of 37 samples (3.7%) of fresh beef and 8 out of 30 ground beef samples (26.7%)(Sallam, Mohammed, Ahdy & Tamura, 2013). A recent study showed that only one out of 150 samples from vacuum packaged beef in Canada were positive for non-O157:H7 (Jones et al., 2014). No *E. coli* O157:H7 was detected in a US study from 2006, sampling 1 199 subprimal beef cuts before enhancement process, and the incidence was therefore reported to be less than 0.083% (Kennedy, Williams, Brown, & Minerich, 2006). In another US study from 2006, occurrence of *E. coli* O157:H7 on beef subprimals collected from six packaging facilities and surface concentrations on positive samples was 0.2% and below 0.375 cfu/cm2, respectively (Heller, et al., 2007). In the same study, transfer of STEC from outer surface to the inner surface of beef during moisture enhancement was investigated using surface inoculated beef cuts. For meat injected with distilled water, 1.94% of the STEC on the surface could be recovered from the internal meat. In another study beef was contaminated with *E. coli* O157:H7 before needle injection with distilled water (Echeverry et al., 2009). After vacuum pack storage for 14-21 days under refrigerated conditions, the inner muscle contained 3-4 log cfu/g with an initial surface contamination of about 7 log cfu/cm2. The surface contamination level in these studies was much higher than what is expected in real beef cuts, but demonstrates that the needle injection process itself may contribute to transfer of microorganisms from the outer surface into the core of beef cuts.

Reservoirs of Stx phages can be of both human and animal origin. A recent study reported infective Stx phages to be common in feces from healthy persons (Martinez-Castillo et al. 2013). There are few studies on the prevalence of Stx phages in cattle and beef. In an investigation on the prevalence of Stx phages in beef purchased from retailers in Spain (36 samples, freshly minced on request at local supermarkets) all samples were positive using qPCR for detection (Imamovic & Muniesa, 2011). The number of Stx phages was high for some of the samples (~106/25g) but the number of infectious phages was difficult to estimate. The same group did an estimate in another study (Imamovic, Serra-Moreno, Jofre & Muniesa, 2010) where the amount of infectious phages detected by their ability to infect host strains was 1-3 log below the numbers detected by qPCR and electron microscopy. Oot et al (2007) also found that phage numbers were difficult to estimate and concluded that there might be a large underestimation of infectious O157:H7 phages in livestock of cattle. In conclusion, it is not unlikely that Stx phages are present on beef used for injected products.

## 3.2 Marinade as a vector for microbial cross-contamination

During the enhancement process there is a significant risk of cross-contamination since the marinade is often recycled and the needles are not cleaned between injections. Microorganisms and bacteriophages from contaminated beef cuts may be transferred to the needles and/or released into the marinade and injected into subsequently injected meat cuts. A recent study showed no reduction in numbers of *E. coli* O157:H7 in distilled water, or marinades based on sodium chloride (NaCl) or combinations of Sodium chloride and phosphate during storage at 4 and 15oC (Adler, et al., 2011). If meat homogenate was added to simulate used marinade as in a recirculation process, growth was recorded. Increase in total bacterial numbers in the marinade and injected beef steaks for each recycling round in a pilot scale enhancement process was reported by Paulson et al. (Paulson, Wicklund, Rojas, & Brewer, 2010). From an investigation of commercial production of moisture-enhanced pork, it was concluded that recirculated marinades can harbor large populations of spoilage bacteria and *L. monocytogenes* (Greer, Nattress, Dilts, & Baker, 2004). The bacterial numbers increased with 1-2 log cfu/ml during a production period of 2.5 h to a final level of 4.5 log cfu/ml. The injection marinade used was a commercial marinade containing Sodium chloride, phosphates and lemon juice. As far as we know, similar studies have not been done for production of enhanced beef.

Bacteriophage activity affected by the marinating process and transfer of Stx phages to the interior of beef may also represent a risk, but has to our knowledge not previously been studied. Results obtained in the ProSafeBeef project indicated that the infectious stability of phage particles was gradually reduced in marinade, but the process is slow and it cannot be excluded that they can infect *E. coli* present in the marinade or meat. The Stx phage in the study originated from a human case isolate of *E. coli* O103:H25 (NIPH-11060424) from a Norwegian outbreak in 2006 (Schimmer, et al., 2008; Sekse, et al., 2009). Prior to the study, the phage was modified by substituting the *stx* gene with the antibiotic resistance gene chloramphenicol acetyl transferase (*cat)*, (∆ *stx*::*cat).* The phage was transformed to *E. coli* C600 (Serra-Moreno et al., 2006). This lysogen was used for production of phage particles. Infectious phage particles were enumerated by plaque assay as described previously (Rode, et al., 2011). Phage particles were inoculated into marinade (Control: 6% Sodium chloride and 3% glucose, Acified: lactate/acetate (pH 5.7, 4%, Opti-Form SD4, Purasal, PURAC America, Lincolnshire, IL) plus ascorbate (0.5 g/L) and dextrose (3%)) at a start level of 106/ml and stored at 20oC. After one day of storage, 1 log reduction was observed in the control marinade, while a 3 log reduction was seen in the marinade with organic acids. At the second day, no phages were detected, using the plaque assay, in the acidified marinade. For the control marinade an additional one log reduction was observed from day 1 to 2, but no phages were detected after seven days. An equivalent experiment was done adding phages and *E. coli* together to the marinade, both in the control and acidified marinade. The survival of phages was not affected by the acidity in the marinade, and phages were detected after 7 days, but not after 14 days. The same phage filtrate showed a very low reduction when stored in LB-medium under the same conditions, < 1 log. We have earlier shown that the stability of phages dropped 3 log the first day of storage at 4°C compared to approximately no reduction when stored at 24°C (Rode, et al., 2011). This was in LB broth, but indicates that storage of marinade at chilled temperature can reduce phage stability. In the same study we also showed that pH (5–9) has little effect on the stability of Stx2 phages. No infectious phages were present after 10 min at pH 3, and a 3–4 log reduction was recorded after 24 h at pH 4 and 10. In conclusion, using a marinade with low pH and storage at cool temperatures will probably reduce bacteriophages in a marinade used in an enhancement process.

**3.3 Role of process equipment in microbial contamination**

The brine injection equipment may represent a contamination source if not sufficiently cleaned and disinfected. Indeed, brine injection equipment has been identified as a source of contamination of enhanced pork (Berzins, Hellstrom, Silins & Korkeala H. (2010). Contamination of beef due to inadequate sanitation in industrial production has not been reported and is a topic for further investigations.

# 4. Microbial activity and Stx phage stability in moisture enhanced beef

## 4.1 Survival of STEC and stability of Stx phages

There are only few studies on survival of *E. coli* during storage of enhanced beef. The studies show that *E. coli* in the interior of enhanced beef stored at cooling temperatures can survive throughout the storage period. Organic acids in the enhancement solutions may reduce, but do not eliminate *E. coli* (see chapter 6.1 for details on effects of antimicrobial ingredients in marinades). High survival of *E. coli* K12 (106 cfu/ml) injected in beef strip steaks with enhancement solutions with salt (0.3%) and phosphate (0.3%) was reported in two studies by the same research group (Paulson, Wicklund, Rojas, & Brewer, 2007; Wicklund, Paulson, Rojas, & Brewer, 2006). After 7 and 14 days of vacuum packed storage at 4oC, no reductions and <1 log reduction in coliform counts, respectively were found. Lower counts were reported after 7 days (about 1 log reduction), but no further reduction was obtained after 14 days when organic acids (0.3% lactate or 0.3% lactate plus 0.25% acetate) were added to the enhancement solution. The same research group reported growth of *E. coli* K12 in vacuum packaged enhanced beef (110%, 0.3% salt plus 0.3% phosphate) stored at 4oC when a lower inoculation level of bacteria (104 cfu/ml) was used (Wicklund, Paulson, Rojas, & Brewer, 2007). By adding 3% lactate in the enhancement solution growth was inhibited for 14 days of storage, but regrowth was recorded after 21-28 days. Use of 0.25% diacetate led to numbers of coliform bacteria below the detection limit. Since *E. coli* K12 is not expected to multiply at 4oC and coliform medium was used for detection of the strain added, one may speculate that the growth observed was due to other coliforms present on the beef and not the inoculated *E. coli* strain. Ponrajan et al. (2011) studied the effect of adding diacetate and citrate or buffered vinegar to the enhancement solution. Beef was surface inoculated with *E. coli* O157:H7 and injected with marinade (110%, 0.5% sodium chloride plus 0.4% sodium triphosphate) containing a sodium diacetate (0.8%) and sodium citrate (0.2%) or vinegar (2%). The enhanced beef was stored vacuum packed for 10 days at 4oC before microbial analysis. Addition of organic acids or vinegar resulted in a similar and significant reduction (approx. 90 %) of the STEC strain.

Stx phages have been reported to maintain their infectivity in foods and under food-processing conditions but the stability and infectivity of Stx phages in stored fresh meat or moisture enhanced meat has not been reported earlier. Rode et al. (2011) showed that phage stability and infectivity under various food related conditions were dependent on a number of factors. The stability of the phages at different temperature, food matrix and storage time were maintained under various relevant conditions, and they were able to infect *E. coli*. In the ProSafeBeef project, survival of a cocktail of STEC (O157:H7, O103:H25, O26, O145 and O113 added in equal amounts, see Table 1) and Stx phage in marinated beef (*Longissimus dorsi*) under retail storage conditions was investigated. Beef loins were injected (110%) with marinade (6% sodium chloride plus 3% glucose) without and with organic acids (lactate/acetate (4%, Opti-Form SD4, Purasal, PURAC America, Lincolnshire, IL), ascorbate (0.5 g/L) and dextrose (3%) instead of glucose). The pH of the acidified marinade was 5.7. Marinades were not inoculated or inoculated to a final concentration of 5\*107 cfu/ml with cold-adapted STEC (stored at 4oC for 24 h) or 106 /ml Stx phages before injection. The steaks were vacuum packaged and stored at 4oC for 2 days. The loins were cut in three steaks: One steak was sampled immediately, and the remaining steaks vacuum packaged and stored at 4oC for further 7 days. After 7 days, one steak from each treatment combination and loin were taken out for microbial analysis (Tryptone Soy Agar added Rifampicin (100 µg/ml, Oxoid, Basingstoke, UK). The remaining steaks were wrap-packed to simulate wrap-packaging in the retail. These steaks were further stored at 4oC for 7 days before analysis resulting in a total of 14 days storage after injection. As shown in Figure 1, no reduction in STEC strains was found during storage, even using enhancement solution with organic acids.

An overview of the surviving phages in meat, meat juice and marinade during 14 days of storage is shown in Figure 2. The Stx phages in the stored meat dropped approx. 3 log after 2 days, and almost 5 log reduction of phages was evident after 7 days. There were small differences between the stability of phages in the enhanced beef using reference or acidified marinade. After 14 days, only one out of three parallel samples of beef enhanced with acidic marinade contained some infective phages. The stability of phages was much higher in the meat juice compared to the enhanced beef. Most of the sampled meat juice was probably marinade that had seeped out from the meat during storage. The number of infective phages after 7 days was at the same level as detected in the enhanced beef after 2 days. After 14 days phages were detected in all samples of meat juice in the reference marinade.

**4.2 Growth and survival of spoilage bacteria**

Enhanced meat has been proven to be more susceptible to microbial spoilage than intact meat, especially by lactic acid bacteria (Vihavainen & Bjorkroth, 2007). Paulson and co-workers showed that the level of aerobic counts in the injection solution and the enhanced beef increased for each round of recycling of marinade (Paulson, Wicklund, Rojas & Brewer, 2010). Ponrajan et al. (2011) demonstrated that the psychrotrophic flora in injected beef increased from about 4 log cfu/cm2 to almost 10 log cfu/cm2 during a 21 day period of aerobic storage. The growth could be delayed by adding sodium diacetate (0.8%) and sodium citrate (0.2%) or 2% vinegar to the enhancement solution. In the study on injected beef performed in the ProSafeBeef project (see 4.1 for experimental description), it was found that during storage, the microbiota in enhanced beef increased with about 5 log units during 14 days of storage at 4oC (Figure 3). The final microbiota was dominated by psychotropic bacteria, mainly lactic acid bacteria. Organic acids (diacetate/lactate, 4%, Opti-Form SD4) inhibited growth of mesophilic bacteria, but not the dominating microbiota.

In a Finnish study of enhanced beef rejected in the quality control of commercially produced beef products, the predominant species were *Leuconostoc gasicomitatum*, *L. gelidum*, *Lactobacillus sakei,* *Lb. algidus* and *Carnobacterium divergens* (Vihavainen & Bjorkroth, 2007). The product had been injected (112-115%) with an enhancement solution containing 4.5% corn dextrin, 2.5% sodium chloride, 1.6% sodium di- and tri-phosphates and 1.5% glucose and was stored in high-oxygen modified atmosphere. Assessment of the spoilage potential revealed that *L. gasicomitatum* and *L. gelidum* produced green surface discoloration and buttery off-odors similar to what was found in the spoiled commercial products. Identical genotypes of *L.* *gasicomitatum* have been isolated from a range of processed and marinated meat products. They have a similar contamination pattern in beef, pork and poultry production and have the ability to grow and compete in various meat products regardless of animal species and added antimicrobial components (Vihavainen & Bjorkroth, 2009). However, the spoilage at a given cell concentration is dependent on the conditions such as sugar availability, gas atmosphere and packaging material (Vihavainen & Bjorkroth, 2009). Although *L.* *gasicomitatum* was identified as an important and widespread specific spoilage bacterium in the meat chain in Finland, other studies have indicated that other species of lactic acid bacteria may be as important. For example, in a study of commercially processed enhanced pork in Norway, *L.* *gasicomitatum* was not found and *Lb. algidus* was pointed out as an important spoilage organism (Schirmer, Heir and Langsrud, 2009). Therefore, several species and strains are likely to be important spoilage organisms of enhanced meat.

## 5. Potential risks at the consumer stage

In many cases the microbial safety of raw beef is dependent on a sufficient heat treatment to kill pathogens at the consumer stage. A consumer study in US showed that about 10% of US consumers cook steaks to an internal temperature less than 51oC and 37% below 64oC (Kaplan, Ebel, & Schlosser, 2002). In a survey conducted in the US, 50% of individuals who had consumed steak in the past week reported eating it pink (CDC, 2003). Also, many European consumers prefer undercooked beef (Osornio, et al., 2008; Rossvoll, et al., 2012). Within the ProSafeBeef project European consumer acceptance to moisture enhancement technology was studied (de Barcellos, et al., 2010). Despite a claim in the questionnaire that injection may improve safety of beef consumers did not approve this technology arguing with the risk of contamination in addition to other arguments such as taste and texture. In practice consumers may cook moisture enhanced products as intact beef because they are unaware that the beef has been moisture enhanced by injection or they do not know the possible implications for safety (Sofos & Geornaras, 2010). Also, many consumers use color as an indicator of whether the meat is thoroughly cooked. The cooked meat color depends on a number of factors and is not a good indicator of adequate cooking (King & Whyte, 2006; Sørheim & Høy, 2013). This implies that one cannot expect that consumers cook moisture enhanced beef to an internal temperature resulting in sufficient kill of STEC.

Most studies on thermal destruction of *E. coli* in meat are done in ground beef models. In ground beef, the expected D-values at temperatures below 65oC are of minutes of magnitude. Below 55oC, the holding time needed to obtain 5 log reductions would be of hours of magnitude (Juneja, Snyder, & Marmer, 1997; Wiegand, Ingham, & Ingham, 2012). The actual and measured thermal destruction of *E. coli* is affected by a number of factors before, during and after heat treatment (O'Bryan et al., 2006). Stressed cells may become more or less resistant to heat depending on the stress applied. For example, cycles of freezing and tempering render *E. coli* more susceptible to heat stress (Byrne et al., 2002) while acid-adapted cells are more resistant (Shen et al, 2011). The composition and structure of the meat will influence the thermal lethality and data from one food matrix cannot necessarily be extrapolated to other foods (O'Bryan, et al., 2006). Indeed, *E. coli* O157:H7 appears to be more heat tolerant in non-isothermal cooking of whole-muscle products than in ground meat (Wiegand, et al., 2012). Therefore, cooking temperatures above 65oC and minutes of holding time were necessary to eliminate (5 log reduction) pathogenic *E. coli* inbeef injected with a marinade without antibacterial agents (Wiegand, et al., 2012). In another study more than 7 log reduction of a cocktail of *E. coli* O157:H7 in the interior of moisture enhanced beef heated to 64oC for 2 min was found (Gill, Moza, & Barbut, 2009).

Little is known about survival of Stx phages after heat treatment of beef. In an earlier study we found that in ground beef, 5 log reduction of Stx phages was obtained at 60 °C after a holding time of 10 min and 9 log reduction after 30 min (Rode, et al., 2011). Comparing with data on heat inactivation of *E. coli* in ground beef from the literature, these results indicate that heat tolerance of Stx phages and *E. coli* are comparable in meat systems although food matrix has significant effects on Stx phage stability (ACMSF, 2007; Rode et al. 2011).

# 6. New and present eradication strategies

Several potential intervention methods to enhance the microbial safety of non-intact beef products have been studied. These include strategies to improve the safety of the enhancement solutions (brines/marinades), those that can be applied for decontamination of the meat surface prior to injection, and strategies applied on injected product.

## 6.1 Marinade composition

Several studies have reported that *E. coli* survives well in marinades and beef enhanced with marinades containing the basic ingredients water, salt, and phosphates (Adler, et al., 2011; Paulson, Wicklund, Rojas & Brewer, 2007; Ponrajan, et al., 2011; Wicklund, Paulon, Rojas and Brewer, 2007). Natural antimicrobials have possible applications in enhancement solutions and their use will most likely increase in the future. Adler et al. (2011) tested different antimicrobials for their effects on reducing *E. coli* O157:H7 in a standard marinade containing sodium chloride (5.5%), sodium tripolyphosphate (2.75%) and sodium pyrophosphate (2.75%). The tested antimicrobials included cetylpyridinium chloride, sodium metasilicate, potassium lactate, sodium diacetate, lactic acid, acetic acid, citric acid, and combinations of potassium lactate and sodium diacetate, nisin and EDTA, pediocin and EDTA and hops beta acids. Cetylpyridinium chloride (5.5%) and sodium metasilicate (2.2%) showed immediate effects and reduced the pathogen to undetectable levels within 4 h (≥2.4 log reduction). Among the three organic acids tested (3.3%), lactic acid was the most effective, reducing pathogen counts to below the detection limit within 12 h at both temperatures. The antimicrobial activities were temperature and time dependent and the reduction increased during storage. Marinades containing sodium citrate together with sodium diacetate or buffered vinegar and injected into *E. coli* O157:H7 surface inoculated beef provided 0.6 to 1 log reductions in *E. coli* counts after 10 days vacuum storage at 4°C. Growth of psychotropic organisms was also restricted in a combination of sodium diacetate and sodium citrate as well as buffered vinegar samples. Paulson et al. (2007) showed that enhancement solutions containing combinations of sodium lactate (3%) and sodium diacetate (0.25%) were more effective in controlling bacterial growth of *E. coli* K12 in enhanced beef than SL alone. Although the effect was limited (approx. 1 log reduction in total plate counts after 7 days), it illustrates that antimicrobial combinations with synergistic effects can be obtained. The effect of marinade composition and storing conditions on the survival and virulence of pathogens at a later stage should also be evaluated. Stressed cells may become more or less susceptible to the final heat treatment at the consumer stage (Byrne et al., 2002, Shen et al., 2011) and there is a need for more research on this area.

Eradication of bacteriophages in marinade has not been reported earlier but previous investigations from other environments may give some indications of potential methods. A study of Stx phages in aquatic environments showed that the reduction of phages were lower than the reduction of bacteria in river water (Muniesa, Lucena, & Jofre, 1999) indicating that reduction in bacteria is not a guarantee for reduction in viable phage particles. Many marinades are acidic and at low pH they will probably have a good effect in lowering the stability of phages. Earlier investigations showed that no infectious Stx phages were present after 10 min exposure to pH 3 (LB-broth). In fruit juices at pH 3.7-3.9 at 20°C, no infective phages were present after 24 h exposure (Rode, et al., 2011). This corresponds with a previous study reporting that low pH and low temperatures prevented transduction in food matrices (Imamovic & Muniesa 2009). Traditional preservatives such as sorbate, benzoate and propionate can inhibit both free Stx phages and lysogenic phages in *E. coli*, but their efficacy in marinade has not been studied (Subils, Aquili, Ebner & Balague, 2012).

A number of limiting factors should be taken into account when considering, evaluating and implementing the use of antibacterial agents in the enhancement solution. The lethal and inhibitory effects of antimicrobial ingredients in marinades will often be different in the enhancement solution and in the marinated product. This is due to e.g. the dilution effects of the antimicrobial ingredients once injected into the meat and interfering effects of meat residues (Adler et al., 2011). Temperature and pH also affects the activity of most antimicrobial agents. Therefore, results from laboratory studies using bacteria suspended in standard nutrient broth added antimicrobials and temperatures above cooling temperature may be misleading. This may have value in an initial screening study, but it is important that potential antimicrobial ingredients are tested in real meat products. Another aspect is sensory properties of antimicrobials. Many natural ingredients, such as essential oils have a strong flavor and this limits the concentration range in marinades. The addition of organic acids decrease, while sodium metasilicate increase the pH of the marinades, and this will probably affect sensory properties (Adler, et al., 2011). Wicklund, Paulson, Rojas & Brewer (2007) reported that marinades with sodium lactate reduced the red color of enhanced meat compared to beef enhanced with standard marinade. However, organic acid salts may also stabilize the color of moisture-enhanced beef (Suman, Mancini, Ramanathan, & Konda, 2009). A strategy could be to combine several antimicrobials at low concentrations with other preservation techniques according to the hurdle technology concept. This concept could be used through the processing of enhanced meat and involve strategies for improved marinade formulations (e.g. optimal combinations of antimicrobials), raw meat quality (e.g. decontamination) and post processing strategies (e.g. packaging and storage). More examples of some relevant hurdle combinations were recently presented by Chen et al. (2012).

## 6.2 Beef raw materials

Several interventions have been tested for obtaining raw meat ingredients with improved bacteriological quality prior to the enhancement process (Echeverry et al., 2009; Heller et al., 2007). These include meat surface trimming, hot water spray, lactic acid bacteria spray and different surface treatments and combinations involving antimicrobials (organic acid spray, lactoferrin, sodium hypochlorite, acidified sodium chlorite). Washing with water is a simple decontamination method of meat although effects are highly dependent on the water temperature. Room tempered water provided <1 log cfu/cm2 reductions in *E. coli* O157 on beef subprimals (Lemmons, Lucia, Hardin, Savell, & Harris, 2011). Hot water (85°C) was the most effective intervention tested providing STEC reductions of 3.2 to 4.2 log cfu/cm2 on beef (Kalchayanand, et al., 2012).

Use of coliphages represents another potential measure to reduce incidence of STEC on beef.

Surface inoculation of beef with coliphage provided 2-4 log reductions of *E. coli* O157:H7, depending on phage concentration and storage conditions (Hudson, Billington, Cornelius, Wilson, On & Premaratne, 2013). In another study, a small, but statistically significant reduction of STEC (0.8-1 log reduction) was obtained by surface treatment of beef with a bacteriophage isolated from feces (Tomat et al, 2013).

Heller et al. (2007) tested the efficacy of 5 interventions applied 5 min before moisture enhancement for minimizing translocation of *E. coli* O157:H7 during the process. Trimming of outer surface or surface decontamination (hot water (82 °C), warm organic acids (55 °C) 2.5% or 5% lactic acid or 2% lactoferrin and 5% lactic acid) of STEC-contaminated meat reduced the number of STEC recovered from the inner surface after needle injection from 1.94% to 0.25-0.46%. Full trimming had the best efficacy on internal loads of STEC O157:H7. Echeverry et al. (2009) showed that pretreatment of beef surfaces with three sprays (lactic acid bacteria, acidified sodium chlorite and lactic acid) followed by storage for 14 or 21 days before marinade injection reduced internal *E. coli* loads between 0.8 and 3 log units. They also observed that once *E. coli* O157:H7 was internalized within the steaks it was quickly inactivated in the new environment. They hypothesized that this was due to the combinations of exposure to the antimicrobial in conjunction with the absence of oxygen in the internal muscle as the treatment alone in the presence of oxygen allowed better survival. In the same study, this phenomenon was not so noticeable with *S. Typhimurium* DT104 and thus requests the effects on other meat pathogens and spoilage microbes. The combinations of antimicrobial treatments with atmospheric conditions should be further investigated for obtaining effective combinatory interventions in the control of bacteria in enhanced meat.

## 6.3 Process

High pressure needleless injection is an emerging process for moisture-enhancement of meat, but *E. coli* O157 is transferred from the outer surface to the inner meat muscle and recirculation of the marinade leads to similar cross-contamination issues as for needle injection (Jefferies, Hansen, & Steele, 2012).

# 7. Conclusion and future challenges

Due to consumer preferences for rare done beef one cannot rely on proper cooking of moisture enhanced meat at the consumer stage. Although the incidence of STEC on beef is low, one cannot rule out the possibility of more outbreaks related to these products. So far, technology to eliminate STEC and Stx phage in these products has not been established. Future research should focus on optimization and streamlining of combined enhanced meat processes to obtain improved food safety and high microbial and sensory quality products. This work could also include the implementation of novel intervention technologies along the meat chain including potential novel processing technologies of meat (e.g. high pressure processing), marinades and novel packaging technologies.

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## Figure legends

**Figure 1** Survival of STEC during storage of beef injected with reference marinade (white) and acidified marinade (black) and stored vacuum-packed at 4oC for 7 days and then wrap-packed to simulate the retail packaging for 7 days. Samples were taken from the interior of the steaks and mean values for three steaks for each treatment and day is showed.

**Figure 2.** Detection of bacteriophages in beef, meat juice and in marinade. The start level (white bars) of phages in the marinade was 10% higher in the marinade compared to the others as the marinade was diluted 1:10 (vol:weight) in beef. Both reference and acidified marinade was used. The marinade was stored at room temperature 20°C (± 2°C), while the beef was stored at 4oC, first as vacuum-packed for 7 days (dark grey) and then wrap-packed to simulate the retail packaging for 7 days (14 days: black). Samples were also collected after 2 days of storage (light grey). Samples were taken from the interior of the meat and mean values for three steaks for each treatment and day are shown. The meat juice was collected from the juice seeping out of the meat during storage.

**Figure 3.** Total bacterial counts: ◇, TSB 30°C and □, TSB 15°C, and lactic acid bacteria (△, MRS) during storage of moisture enhanced beef with reference marinade (open symbols) and acidified marinade (closed symbols) and stored vacuum-packed at 4oC for 7 days and then wrap-packed to simulate the retail packaging for 7 days. Samples were taken from the interior of the steaks and mean values for three steaks for each treatment and day are shown.