DOCTOR OF PHILOSOPHY

Use of novel chitosan derivatives for the control of food-borne pathogens

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ABSTRACT

Outbreaks of *E. coli* O157 infection, primarily transmitted through meat products, have increased in recent years. There are many methods of preserving food, but strong consumer demands currently seek effective but natural preservatives. One example of such a preservative is chitosan (Ch). Although shown to possess antimicrobial properties, the exact antimicrobial action of chitosan and its derivatives under different conditions remains unclear. Additionally, little research has been done on applications of chitosan as a packaging material and its optimization as an agent for food preservation and enhancing food safety. This was the focus of this PhD study.

In the first experiment, the concentration of chitosan in solution and the pH values of its solvent were manipulated. Results showed that lower pH values exerted a stronger bactericidal effect against *E. coli* O157 under low chitosan concentration, with higher concentrations showing a stronger effect at more neutral pH. Chitosan only played a minor role in inhibition of the test pathogen, whilst pH exerted the predominant bactericidal influence. Further studies investigated chitosan-arginine (Ch-arg), a water-soluble derivative of chitosan. The second experiment showed that, when applied to meat juice, Ch-arg significantly reduced pathogen cell count and metabolic activity in a dose-dependent manner, with greater inhibition at higher concentrations, and suppression of general food spoilage bacteria. Experiment three evaluated whether the age of pathogenic *E. coli* O157 cells altered the effectiveness of Ch-arg in meat juice. It was found that Ch-arg was most bioactive against cells in the lag and exponential phases, significantly decreasing cell activity and density. In comparison, a reduced, although still significant, inhibitory effect was observed in the stationary phase. The final experiment investigated the use of cellulose chitosan arginine film (Ch-arg) as an antimicrobial additive to film packaging. High concentration of Ch-arg within film had a significant effect on cell count of *E. coli* O157 in chicken juice, but not in beef juice. Taken together, these studies suggest that Ch-arg may offer potential for limiting the growth of spoilage and pathogenic bacteria in food; however, further research is needed to establish the practicality and costs relative to other measures that are available to the food industry.

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DEDICATION

To the first witness who ever saw,

My very first smile and many more.

On his shoulders he bore my worries and cares,

My problems and trials he willingly shared.

His was the first name I ever spoke,

Picked up the pieces of anything I broke.

Assisted my first steps to steady me,

A finer father there could never be.

My thoughts of endearment are so sincere,

I dedicate this poem to the soul of my father so dear.

Father I miss you as is clearly shown,

To the greatest man I have ever known.

……

In the hope that he will know

RABYA

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Abbreviations

Ch	Chitosan
Ch-arg	Cellulose-Chitosan arginine
VTEC	Verocytotoxin-producing *Escherichia coli*
DD	Degree of deacetylation
pKa	Acid dissociation constant
CFU	Colony forming units
CDC	Centres for Disease Control and Prevention
NH_3^+	Amino group
LB	Luria-Bertani broth
RLU	Relative light units
CT-SMAC	Cefixime-tellurite-sorbitol-MacConkey agar
mTSB	Modified Tryptone Soy Broth
TOC	Total organic carbon
TON	Total organic nitrogen
TVC	Total viable counts
WC	Woolcool® lined
WCUN	Woolcool® unlined

1.1. Introduction

The increasing number and severity of food poisoning outbreaks on a global scale have considerably increased public awareness of food safety (Food Standards Agency, 2012). Well publicised cases of *Escherichia coli* (*E. coli*) serotype O157:H7 infections in particular are of concern due to the potential severity of symptoms (HPA, 2013). Although *E. coli* O157:H7 was only first recognized as a cause of foodborne illness just over 30 years ago (Forsythe, 2010), it has been implicated in sporadic cases and large outbreaks of haemorrhagic colitis and fatal haemolytic uremic syndrome (Karmali et al., 2010). This serotype is regarded as being more transmissible than other *E. coli* serotypes (O26:H11, O111:H11), for a number of reasons, including its increased tolerance to acid, which allows it to easily survive the acidic conditions of the stomach. This bacterium also produces Shiga toxins, which are heat stable, and therefore unaffected by conventional pasteurization methods (Rasooly & Do, 2010). Small doses of fewer than 10 cells may lead to infection (Forsythe, 2010). Collectively, these factors make the control of *E. coli* O157:H7 an important issue in recent times for the food sector.

The main causes for concern and product recalls associated with *E. coli* O157:H7 are meat products (Mor-Mur & Yuste, 2010). In particular, cattle and sheep are major reservoirs for this pathogen (Nastasijevicl et al., 2008; Hutchinson et al., 2005). Contact with those ruminants or their faeces can lead to ingestion of the organisms and subsequent human illness. Further, contamination of carcasses and food products by animal faeces can lead to transmission of foodborne pathogens to consumers (Oliver et al., 2008). Numerous interventions to be applied at the farm level have been investigated over the past 20 years, but most have proven to be ineffective and/or impractical (Soon et al., 2011). Furthermore, the conflict between demands for minimally processed foods and the modern requirement

of long shelf-life and food safety is an issue for the food industry. This has led to interest in the use of natural antimicrobial products.

One promising natural preservative is chitosan, a marine-based carbohydrate polymer. It is derived from chitin, which is found in *Zygomycetes* fungi, chlorella and insect cuticles. Chitosan has been reported to exert antimicrobial effects in ground beef (Juneja et al., 2006). It is versatile in application, being biodegradable, biocompatible, non-toxic and having many other useful physical and chemical properties, including potential for use in antimicrobial packaging (Alishahi et al., 2012). However, the mechanisms underlying the antimicrobial activity of chitosan have only been studied relatively recently. Although more information is now becoming available, only a few reports are supported by experimental evidence (Raafat & Sahl, 2009).

The antimicrobial activity of chitosan is greatly dependent on its physical characteristics, most notably molecular weight (Mw) and degree of deacetylation (DD). Chitosan is water-insoluble but soluble in weak organic acid solutions. In previous studies, chitosan usually shows greatest antibacterial activity in acidic media (Liu et al., 2004; No et al., 2002). However, it is possible that the reported antimicrobial activities might be the effect of the acidic media itself. The poor solubility of chitosan at high pH also limits potential applications. In this context, a novel water-soluble chitosan derivative, chitosan-arginine, has been produced. This chitosan derivative is obtained by modification with arginine groups to form stable peptide bonds between the arginine carboxylic acid and the amine on glucosamine (Tang et al., 2010).

Direct surface application or incorporation of bactericidal agents such as chitosan into food formulations has limited benefits (Quintavalla & Vicini, 2002). Recent years have witnessed the development of antimicrobial packaging as one promising application

method. In particular, packing or coating foods with chitosan-based biocomposites may significantly enhance microbial safety and extend shelf life (Lee et al., 2003).

Food preservation has become an important issue not only for manufacturers, but also for health-conscious consumers. This PhD research aims to provide solutions in the field of food microbiology which will have practical value to the food industry.

1.2. References

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2.1. Clinical aspects of *E. coli* O157:H7

In comparison with *Salmonella* and *Camplyobacter* there are considerably fewer human infections caused by *E. coli* O157. However, verocytotoxin-producing *Escherichia coli* (VTEC) is one of the most important causes of diarrheal episodes on a global scale (Bottero et al., 2004). A subgroup of *E. coli*, serotype O157:H7 (*E. coli* O157:H7), first identified as a human pathogen after the 1982 outbreak of hemorrhagic colitis (HC) in Oregon and Michigan (Riley et al., 1983), has been implicated in many cases of food contamination across the world (Barlow et al., 2006; Tauxe, 1997). VTEC infection rates have steadily increased in North America and the UK over the last 30 years. More than 90% of VTEC strains isolated in North America and the UK belonged to serotypes O157:H7 or O157:H^{-} (Chapman, 1995).

Infection with *E. coli* O157:H7 is asymptomatic in a large proportion of cases, but can also engender a wide range of clinical symptoms ranging from non-bloody diarrhoea to hemorrhagic colitis and other life-threatening complications. Serious health effects arising from infection can involve acute renal failure caused by haemolytic uraemic syndrome (HUS), and neurological problems in the form of thrombotic thrombocytopenic purpura (TTP; Duffy et al., 2006). When HUS manifests, health effects are primarily related to the kidneys (Rahal et al., 2012). Other rare complications include pancreatitis, diabetes mellitus, and pleural and pericardial effusions (Mead & Griffin, 1998). Occasionally, patients infected with *E. coli* O157:H7 suffer damage to their central nervous system due to TTP, typically including seizures arising from hypertensive encephalopathy. Untreated TTP can have a mortality rate as high as 95%. Symptoms may include thrombocytopenia, fever, renal insufficiency, neurological deficit, microangiopathic haemolytic anaemia, headache, fatigue/malaise, altered mental status, and hemiplegia (Rahal et al., 2012).

2.2. Epidemiology

VTEC has become the most frequently reported cause of bacteraemia in England, Wales and Northern Ireland (HPA, 2007). A report from HPA (2012; Figure 2.1) suggests almost a 100% increase, from 595 to 1182, in the annual totals of VTEC infections in England & Wales between 2002 and 2011. To date, many parts of the world have witnessed outbreaks of VTEC infections involving *E. coli* serotype O157 (Duffy et al., 2006). Infection rates differ widely between geographical regions. In Europe, Scotland possesses the highest infection rates with approximately 4 cases per 100,000 (Duffy et al., 2006), while in Northern Europe infection rates are very low (e.g. 0.04 per 100,000 in Norway and Finland). In North America, the infection rate for *E. coli* O157:H7 was 0.9 per 100,000 in 2004. In Asia, Japan has experienced the most problems related to *E. coli* O157:H7 (2.74 per 100,000 averaged between 1999 and 2004; Duffy et al., 2006). An estimated 73,500 cases of illness, 2000 hospitalisations and 60 deaths occur each year in the USA due to *E. coli* O157 infection (Mead et al., 1999), costing approximately $1 billion a year in medical costs and lost productivity (Wilks et al., 2005).

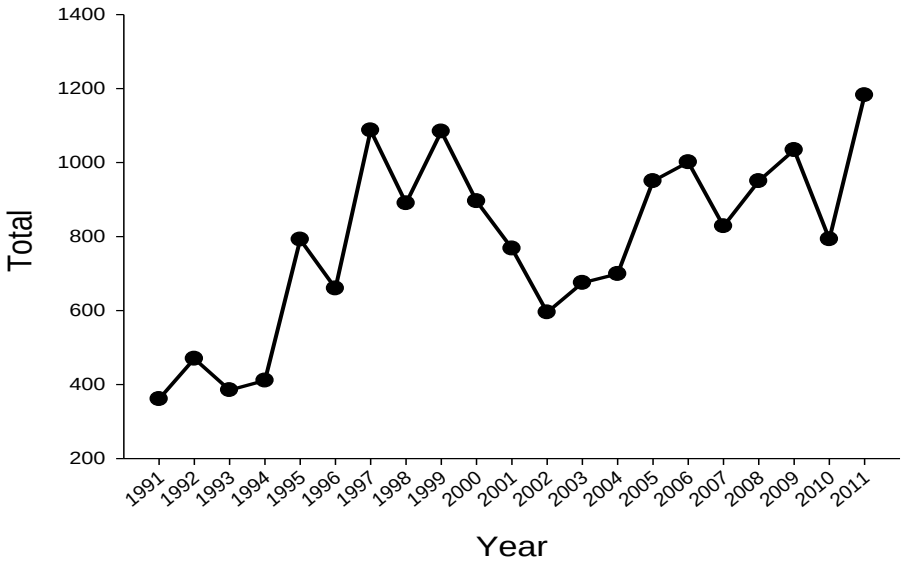

Figure 2.1. Annual totals of VTEC (*E. coli* O157:H7) infections in England & Wales (HPA, August 2012).

2.3. Sources of infection

E. coli O157:H7 infections have been associated with a variety of sources and routes (Duffy et al., 2006). Apart from animal-to-person and person-to-person transmission, consumption of *E. coli* O157:H7-contaminated food, particularly in public places such as day care centres, is an important mode of transmission that has attracted much attention in recent years (Chang & Fang, 2007; Duffy et al., 2006; EFSA, 2007; Liu et al., 2009; Meyer-Broseta et al., 2001). To date, research has investigated survival of the pathogen in a wide range of foods, including meat and meat products (Hwang et al., 2009; Rhoades et al., 2009), dairy products (Voitoux et al., 2002), lettuce (Koseki et al., 2004), apples (Du et al., 2003), tomatoes (Eribo & Ashenafi, 2003), chocolate and other confectionery (Baylis et al., 2004), and drinking water (Schets et al., 2005).

Farm livestock, particularly ruminants like cattle, sheep, and goats, are regarded as the primary reservoirs for VTEC (Heuvelink et al., 1998). Numerous studies have investigated *E. coli* O157:H7 prevalence, transmission, survival and control in cattle and beef (Duffy et al., 2006; Rhoades et al., 2009). Among others, the review by Rhoades et al. (2009) discussed factors that influence the prevalence of three important pathogens, VTEC *E. coli*, *Salmonella enterica*, and *Listeria monocytogenes*, in the whole process of meat production. The most severe cases of food-borne disease have been reported to be attributable to various foodstuffs containing beef. For instance, Adak et al. (2005) indicated that in England and Wales, 7% of the 1.7 million cases of food-borne disease in the period 1996-2000, including 67 deaths, were associated with beef. In the Netherlands, undercooked ground beef and raw milk have most often been implicated in food-borne infections (Heuvelink et al., 1998). Different countries may present different situations of food-borne disease, depending on factors such as the pathogen load in the beef products consumed and the cooking and consumption habits of the country concerned (Rhoades et

al., 2009). Products such as lightly-cooked burgers may be eaten more frequently in the USA, while people in France and the Netherlands consume more steak tartare than people in the UK and Greece.

2.3.1. Meat

E. coli O157:H7 exists as a normal coloniser of the gastrointestinal tract of cattle (Heuvelink et al., 1998; Nastasijevic et al., 2009). A number of studies have focussed on the prevalence of *E. coli* in the meat chain starting from the farm, to the slaughterhouse, to the final, ready-to-eat products (Nastasijevic et al., 2009, Rhoades et al., 2009). The spread of *E. coli* O157:H7 has been identified in farm housing and faeces (Jones, 1999) and the pathogen is known to survive for considerable periods in faeces and slurry (Avery et al., 2004). This bacterium may readily leach from sheep and cattle faeces during rainstorm events thus leading to further infections (Williams et al., 2005). Pigs and poultry can also be a source of O157 VTEC strain. Heuvelink et al. (1999) found that *E. coli* O157:H7 were isolated from 1.4% of 145 pigs and from 1.3% of 459 pooled faecal samples from Turkey flocks but was negative in faecal samples from chicken flocks. In a similar study, Kijima-Tanaka et al. (2005) isolated shiga toxin-producing *Escherichia coli* (STEC) from 23% of 62 bovine faecal samples and 14% of 25 swine samples and again there was no isolation from chicken samples. A Korean study by Jo et al. (2004) reported a higher prevalence of *E. coli* O157:H7 in cattle than in pigs (8.4% versus 0.3%) and none in chicken. At slaughter, transmission of *E. coli* from faecal material and hides to carcasses varies from 4.5% to 56% and from 1.1% to 43.4% respectively, which poses a great threat for the contamination of raw meat with this pathogen (Nastasijevic et al., 2009). In addition, contamination may occur during the dressing, skinning and evisceration phases (Nastasijevic et al., 2009). Reinstein et al. (2009) examined the prevalence of *E. coli* O157:H7 in organically and conventionally raised beef cattle at slaughter and found 14.8%

and 14.2% positives, respectively. An Irish study recovered *E. coli* O157 from 2.4% of beef trimmings samples, 3.0% of beef carcasses and 3.0% of head meat samples (Carney et al., 2006). The probability of *E. coli* O157:H7 spreading during the mincing process may be highest in the meat chain (Hawker et al., 2001). One carcass contaminated with *E. coli* O157:H7 may quickly spread the bacterium across the whole batch of minced meat from uninfected cows. Cagney et al. (2004) detected *E. coli* O157:H7 in 2.8% of minced beef and beef burgers, both frozen and fresh, in the Republic of Ireland. Magwira et al. (2005) investigated 400 meat samples (134 meat cubes, 133 minced meats, 133 fresh sausages) collected from 15 supermarkets and butcheries in Botswana and found prevalence rates of *E. coli* O157:H7 were 5.2 % in meat cube samples, 3.8 % in minced meat samples, and 2.3 % in fresh sausages. In South Africa, the prevalence of *E. coli* O157:H7 was identified on selected meat and meat products, including 45 samples each of biltong, cold meat, mincemeat, and polony (Abong'o, Momba 2009). Strains of *E. coli* O157:H7 were isolated by enrichment culture and confirmed by polymerase chain reaction (PCR). Also investigated were the antibiogram profiles of the *E. coli* O157:H7 isolates. Five (2.8%) out of 180 meats and meat products examined were positive for *E. coli* O157:H7. A parallel study in Switzerland (Fantelli & Stephan, 2001) was conducted on minced meat (beef and pork) samples to test for the presence of STEC. STEC was isolated from 2.3% minced beef samples and 1% minced pork samples.

2.3.2. Dairy products

Outbreaks of *E coli* O157:H7 illnesshave been found to be linked with consumption of raw milk and cheeses made from unpasteurized milk (Elhadiay & Mohammed, 2012; Vernozy-Rozand, 2005). In 1999, more than 11% of the total number of *E. coli* O157:H7 infections in England and Wales were due to unpasteurized milk and dairy product (CDSC, 2000), most probably due to faecal contamination during milking (Hussein & Sakuma,

2005). Conedera et al. (2004) noted that although the prevalence of VTEC O157 in raw milk and cheese is low, the organism appears to be able to survive the various stages of the cheese-making process. They found that the heat treatment of milk at the beginning stages of cheese production is usually not sufficient to kill the contaminating vegetative bacteria which can later survive the manufacturing and curing procedures. For example, *E. coli* O157:H7 was found to survive during the manufacturing process of soft Hispanic-type cheese (Kasrazadeh & Genigeorgis, 1995). *E. coli* O157:H7 is characterized by its ability to survive in acidic environments (e.g. in cheddar cheese after a curing period of more than two months; Reitsma & Henning, 1996). Furthermore, fermented dairy products made from raw milk contaminated with *E. coli* O157 can pose a risk to human health (Vernozy-Rozand et al., 2005). Marek et al. (2004) examined the survival of *E. coli* O157:H7 in pasteurized and unpasteurized Cheddar cheese whey. Five strains of *E. coli* O157:H7 were used for the study and were inoculated into 100 ml of fresh, pasteurized or unpasteurized Cheddar cheese whey at 10^5 or 10^2 CFU ml^{-1}, and stored at varying temperatures. Results showed that survival of *E. coli* O157:H7 was significantly higher in the pasteurized whey compared to that in the unpasteurized samples at all storage temperatures. Stringent sanitary practices should therefore be undertaken, particularly during the storage and handling of whey, and pasteurized milk should be used for cheese manufacture.

2.3.3. Vegetables and fruits

In the past ten years, an increased number of *E. coli* O157:H7-related outbreaks have been associated with fresh produce such as lettuce, cantaloupe, and alfalfa sprouts (Doyle & Erickson, 2008; Silagyi et al., 2009; Pathanibul et al., 2009). This growing tendency could be due to increased consumption of potentially risky fresh-cut pre-packaged products (Doyle & Erickson, 2008). Four separate outbreaks of food-borne *E. coli* O157 infections were recorded in USA in 2006 (Doyle & Erickson, 2008). Common

vehicles of the disease noted were fruits and vegetables such as green-based salads, potatoes, lettuce, unspecified fruits, and sprouts (Doyle & Erickson, 2008). Among the reported outbreaks, lettuce was the single most frequently mentioned produce (Ackers et al., 1998; López-Gálvez et al., 2009). Ackers et al. (1998) found 40 Montana residents to be infected with *E. coli* O157:H7 due to the consumption of purchased leaf lettuce. In addition, Eribo and Ashenafi (2003) demonstrated that *E. coli* O157:H7 could be found in tomato and processed tomato products as well as products containing vinegar. *E. coli* O157:H7 showed the ability to grow during germination a sprouting of alfalfa (Castro-Rosas & Escartin, 2008) and in acidic foods such as fermented Spanish-style table olives (Spyropoulou et al., 2001).

2.4. Resilience of *E. coli* O157 to environmental conditions

Environmental conditions such as temperature, pH value, water activity, and sodium chloride have important implications in the survival and growth rates of *E. coli* O157:H7 in foodstuffs. The bacterium is known to have resistance to heat (Kaur et al., 1998). It can proliferate at a temperature range of 8-44.5 °C, with the optimal temperature for growth at 37 °C (Edwards & Fung, 2006). Cooking beef thoroughly to 71 °C for 15 seconds is effective in eliminating the organism (Public Health Agency of Canada, 2012), although slow cooking of meats may not eradicate the organism as effectively as rapid cooking (Edwards & Fung, 2006; Kaur et al., 1998). Regardless of pH and water activity, survival of *E. coli* was found to be better at 5 °C than at 20 or 30 °C in tryptic soy broth (TSB; Rocelle et al., 1996). Moreover, *E. coli* was found to survive but not grow during fermentation, drying, or subsequent storage at 4 °C for 2 months (Glass et al., 1992). The heat-resistant property of *E. coli* O157:H7 is relative as it can be influenced by many other environmental factors, including growth phase, the amount of heat applied, the rate of heating and the water activity (Kaur et al., 1998). For instance, at 30 °C, inhibition of

growth of *E. coli* O157:H7 in TSB was enhanced by reduction of the water activity (Rocelle et al., 1996) as well as increase of sodium chloride concentration (Jordan & Davies, 2001).

Much evidence has shown that pH value plays a primary role in the growth rates of *E. coli* O157:H7. For instance, growth rates are similar at moderate pH values (pH 5.5-7.5), but decrease significantly at lower pH values (Edwards & Fung, 2006). Yet, Benjamin & Datta (1995) found the organism to be acid tolerant under the optimal temperature (37 °C), surviving at pH 2.5 for up to 7 h. The pathogen is capable of acid-adaption and adapted cells have shown increased survival in shredded dry salami and apple cider (Leyer et al., 1995). *E. coli* O157:H7 has been reported to survive for months in acidic foods, such as fermented sausages (CDC, 1995) and apple cider and apple juice (Du et al., 2003); even though products such as fermented sausage may also lead to water stress in bacteria. The resilience of the organism to a combination of factors such as temperature, pH, water activity and sodium chloride can all contribute to the survival and growth of *E. coli* O157:H7 in foodstuffs. Its ability to withstand low pH environments is also crucial during passage through the gastrointestinal tract of livestock and humans.

E. coli O157 can survive and grow in both aerobic and anaerobic conditions as well as modified atmospheres used for food packaging (Bromberg et al., 1998). Therefore, as a facultative anaerobe, the heat resistance of this pathogen can vary. For instance, it has been documented that holding the pathogen under anaerobic conditions may allow heat-injured cells to grow when oxygen is present. However, under aerobic conditions, *E. coli* O157 showed reduced heat-resistance. (Bromberg et al., 1998). Consequently, this has important implications in food packing. There may be increased risk of *E. coli* O157:H7 surviving during heating treatments of foodstuffs that are packed under vacuum or reduced oxygen atmospheres (George et al., 1998).

2.5. Control of *E. coli* O157

The increase in number of food-borne pathogenic infections has generated considerable efforts in the control of organism such as *E. coli* O157 in food. Many preventative measures have been introduced and targeted at all stages of the food chain, from the farm, to the slaughterhouse, and to the preparation of food at home (Vernozy-Rozand et al., 2002; Zhu et al., 2009).

Although total elimination of *E. coli* O157:H7 carriage in livestock appears unlikely, pathogen transmission can be reduced through a number of farm management practices, such as to forbidding farmers from applying slurry and animal manure to ready-to-eat vegetables and fruit plants (Jones, 1999). Good hygiene practices such as careful preparation and cooking of food and interventions such as pasteurization, organic acid washes, and steam vacuuming, as well as the use of antimicrobial solutions (e.g. dilute lactic acid, trisodium phosphate and chlorine) can be effective means to eliminate *E. coli* O157:H7 from food (Marshall et al., 2005; Rhoades et al., 2009; Vernozy-Rozand et al., 2002). For instance, to prevent minced meat from contamination with *E. coli* O157:H7 during the mincing process, cooking at a high temperature can destroy *E. coli* O157:H7 cells (Abong'o & Momba, 2009). To prevent contamination of apple cider, it is suggested to wash and brush apples and preserve the cider with sodium benzoate (Zhao et al., 1993) or aqueous commercial cleaner (Kenney & Beuchat, 2002). To reduce the number of VTEC on salad vegetables, storing salad vegetables at 4 °C can be an effective means (Abdul-Raouf et al., 1993).

In recent years, advanced technologies have also been explored in the produce industry to reduce *E. coli* O157:H7 as well as to maintain the sensory quality of the produce itself. One study by Selma et al. (2008) showed the combined application of gaseous ozone and hot water could effectively control microbial growth in cantaloupe

melon as well as maintain its initial sensory quality such as aroma and texture. However, this study failed to point out the specific action of ozone in inactivating *E. coli* O157:H7. Additionally, a study by Purnell et al. (2004) found a hot wash treatment at 70 °C at 40 second, to significantly inhibit pathogens on raw chicken surfaces, relative to an untreated control, without causing the chicken skin to tear during processing. Mahmoud (2010) explicitly demonstrated the efficacy of X-ray on inoculated *E. coli* O157:H7 (also including *L. monocytogences*, *S. enterica* and *S. flexneri*) on shredded iceberg lettuce. By treating iceberg lettuce with 1.0 and 2.0 KGy X-ray, the study detected significant reductions of *E. coli* O157:H7 population in both conditions. This approach also showed its promising application because the sensory quality (i.e., visual colour) of leaves was not adversely affected during subsequent storage.

Although controlling *E. coli* O157:H7 in food through thermal treatment, chemical destruction and preventative interventions have showed some due efficacy, some studies also report negative findings. For instance, organic acids such as lactic acid and citric acid were reported to be ineffective in controlling *E. coli* O157:H7 in beef burgers, even when combined with freezing at -20 °C for 2 hours (Bolton et al., 2002). Another study on traditional Iranian barbecued chicken (TIBC) reported that although essential oils of oregano and nutmeg showed effectiveness in inhibiting the growth of *E. coli* O157:H7 in a broth culture system, they reported no inhibitory effect against this pathogen in ready-to-cook TIBC, suggesting that *in vitro* investigation may not necessarily be applicable to food conditions (Shekarforoush et al., 2007). Although the importance of temperature control and protective packaging has been emphasized in reducing pathogen growth on raw meat, inoculated *E. coli* O157:H7 strain NCTC 12900 could still increase when lamb chops were kept air packed at 4 °C for 12 days (Barrera et al., 2007).

Many of the intervention measures described are still effective at experimental stage and are unlikely to be widely implemented in the foreseeable future due to a lack of commercial viability, geographical differences in the regulatory framework, or a lack of acceptance by consumers. Elevated public concerns about the adverse consequences of chemically synthesized preservatives used in food industry have diverted research to the application of natural antimicrobials to inhibit *E. coli* O157:H7 growth and activity (Raybaudi-Massilia et al., 2009). Numerous studies have been done using antimicrobials of animal, plant and microbial origin to prevent or control microbial contamination in fresh-cut fruits and fruits juice (Raybaudi-Massilia et al., 2009). For instance, essential oils containing thymol, eugenol, and carvacrol can disrupt the cellular membrane and cell walls of microorganisms (Raybaudi-Massilia et al., 2009). Cinnamic aldehyde extracted from cinnamon shoots also showed antimicrobial activity and significantly reduced *E. coli* O157:H7 numbers at 37 °C after 12 hours (Kim et al., 2004).

Recent years have witnessed emerging interest in the use of chitosan in the food industry due to its antimicrobial activity against a wide range of food-borne bacteria (No et al., 2007; Goy et al., 2009). In particular, chitosan has attracted attention as an antimicrobial additive in packaging to assist the preservation and extend the shelf life of perishable foods (Lee et al., 2003).

2.6. Chitosan and its applications

Chitosan (Ch), a partially deacetylated derivative from chitin, is a linear polysaccharide composed of 2-amino-2-deoxy-d-glucose (glcn) and 2-acetamide-2-deoxy-d-glucopyranose units linked by β (1-4) glycosidic bonds (Figure 2.2; Chung & Chen, 2008). It is a versatile nontoxic, biodegradable film-forming polymer widely used in food, biomedical and chemical industries (Shahidi et al., 1999) and has been recognized as

Generally Regarded As Safe (GRAS; FDA, 2001). Sources of chitosan include crustacean shells, insects, and the cell walls of certain fungi such as *Mucor rouxxi* (Bento et al., 2009). With many unique biological properties, chitosan exhibits a wide spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as fungi (Dutta et al., 2009; Helander et al., 2001; Ziani et al., 2009); though generally has a stronger impact on Gram-negative organisms (Chung & Chen, 2008; Devlieghere et al., 2004).

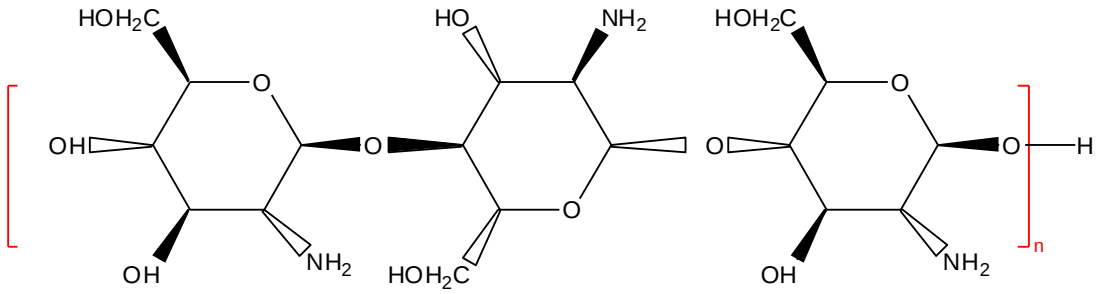

Figure 2.2. Polymer of β-(1-4)-D-glucosamine units

Chitosan is a natural bactericide (Chung & Chen, 2008). The presence of the positively charged amino groups in C2 position (below pH 6) is suggested to provide major functional structure (NH_3 groups of chitosan acetate) expected to interact with the predominantly negatively charged cell surface of bacteria (Holappa et al., 2006; Liu et al., 2004; Nikaido, 1996). Through binding and disrupting the normal functions to cell membranes, chitosan promotes the leakage of intracellular components (including enzymes and nucleotides; Chung & Chen, 2008) through damage of the cell membrane (Liu et al., 2004) and cell wall (Chung et al., 2004) and also by inhibiting the transport of nutrients into the cells (Chen & Chou, 2005). However, the exact mechanism of the antimicrobial effect of chitosan is still inconclusive. Chung and Chen (2008) suggest a two-step sequential mechanism of the antimicrobial activity of chitosan on an *E. coli* cell, initially

separating the cell wall from its cell membrane, and then destroying the cell membrane. Li et al. (2010) however, suggested that chitosan increases the permeability of the outer membrane and inner membrane of *E. coli*, ultimately disrupting the cell membrane and releasing cellular contents inside the bacteria.

The antimicrobial effect of chitosan depends on several factors such as the target microorganisms (Gram-positive or Gram-negative bacteria), the kind of chitosan (deacetylation degree and molecular weight [MW]) used, the pH of the medium, the temperature, and the presence of different food components (Devlighere et al., 2004; Fernandes et al., 2008). Chung et al. (2004) found that most negatively charged cell surfaces had a greater interaction with chitosan and its inhibition efficiency showed a high correlation with the amount of absorbed chitosan. The antibacterial activity against *E. coli* has been shown to decrease when the molecular weight (MW) of hydrolyzed chitosan decreases (Hongpattarakere & Riyaphan, 2008). The bactericidal action of chitosan is further complicated by the interaction between MW and target microorganisms, with lower MW chitosan exerting more inhibition in the case of the Gram-negative bacteria and the reverse in the case of the Gram-positive ones (Fernandes et al., 2008). The antimicrobial activity of chitosan increases with increasing degree of deacetylation, due to the increasing number of ionisable amino groups (Liu et al., 2001). Furthermore, chitosan is found to be more effective as a food preservative at low pH and in low protein foods (Fernandes et al., 2008). This is because decreasing pH (below 6) makes the amino groups of chitosan ionized with a positive charge (Figure 2.3; Kumirska et al., 2011) thus increasing its antimicrobial activity (Yang et al., 2005); unmodified chitosan is not antimicrobial at pH 7, because it does not dissolve to carry any positive charge on the amino groups (Chung et al., 2005). Devlieghere et al. (2004) found that the microbiological load on chitosan-dipped strawberries and lettuce was lower stored at 7 °C for 4 days compared with that on control

samples (pH value equivalent with that of products). The antimicrobial effect of chitosan on strawberries lasted longer than that of lettuce (12 days versus 4 days), indicating that the effect of chitosan may vary among different food components.

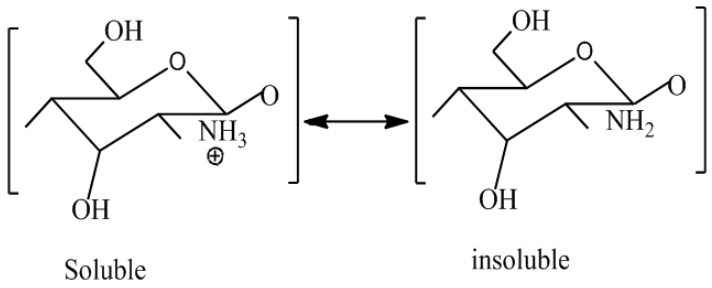

Figure 2.3. The properties of chitosan.

Potential applications of chitosan as a biopreservative have been investigated in various food products (Giatrakou et al., 2010). For instance, chitosan coatings effectively maintained the sensory quality of peeled litchi fruit and extended its shelf-life by inhibiting the increase in activities of polyphenol oxidase and peroxidise (Dong et al., 2004). The use of chitosan at concentrations up to 1 g l^{-1} could extend the shelf-life of fresh orange juice by reducing enzymatic and non-enzymatic browning and controlling the spoilage while maintaining the nutritional value of juice (Martin-Diana et al., 2009). Moreover, the antimicrobial effect can be enhanced when being modified through chemical and enzymatic treatment (Kanatt et al., 2008), or combining with other natural antimicrobials such as thyme oil (Giatrakou et al., 2010) and nisin (Lee et al., 2003). By heating chitosan with glucose (chemical modification), Kanatt et al. (2008) developed a novel food preservative, chitosan glucose complex (CGC), which showed excellent antioxidant activity and antimicrobial activity against common food spoilage and pathogenic bacteria such as *E. coli*, *Pseudomonas*, *Staphylococcus aureus* and *Bacillus cereus*; whereas chitosan alone did not have any significant antioxidant activity. CGC increased the shelf

life of lamb meat and pork cocktail salami by more than 2 weeks and 28 days, respectively, during chilled storage. Chitosan-lysozyme (CL) films and coatings (enzymatic treatment of chitosan) reduced populations of *E. coli*, *P. fluorescens* and *L. monocytogenes* in Mozzarella cheese and completely inhibited the growth of mould (Duan et al., 2007). In addition, the shelf life of a ready to cook chicken-pepper kebab (skewer) treated with chitosan and thyme oil was extended for a period of 3 days at 4 ± 0.5 °C of storage; the combination of chitosan and thyme oil was most effective in inhibiting the growth of the microorganisms such as lactic acid bacteria, *Brochothrix thermosphacta* and *Enterobacteriaceae* (Giatrakou et al., 2010). Finally, chitosan-coated paper was more effective against *E. coli* O157:H7 than nisin-coated paper and the coating of combined nisin and chitosan could improve the microbial stability of milk and orange juice stored at 10 °C (Lee et al., 2003).

However, other results have raised the issue about the efficacy of chitosan as an antimicrobial agent. For instance, Bajpai et al. (2010) found no bactericidal action of chitosan based ZnO films against *E. coli*. The authors explained this on the basis of the films being made by a chitosan with high molecular weight (1.42×10^6) which has been previously argued to be ineffective against bacterial growth (Bajpai et al., 2010). Another study found that the addition of chitosan to apple juice delayed spoilage by yeasts but enhanced the survival of *E. coli* O157:H7 (Kisko et al., 2005). Nevertheless, the said study admitted the possibilities that the underestimation of viable counts of *E. coli* O157:H7 resulted from direct plating on selective media and the nourishing contents of apple juice used in the experiment that contained a natural, mixed microflora.

The biocidal properties of chitosan-based biocomposites have been noted for many years (Fernandez-Saiz et al., 2008). Chitosan incorporated polymeric materials are particularly effective in food preservation (Dutta et al., 2009). Chitosan based edible films

and coatings, such as chitosan-tapioca starch, showed advantageous properties in terms of water vapour permeability and solubility (Vásconez et al., 2009). Vásconez et al. (2009) also found that the chitosan solution was the best coating for a salmon slice because it reduced aerobic mesophilic and psychrophilic cell counts and maintained an acceptable pH and weight loss throughout refrigerated storage. Moreover, incorporating chitosan into an insoluble biopolymer matrix can generate novel chitosan-based antimicrobial materials with potential advantages as active food packaging applications (Fernandez-Saiz et al., 2008). Recently, the combination of chitosan with food packaging materials synthesized from bio-derived polymers has showed a promising strategy to elevate the antimicrobial property of the packaging materials. The polymer films already in use for food packaging are polyethylene (PE), polyvinyl chloride (PVC), polyvinyl alcohol (PVA), and polylactic acid (PLA; Kosior et al., 2006), which are renewable thermoplastic polyesters and were approved by the Food and Drug Administration (Rasal et al., 2009). Since bio-derived monomer materials generally offer a range of flexible, thermo-mechanical and barrier properties, and good biocompatible properties (Kosior et al., 2006), if combined with chitosan, the resulting packaging should exhibit advantageous physical and antimicrobial properties. Tripathi et al. (2009) have developed an antimicrobial film based on chitosan and poly (vinyl alcohol; PVA) cross-linked by glutaraldehyde (GA). When applied to minimally processed tomato, this material demonstrated strong antimicrobial activity against food pathogenic bacteria including *E. coli* O157, *S. aureus* and *B. subtilis*.

2.7. Aims and objectives

Based on the literature review above, several important issues emerge that call for further investigation. Firstly, the exact antimicrobial action of chitosan under different conditions remains unclear, particularly against *E. coli* O157:H7, with conflicting results

evident in the literature (Zheng & Zhu, 2003). Secondly, little research has been done on food applications of chitosan as biocomposites incorporated into packaging materials, and thus optimization of chitosan as a food preservative is worth exploring. The overall objective of the proposed study is therefore twofold:

1. To quantify the antimicrobial effect of chitosan and chitosan derivatives at varying concentrations and pH values on *E. coli* O157:H7, from which it is hoped to find the optimal combination of concentration and pH of chitosan for controlling *E. coli* O157:H7.
2. To determine the effects of chitosan-arginine based biocomposite films on controlling *E. coli* O157 in beef and chicken meat.

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Abstract

Chitosan is a natural polysaccharide biopolymer heralded as a promising preservative agent for use in the food industry. The antimicrobial actions of chitosan are thought to be concentration-dependent as well as medium pH–dependent. However, limited attention has been paid to the combinatory effects of both factors on the bactericidal ability of chitosan. The aim of the present study was to investigate what concentration-pH combination would optimize the antimicrobial action of chitosan solution against *E. coli* O157:H7. Chitosan concentrations ranging from 0-1 gl^{-1} and medium pH values from 4-7 were combined. Results showed that lower pH values exerted a stronger bactericidal effect against *E. coli* O157 when the chitosan concentration was low. Higher chitosan concentration showed a stronger bactericidal effect when more neutral pH values were used. At all concentrations, chitosan only played a minor role in the inhibition process against the test pathogen, whilst pH exerted the predominant bactericidal influence. These results implied a complex interaction between chitosan concentration and pH. Further investigation is needed to confirm the relationship between the two factors and the influence of other solutes.

Keywords: Acetic acid, biological activities, food-borne, food poisoning, in vitro preservation, pathogen, polycationic

3.1. Introduction

Escherichia coli O157 is an important food-borne bacterial pathogen closely associated with many severe human illnesses such as haemolytic uremic syndrome (Pennington, 2010). The primary route for food-borne infection with *E. coli* O157 is via consumption of contaminated meat (Rhoades et al., 2009). Much effort has been made by industry to control the bacterium at all points along the food chain, including in food packaging.

An increasing number of reports have indicated the promise of using chitosan and its derivatives as a potential antimicrobial agent against food spoilage organisms and human pathogens, including *E. coli* O157 (Rodríguez et al., 2003; Helander et al., 2001). Chitosan is an aminopolysaccharide biopolymer primarily derived from chitin. It consists of two monosaccharides, *Glc*NAc and D-glucosamine (GlcN), linked together by β - (1→ 4) glycosidic bonds (Raafat & Sahl, 2009). Chitosan possesses many unique chemical features which make it a suitable functional material. Some of these include its biocompatibility with other materials, its biodegradability and its strong adsorption properties, including a high charge density, reactive hydroxyl and amino groups as well as extensive hydrogen bonding (Raafat & Sahl, 2009; Ravi Kumar et al., 2004). The antimicrobial function of chitosan is suggested to result from a sequence of molecular processes characterized by multiple detrimental reactions with the cell membrane of the chitosan–treated bacterium (Raafat & Sahl, 2009). A commonly recognized basis for this function is its polycationic structure brought by the positively-charged NH_3^+ groups of glucosamine, which may interact with negatively charged surface components of many bacteria.

The antimicrobial effect of chitosan is likely to depend on many factors, including the target microorganism (Gram-positive or Gram–negative), the kind of chitosan used (deacetylation degree and molecular weight [MW]), solution pH, and temperature during incubation (Devlighere et al., 2004; Fernandes et al., 2008). No et al. (2002) found that the antimicrobial activity of chitosan was inversely related to pH, with greater activity found at lower pH, whilst Chung et al. (2004) found chitosan was absorbed more readily by bacteria at pH 4 than pH 5. In addition, higher antibacterial activity was observed at higher chitosan concentrations (Liu et al., 2004). However, so far, scant attention has been paid to the investigation of co-factorial influence of chitosan concentration and medium pH in optimizing the efficacy of chitosan.

The present study set out to explore the optimal combination of chitosan concentration and environmental pH for the antimicrobial control of *E. coli* O157:H7. Varied chitosan concentrations (0-1 g l^{-1}) and LB broth pH values (4, 5, 6, and 7) were combined and compared. The outcome measures included both *E. coli* O157:H7 cell counts and its metabolic activity.

3.2. Materials and methods

3.2.1. Preparation of chitosan solutions

A low molecular weight form of chitosan [75-85% deacetylated chitin, poly (D-glucosamine), viscosity 20-200 cps] was sourced from Sigma-Aldrich (448869-250G, Aldrich). Chitosan solutions were prepared in 2.5% (v/v) acetic acid at concentrations of 0 (control), 0.1, 0.2, 0.4, 0.6, 0.8 and 1 g l^{-1} on a dry weight basis before being applied to Luria-Bertani (LB) broth, prepared as described below.

3.2.2. Preparation of E. coli *O15 inoculum*

A strain of *E. coli* O157 (strain 3704 Tn*5 luxCDABE*; Ritchie et al., 2003) was prepared from a fresh overnight LB broth (Difco Ltd, Teddington, Surrey, UK; 37°C, 18 h, 150 rev min^{-1}; Williams et al., 2008). Cells were washed and concentrated by centrifugation as described in Avery et al. (2005). The strain has been proven to be non-toxigenic due to the absence of toxin activity (by Verocell assay) and toxin genes (by PCR; Ritchie et al., 2003); however, it is still thought to reflect the survival pattern of a toxigenic strain (Ritchie et al., 2003; see overall discussion for further details). The concentration of *E. coli* O157 in the inoculum was 10^7 CFU ml^{-1}, determined by plating out in duplicate onto cefixime-tellurite-sorbitol-MacConkey agar plates (CT-SMAC; Oxoid, Basingstoke, UK) and enumeration of colonies with the characteristic appearance of *E. coli* O157 following incubation (18 h, 37°C).

3.2.3. Antibacterial testing

For the control treatment, LB broth with 2.5% (v/v) acetic acid and LB broth with ¼-strength Ringer solution only were prepared. Chitosan solution (concentrations at 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 g l^{-1}) were added to the LB broth (n = 3) and the pH was then adjusted to pH 4, 5, 6, or 7 using either 1 M HCl or 1 M NaOH. pH values of all the samples were checked midway through and at the end of the experiment to verify that it remained constant. Samples were taken at 0 h (immediately after inoculation), 2, 6, 12, 24, 48 and 72 h post-incubation and numbers of *E. coli* O157:H7 determined by plating in duplicate onto CT-SMAC plates and incubated as described previously. Non-sorbitol-fermenting colonies were examined via latex agglutination (Oxoid DR620).

3.2.4. Luminescence of E. coli *O157*

Luminescence of *E. coli* O157 was measured at 0 h (immediately after inoculation), 2, 6, 12, 24, 48, 72 h post-inoculation. At each time point, a 1-ml aliquot was placed in triplicate into a plastic luminometer cuvette and its luminescence (relative light units [RLU] determined using a SystemSURE 18172 luminometer [Hygiena Int., Watford, UK]).

3.2.5. Data analysis

Data were analyzed through IBM SPSS Statistics version 16.0 for Windows. All plate count and luminescence data for *E. coli* O157 were $\log_{10}(y + 1)$ transformed prior to analyses to meet the assumptions of ANOVA. A full 3 × 2 factorial model was used to analyze the effects of concentration, pH, and time (fixed factors) on transformed luminescence and cell counts. Post-hoc tests using Tukey HSD at $p < 0.05$ was adopted to identify significant differences between each treatment condition.

3.3. Results

3.3.1. Co-factorial influence of chitosan concentration and medium pH on E. coli *O157 cell counts*

Figure 3.1 depicts the effect of medium pH on *E. coli* O157 cell count at varying chitosan solution concentrations. The two control groups (i.e. without chitosan and with 2.5% acetic acid) did not differ from each other during the first 12 h ($p > 0.05$). Thereafter, numbers of viable *E. coli* O157 cells treated with 2.5% (v/v) acetic acid were actually greater than in the samples with no treatment. The effects of incubating at pH 6 and 7 were similar to the control treatments with the exception of chitosan solutions at 0.8 g l^{-1} where the pH 6 treatment caused a larger reduction in the number of viable bacterium at 72 h ($p < 0.05$).

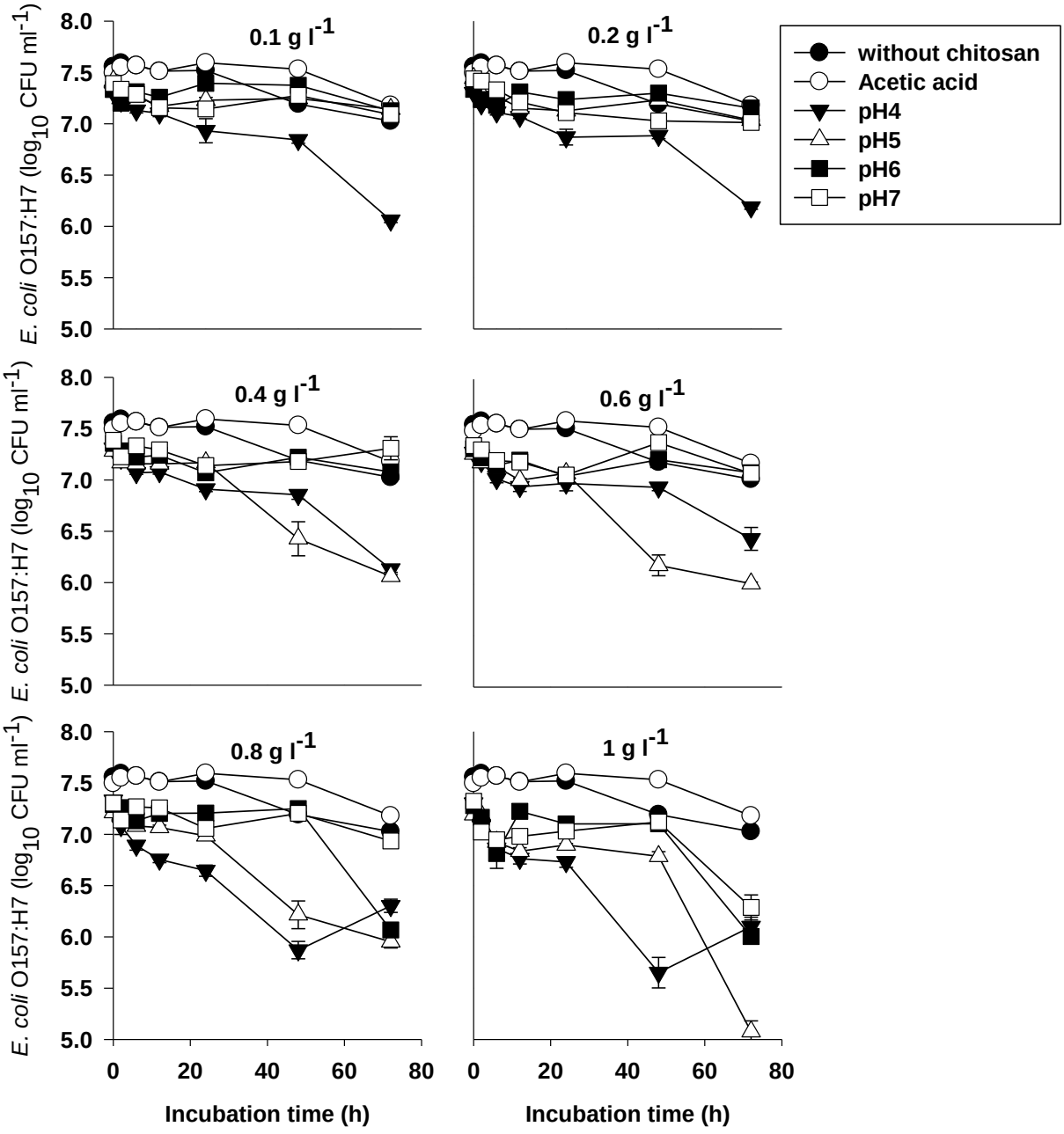

Figure 3.1. The effects of solution pH (4, 5, 6, and 7) on viable cell counts of *E. coli* O157 under varied concentrations of chitosan (0.1, 0.2, 0.4, 0.6, 0.8 and 1 g l^{-1}). The error bars represent standard error of the mean (SEM).

At lower chitosan concentrations (0.1 g l^{-1} and 0.2 g l^{-1}), the pH 4 treatment resulted in a 1.5-log cell reduction. As chitosan concentration increased, the overall effects of the pH 4 treatment became weaker and pH 5 started to show stronger bactericidal effects than pH 4,

particularly when chitosan concentrations reached 1 g l^{-1}. The resultant effect of incubating at pH 5 was a 2-log cell reduction, whereas pH 4 treatment caused only 1-log cell reduction, similar to that seen at pH 6 and 7. These results indicate that the lower the pH value of the medium, the stronger bactericidal effect it has when the chitosan concentration is relatively low; vice versa, the higher the chitosan concentration in solution, the stronger its bactericidal effect, as long as the pH value of the medium is relatively high.

It appeared that the combination of medium pH and chitosan concentration at around pH 5 and 1 g l^{-1} provided the optimal bactericidal environment for reducing the viable cell count of *E. coli* O157 by around 5-log cell count.

3.3.2. Co-factorial influence of chitosan concentration and medium pH on E. coli *O157:H7 luminescence.*

The effects of medium pH on the luminescence of *E. coli* O157:H7 at varying chitosan concentrations are presented in Figure 3.2. Incubating at pH 4 resulted in a persistent and strong inhibitory effect on bacterial activity, with a very minor co-factorial influence from chitosan concentrations. The inhibitory effect of pH 4 was observed immediately after exposure to the low pH environment (<2 h). As the concentration of chitosan increased, incubating at pH 5 resulted in a greater inhibition of bacterial activity than pH 4, particularly after 24 h such that the level of *E. coli* O157:H7 activity was reduced to nearly zero.

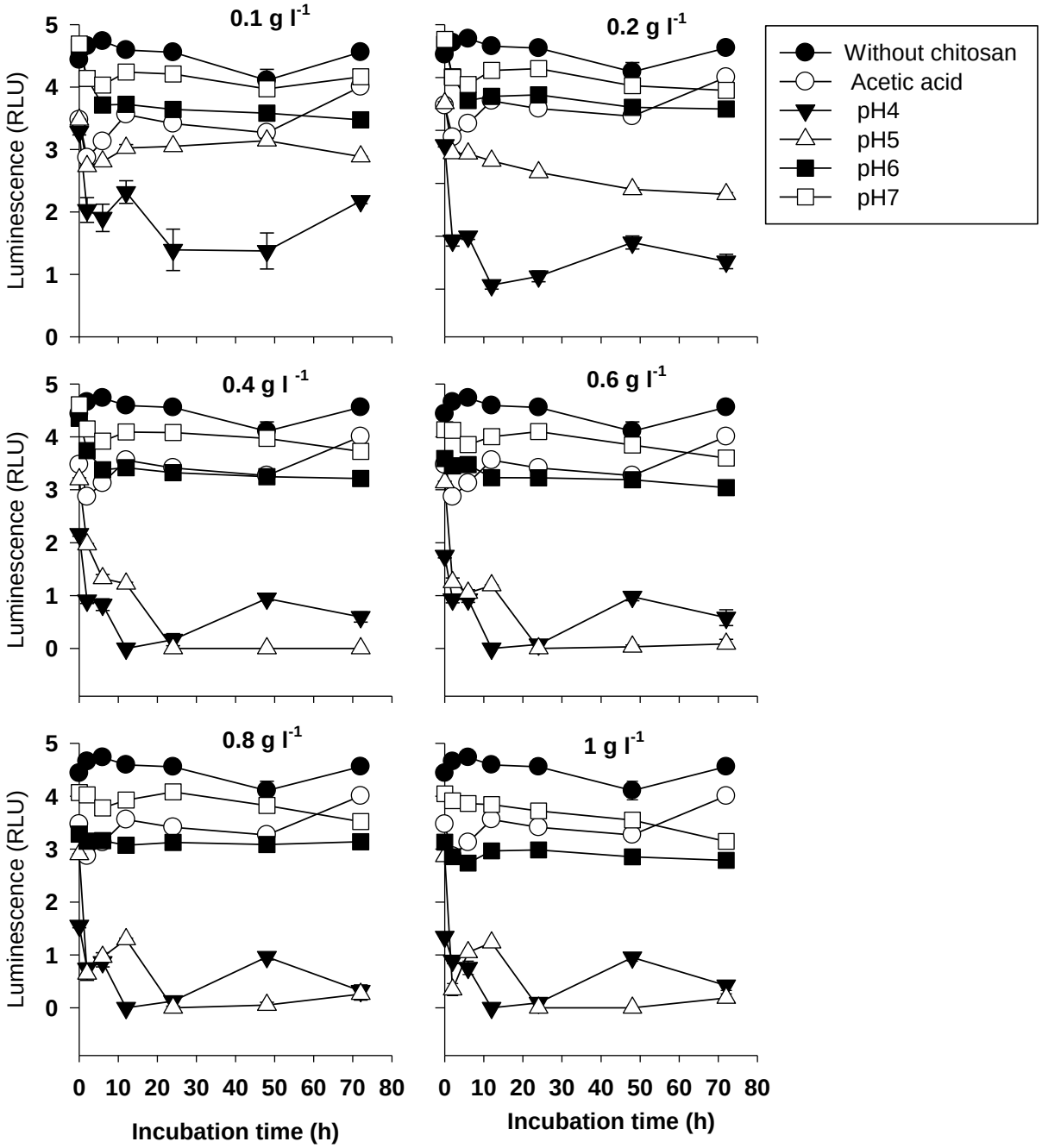

Figure 3.2. The effects of solution pH (4, 5, 6, and 7) on the luminescence of *E. coli* O157 under varied concentrations of chitosan (0.1, 0.2, 0.4, 0.6, 0.8 and 1 g l^{-1}). The error bars represent SEM.

For the other four treatment conditions (two controls and pH 6 and 7), there were no significant difference between them ($p > 0.05$), indicating neither pH 6 nor 7 provided ideal environments to inhibit pathogenic activity, even when the chitosan concentration was very high (1 g l^{-1}). These results imply that a solution pH of 5 or lower exerts a good inhibitory

influence against the activity of *E. coli* O157. Overall, the presence of chitosan only played a minor role in the inhibition process compared to that of acetic acid. Moreover, pH 5 seems able to provide an excellent environment for reducing the activity of the bacterium.

3.4. Discussion

It has been widely observed that the activity of chitosan is inversely related to solution pH, with significantly higher bactericidal properties apparent at low pH (No et al., 2002). In addition, its antimicrobial properties are positively correlated with concentration, with greater microbial growth suppression observed at higher concentrations (Liu et al., 2004). The present experiment considered the potentially more complex co–factor effects of varied chitosan concentrations and pH on the antimicrobial action of chitosan against *E. coli* O157. Our results showed that the impact of chitosan and pH are co-dependent when optimizing their total antimicrobial action.

3.4.1. Effect of Chitosan concentration and environmental pH on E. coli *O157 viability*

One frequently-used indicator of the antimicrobial activity of chitosan is obtained via enumeration of viable bacterial cell counts. Our results from previous studies appeared to imply that concentration influences the antimicrobial action of chitosan in a pH-dependent style. One general claim of the functional mechanism of chitosan is that the positively charged amino groups in the C2 position of chitosan acetate would interact with the predominantly negatively charged cell surface components of the target bacterium, thus increasing the permeability of its inner and outer membranes, and ultimately leading to the leakage of cellular contents inside the bacterium (Li et al., 2010). However, the amount of polycationic chitosan available to bind to a charged bacterial surface was found to be reduced as the

concentration of chitosan increased (Goy et al., 2009). Goy and colleagues (2009) argued that when a larger number of charged sites are present, the polysaccharide chains tend to form clusters by molecular aggregation while they are still in solution. They reported that at higher concentrations the chitosan tended to form a coating over the bacteria, not necessarily attached to the surface. In this respect, higher chitosan concentration may not induce better bactericidal effects if the pH is not adjusted to a suitable level. Our results suggested that pH should be adjusted to higher values in the food environment when chitosan is in solution at higher concentration, in contrast to the prediction made by Goy et al. (2009). Further research is needed to clarify this point.

3.4.2. Chitosan concentration and environmental pH on E. coli *O157 activity*

The present study also employed another indicator to measure the antimicrobial activity of chitosan, i.e. bioluminescence of *E. coli* O157. This indicator directly informs the metabolic activity of the target microorganism (Unge et al., 1999; Williams et al., 2008), which on the part of the infecting bacterium provides a prerequisite for entry into the infected cell (Hale & Bonventre, 1979). Our results showed that there was a predominant influence of solution pH rather than chitosan in the inhibition of *E. coli* O157 activity. This indicates that an acidic environment would inhibit the activity of *E. coli* O157 independent of the variation in chitosan concentration. Since few previous studies could be found to directly compare with our results, further experiments should pay greater attention to this aspect.

3.5. Conclusions

The present study has critically evaluated the co-dependence of two previous claims that have only previously been separately examined: that the bactericidal action of chitosan against *E. coli* O157 is concentration-dependent and that it is pH-dependent. In our study, there was a significant balancing relationship between the antimicrobial activity of polycationic chitosan and hydrogen ions. Chitosan played a minor role in inhibiting *E. coli* O157, but pH exerted a stronger influence. However, the pathogen was not inactivated, so on removal of chitosan and acetic conditions, growth would be re-enabled. Nevertheless, the results are as yet inconclusive due to the various experimental factors involved, such as temperature, and the chemical form of chitosan used, as well as the possible antimicrobial contribution of the chitosan solvent (i.e. acetic acid; Fujimoto et al., 2006). The food industry is increasingly confronted with fundamental challenges regarding the appropriate choice of chitosan formulations. More research is therefore required to better understand the antimicrobial process of chitosan solutions so that the food industry can optimize the use of chitosan as a biopreservative agent against important food and waterborne pathogens. This optimization should also lead to chitosan of greater effectiveness, thus making it a more viable option for industrial use.

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Abstract

Chitosan has attracted much attention in the food industry as a promising bio-preservative and antimicrobial agent. In such cases, acetic acid is used as the solvent for chitosan; however the antimicrobial properties of acetic acid may confound the actual antimicrobial contribution of chitosan. This study aimed to determine the relative antibacterial properties of chitosan and acetic acid against the food-borne pathogen *E. coli* O157. A *lux*-marked strain of *E. coli* O157 was grown in high-nutrient media amended with acetic acid (0.05 and 0.5% (v/v)) in the presence or absence of chitosan (0.05 and 0.5%). The inoculated media, adjusted to either pH 4, 5 or 6, were then incubated at either 4 or 20 °C and the growth and metabolic activity of *E. coli* O157 monitored over time. The results demonstrated that the bactericidal action against *E. coli* O157:H7 was predominantly attributable to the presence of acetic acid at a lower temperature, particularly at lower pH. At higher temperatures, chitosan augmented the biocidal effect of acetic acid, especially at higher pH. This study is the first to suggest that chitosan and acetic acid play different roles in the antimicrobial process, with chitosan tending to be more inhibitory whereas acetic acid is more bactericidal. Further research is needed to establish a strong evidence–base for the antagonistic effect of chitosan solution against *E. coli* O157.

Keywords*:* Acetic acid, food poisoning, food safety, gram negative, in vitro VTEC.

4.1. Introduction

Verocytotoxin-producing *Escherichia coli* (VTEC) are one of the most important causes of diarrheal episodes globally (Taylor et al., 2004). Although the number of VTEC infection has decreased in recent years, the pathogen remains a significant food safety issue. A subgroup of *E. coli*, serotype O157, which was first identified as a human pathogen after the 1982 outbreak of hemorrhagic colitis in Oregon and Michigan (Riley et al., 1983), has since been implicated in many cases of food contamination (Karmali et al., 2010; Tauxe, 1997). More than 90% of VTEC strains isolated in North America and the UK belong to serotype O157 (Chapman, 1995).

As the numbers of food-borne human *E. coli* O157 infections have increased, considerable efforts have been made along the food chain to reduce the risk of food contamination. One promising avenue is the addition of biocidal agents to food packaging, with particular interest in the use of natural antimicrobial agents (Raybaudi–Massilia et al., 2009). Chitosan is a versatile nontoxic, biodegradable polymer, and has shown promising properties as a natural bactericide (Chung & Chen, 2008) as it is considered safe for human consumption (FDA, 2001). Chitosan-based films and coatings are particularly effective in food preservation (Dutta et al., 2009); for instance, chitosan coatings effectively extended the shelf–life of peeled litchi fruit by inhibiting the increase in activities of polyphenol oxidase and peroxidase (Dong et al., 2004) and also the shelf–life of fresh orange juice by reducing enzymatic and non–enzymatic browning (Martin-Diana et al., 2009).

The numerous positively charged C2 position amino groups in chitosan are known to readily associate with the negatively charged surface of bacteria and this has been suggested to

be a major component of its mode of action (Holappa et al., 2006). Through binding and disrupting the normal functions of cell membranes, chitosan promotes the leakage of intracellular components and inhibits the transport of nutrients into the cells (Chung & Chen, 2008). The antimicrobial effect of chitosan is likely to depend on several factors such as the target microorganism (Gram-positive or Gram-negative), the kind of chitosan (deacetylation degree and molecular weight [MW]), the pH of the medium, and temperature (Devlighere et al., 2004; Fernandes et al., 2008).

The pKa value for chitosan is approximately 6.3 and it therefore becomes readily soluble in an acidic environment (<pH 6; Rhoades and Roller, 2000). As chitosan cannot readily dissolve in water, organic acids such as acetic and lactic acid are therefore used to facilitate its solubility and activate positively charged amino functional groups (No el al., 2002; Rabea et al., 2003). However, organic acids have been traditionally used as antimicrobials in the food industry (Raybaudi-Massilia et al., 2009). The presence of acetic acid in chitosan solutions may represent a crucial confounding factor in experiments investigating the antimicrobial activity of chitosan; although this has seemingly received little attention (Fujimoto et al., 2006). Furthermore, the co–influence of the organic acids may be affected by temperature which represents a key regulator of pathogen survival (Conner and Kotrola, 1995; Rocelle et al., 1996).

Apart from the commonly measured cellular viability of a certain pathogen, another important, yet largely neglected, value that provides information about the metabolic activity of the pathogen will be considered in the current experiment. The metabolic activity level represents an important prerequisite for host infection (Hale & Bonventre, 1979). Generally, the higher the metabolic activity of a bacterium, the more harm it will cause to the host.

The primary aim of this study was therefore to critically assess the relative biocidal effect of acetic acid and chitosan towards *E. coli* O157. The secondary aim was to determine the relative effect of acetic acid and chitosan on the growth and activity of the pathogen at a range of pH values and temperatures representative of those typical in food storage environments.

4.2. Materials and methods

4.2.1. Preparation of chitosan solutions

A low molecular weight form of chitosan (50 kDa based on viscosity, 75-85% deacetylated chitin, poly [D-glucosamine], viscosity 20-200 cps) was sourced from Sigma-Aldrich (448869-250G, Aldrich). A stock solution of chitosan was prepared in 2.5% (v/v) acetic acid (from acetic acid glacial 99.7+%, A/0400/PB08, Fisher Scientific).

4.2.2. Preparation of E. coli *O157 inoculum*

A strain of *E. coli* O157 (strain 3704 Tn5 *luxCDABE*; Ritchie et al., 2003) was prepared from a fresh overnight LB broth culture (Difco Ltd., Teddington, Surrey, UK; 37 °C, 18 h, 150 rev min^{-1}; Williams et al., 2008b). The strain has been proven to be non–toxigenic due to the absence of toxin activity and toxin genes; however it still accurately reflects the survival pattern of a toxigenic strain (Ritchie et al., 2003). The *lux*-marked *E. coli* O157 strain used also reports metabolic activity in the form of bioluminescence values. Cells were washed three times in ¼–strength Ringer's solution and concentrated by centrifugation as described in Avery et al. (2005). The concentration of *E. coli* O157 in the inoculum was determined by plating out in duplicate onto cefixime-tellurite-sorbitol-MacConkey agar (CT-SMAC; Oxoid Ltd., Basingstoke, UK) and enumeration of colonies with the characteristic appearance of *E.*

coli O157 following incubation (18 h, 37 °C). Non-sorbitol-fermenting colonies were confirmed as *E. coli* O157 via latex agglutination (Oxoid, DR620).

4.2.3. Antibacterial testing

LB broth was selected as the testing media as it reflects a high-nutrient enrichment environment typical of that in meat juices in which *E. coli* O157 may multiply (i.e. peptide, amino acid and vitamin rich; Williams et al., 2008a). The chitosan–acetic acid stock solution was mixed with the LB broth to yield final chitosan concentrations of 0.05% and 0.5%. Similarly, acetic acid was added to the LB broth but in the absence of chitosan (i.e. final concentrations of 0.05% and 0.5%). For the control treatments, 100 ml LB broth was adjusted to pH 4, 5, and 6 using either 1 M HCl or 1 M NaOH. All solutions (treatments and controls) were subsequently inoculated with approximately 7 $\log_{10}$ CFU ml^{-1} of *E. coli* O157, and then incubated at either 4 or 20 °C. These two temperatures were chosen to reflect the reality when meat is generally stored at 4 °C, and brought to room temperature at 20 °C after purchase. All treatments were performed in triplicate.

The pH values of the media were checked halfway through and at the end of the trial and were found to be stable in all treatments throughout. Samples for microbiological assessment were taken in triplicate at 0 h (immediately after inoculation), 2, 6, 12, 24, 48 and 72 h post-inoculation. Viable counts of *E. coli* O157 expressed as CFU ml^{-1} were determined by the Drop Count Method. For this procedure, aliquots (100 µl) were pipetted into polypropylene tubes containing 900 µl Ringer's solution. Then 3 x 10 µl drops were added in SMAC and colonies grown at 37 °C for 18 h. Luminescence of *E. coli* O157 was measured at the same time points. At each instance, a 1 ml aliquot was placed in triplicate into a plastic

luminometer cuvette and its luminescence (relative light units [RLU]) determined using a Pi-102 Luminometer (Hygiena International Ltd., Watford, UK).

4.2.4. Statistical analysis

Data were analyzed with SPSS 16.0 (SPSS Inc., Chicago, IL). All plate count and luminescence data for *E. coli* O157 were $\log_{10}(y + 1)$ transformed prior to analysis to meet the assumptions of ANOVA. A multivariate model was used to analyze the effects of concentration, temperature, pH, and time (fixed factors) on transformed luminescence and cell count data. Post–hoc tests using Tukey HSD at $p < 0.05$ were adopted to identify significant differences between treatments.

4.3. Results

4.3.1. Effect of chitosan and acetic acid on E. coli *O157 cell counts*

There was evidence for the general claim that the inhibitory effect of chitosan and acetic acid was concentration-dependent, with higher concentrations having stronger antimicrobial activity. However, more complex relationships were found when taking into account both environmental factors such as temperature and pH.

At 4 °C, effects of the acetic acid ± chitosan treatments at pH 4 and pH 5 were very similar over the 72 h experimental period, with only acetic acid at a concentration of 0.5% causing a substantial reduction of a 2.8 log value in cell counts from 48 to 72 h (Fig. 4.1). Overall, there was no additional effect of chitosan over those samples containing just acetic acid alone at either concentration. The control maintained similar numbers of viable cell counts at pH 4 and pH 5 comparing the beginning and end points of the incubation. However, the pH 6 medium environment could not differentiate between the control and the two acetic

acid–only groups. The acetic acid-only groups were less effective compared to those in the presence of chitosan at pH 6 ($p < 0.01$).

The patterns of *E. coli* O157 numbers obtained at 20 °C were generally different from those incubated at 4 °C with an overall drop in *E. coli* O157 numbers observed in all treatments relative to the control after 72 h (Fig. 4.1). Viable cell counts in the control increased roughly 2 log cell counts in all the three pH conditions. The 0.5% acetic acid–only condition showed the strongest bactericidal effect relative to chitosan, reducing *E. coli* O157 numbers 3 log at pH 4 and a very small log value at pH 5. At pH 6, however, the presence of chitosan at the concentration of 0.5% slightly enhanced the bactericidal effect of acetic acid at the concentration of 0.5% ($p < 0.05$). When acetic acid concentration is low, only the low pH value of 4 caused a reduction of cell counts in both acetic acid ± chitosan treatments, whereas the other two pH conditions increased cell counts.

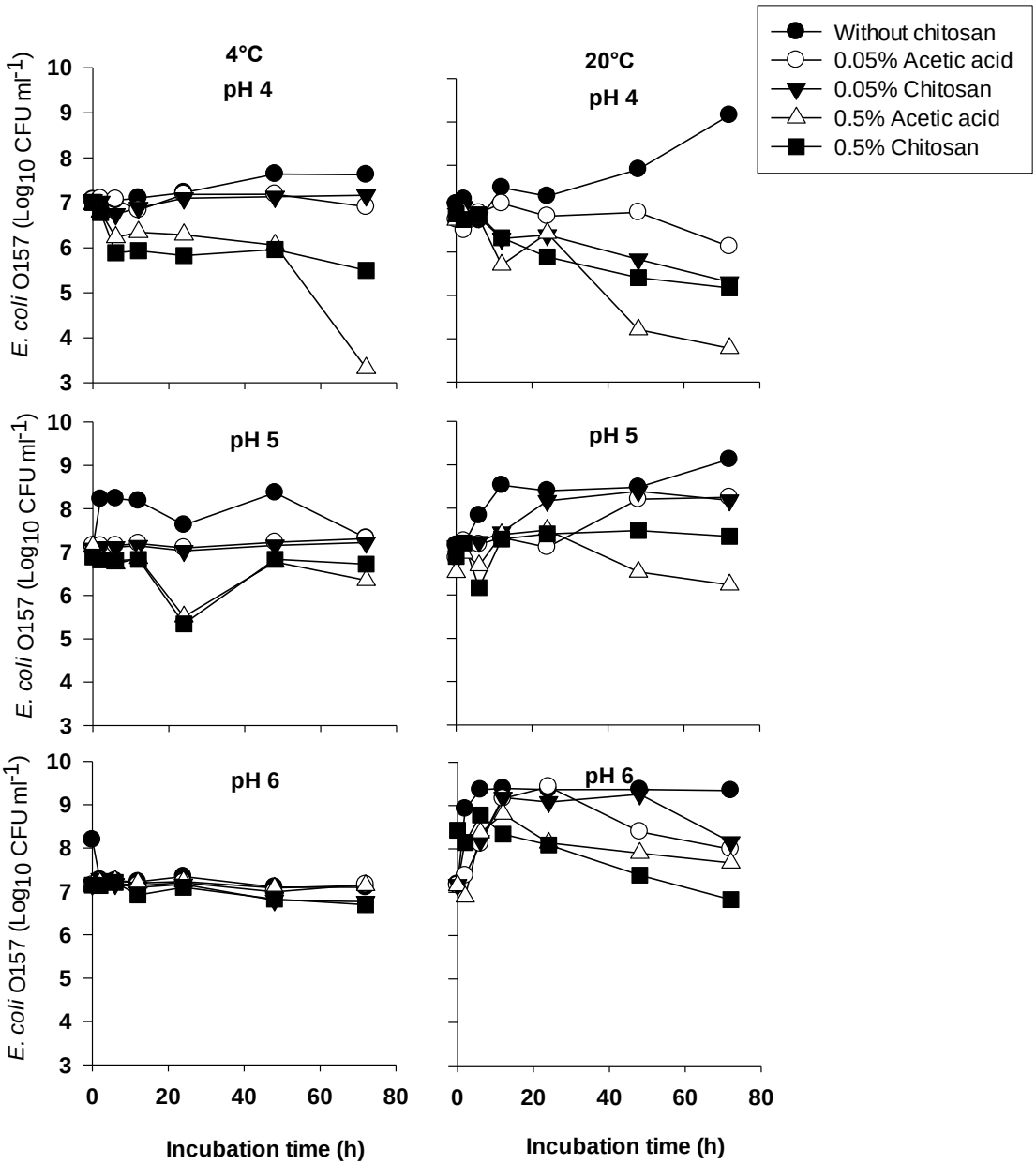

Figure 4.1 Viable cell count of *E. coli* O157 in enrichment media held at 4 or 20°C, across pH 4, 5 and 6, in the presence of acetic acid (0.05 and 0.5%) or chitosan (0.05% and 0.5%). Error bars represent SEM.

4.3.2. Effects of chitosan and acetic acid on E. coli *O157 luminescence*

At 4 °C, the metabolic activity of *E. coli* O157 decreased throughout the whole incubation period independent of pH variations (Fig. 4.2). Overall, the inhibitory influence of acetic acid tends to fluctuate in a similar pattern as its corresponding chitosan solution over the 72 h incubation period (Fig. 4.2). Lower concentrations of acetic acid and chitosan tend to

reduce metabolic activity of the bacterium less than higher concentrations: significantly less at pH 5 ($p < 0.05$). However, lower concentrations of treatments tend to produce a slowly reducing pattern, whereas higher concentrations of treatments tend to have very quick initial inhibitory effects but level off afterwards. In addition, at pH 4 and 5, the activity levels of *E coli.* O157 were immediately much lower at the beginning of the incubation, compared with the pH 6 conditions.

At 20 °C, greater inhibitory effects of chitosan and acetic acid on the metabolic activity of *E. coli* O157 were seen at higher acetic acid and chitosan concentrations (Fig. 4.2). At pH 4, the response of *E. coli* O157 to acetic acid was similar whether or not chitosan was included in the media. The activity of *E. coli* O157 increased in the control at the end of the incubation, differing from the other two pH conditions where the bacterial activity was decreasing in the incubating process. At pH 5, acetic acid at both concentrations showed more pronounced inhibitory effect than its corresponding chitosan solution ($p < 0.05$). At pH 6, chitosan initially increased the metabolic activity of the pathogen relative to the acetic acid only treatment; however, these effects became less apparent over time. It should be noted that at pH 6 in both the low acetic acid and chitosan treatments their presence induced a significant increase in luminescence relative to the untreated control.

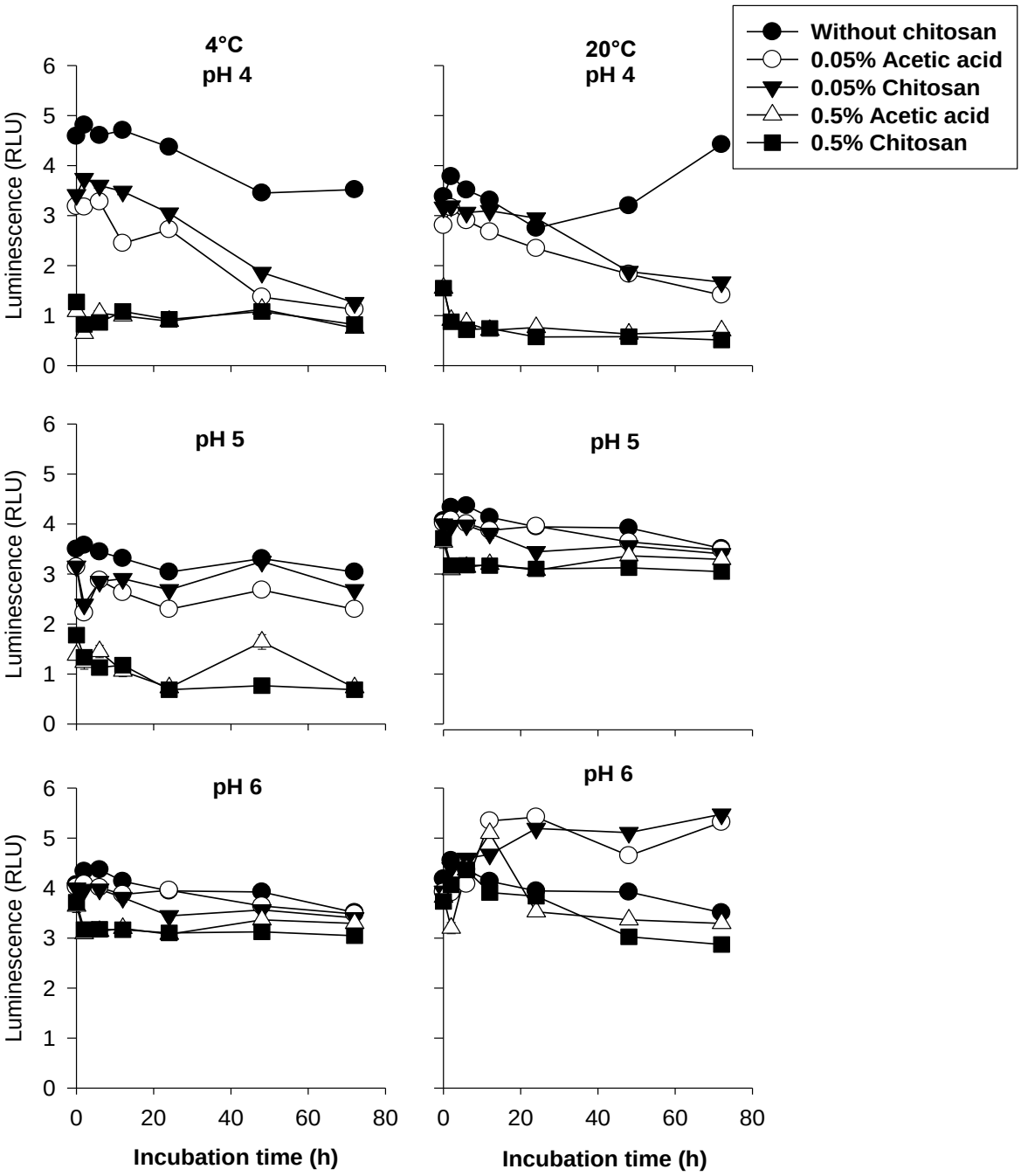

Figure 4.2 Luminescence (activity) of *E. coli* O157 in enrichment media held at 4 or 20°C, across pH 4, 5 and 6, in the presence of acetic acid (0.05 and 0.5%) or chitosan (0.05% and 0.5%). Error bars represent SEM.

4.3.3. Effects of chitosan and acetic acid on the specific activity of E. coli *O157*

At 4 °C, luminescence values expressed on a 'per cell' basis in all treatments were found to overlap over the first 48 h when incubated at pH 4, at a level close to zero, whereas for the other two pH conditions, the graph pattern of each treatment tends to be inconsistent (Fig. 4.3). The initial specific activity of the bacterium in the control differs greatly from the treatment conditions at pH 4 and pH 5 ($p < 0.05$). At pH 5, the activity per cell in the 0.5% acetic acid solutions ± chitosan were similar, whereas in the 0.05% treatment the activity was overall higher. At pH 6, the activity per cell in the 0.5% chitosan treatment overlapped with that of the 0.5% acetic acid only treatment, whereas the specific activity in both the 0.05% acetic acid treatments showed no consistent pattern. At 20 °C, the specific activity of *E. coli* O157 showed no recognizable consistent pattern in any of the chitosan and acetic acid treatments.

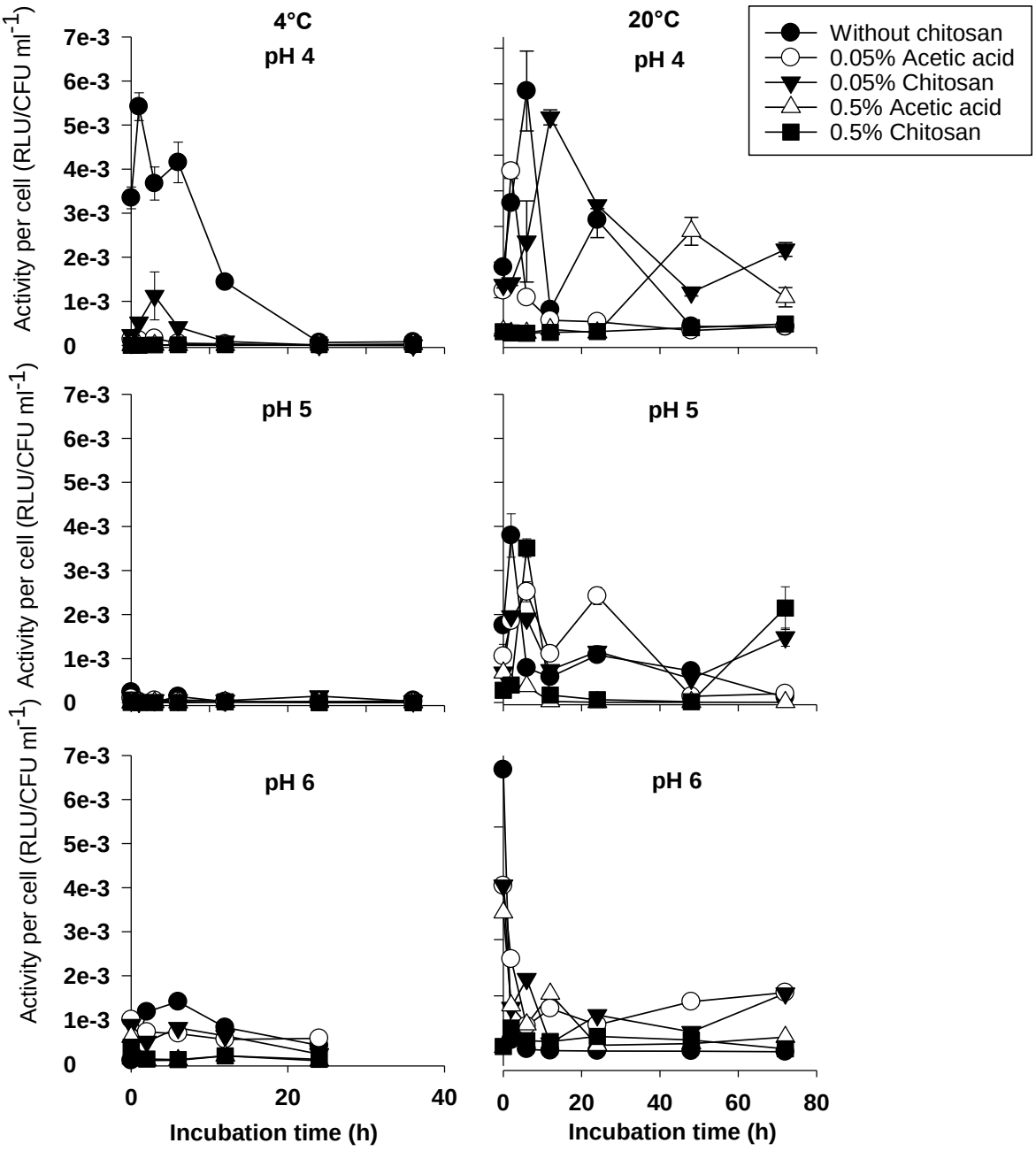

Figure 4.3 Luminescence per cell of *E. coli* O157 in enrichment media held at 4 or 20°C, across pH 4, 5 and 6, in the presence of acetic acid (0.05 and 0.5%) or chitosan (0.05% and 0.5%). Error bars represent SEM.

4.4. Discussion

Chitosan has been investigated as an antimicrobial agent against a wide spectrum of food spoilage organisms (e.g. bacteria, yeast and filamentous fungi) in both in vivo and in vitro experiments (Goy et al., 2009). Such trials have used chitosan in a range of chemical forms and in a wide range of media leading to a range of bactericidal responses (Dutta et al., 2009). Understanding this variability in pathogen response is vital for designing and implementing effective and persistent treatment strategies. It is generally assumed that the positively charged amino groups in the C2 position of chitosan acetate interact with the negatively charged cell surface of bacteria, thus increasing the permeability of the inner and outer membranes of bacterium, and ultimately leading to the leakage of cellular contents (Li et al., 2010). Despite this explanation, most previous research, with the exception of Fujimoto et al. (2006), have failed to exclude the confounding effect of the simultaneously added acetic acid solvent which is also known to increase membrane permeability and induce cell death (via acid stress and through membrane depolarization; Birk et al., 2010; Raybaudi-Massilia et al., 2009).

Reducing the prevalence of *E. coli* O157 in meat is considered paramount in ensuring food safety (Sofos, 2008) and application of chitosan and its derivatives to meat packaging or carcass washwater preservation has been considered as a promising strategy. Since meat is usually stored at low temperatures, but can often remain at room temperature after consumer purchase, our study considered the effectiveness of chitosan at both 4 °C and 20 °C. Our study demonstrated that irrespective of pH, the inhibitory effects of chitosan on *E. coli* O157 numbers at 4 °C were equivalent to the acetic acid solution containing no chitosan over 72 h.

This implied that the bactericidal action against *E. coli* O157 was predominantly attributed to the presence of acetic acid. Our result, however, differs from Fujimoto et al. (2006) who found the antimicrobial action increased with decreasing pH value. Nevertheless, the divergence may be due to the difference in experimental factors between the two studies and particularly their use of 37 °C and lower pH values. At higher temperatures (20 °C), our results showed that the relative bactericidal strengths of chitosan and acetic acid was more pH-dependent, with low pH facilitating the antimicrobial action of acetic acid and neutral pH counteracting this effect. This is presumably due to dissociation of the acetic acid to non-toxic acetate ions which would not interact strongly with cell membranes. It was only under these conditions that chitosan showed any significant impact on *E. coli* O157. As the juice in meat packaging is typically weakly acidic (pH 6.35; Williams et al., 2008a) chitosan has greater potential than acetic acid. Chitosan may also be more preferable to consumers than acetic acid as it has less discernible taste (Harris et al., 2006).

To our knowledge, this study is the first to quantify the effects of the presence of chitosan on cell metabolic activity. Luminescence directly reports on bacterial metabolic activity which represents a prerequisite for host infection (Hale and Bonventre, 1979; Unge et al., 1999; Williams et al., 2008b). Our luminescence measurements show that, in general, chitosan provided little added benefit to that of acetic acid in reducing the metabolic activity of *E. coli* O157, irrespective of pH and temperature. In addition, in some instances chitosan enhanced bacterial metabolic activity in solution suggesting that the chitosan was either negating the impact of acetic acid, acting as a microbial substrate or interacting positively with some other solution component. These results suggest that higher chitosan concentrations may be required to instigate antimicrobial effects; however, this may be impractical on both

chemical (poor solubility) and economic grounds. Future development of other chitosan types may yield those with enhanced antimicrobial properties.

4.5. Conclusions

The present study differentiates between the relative contribution of antimicrobial action of chitosan and acetic acid. It demonstrated that the bactericidal action against *E. coli* O157 was predominantly attributed to the presence of acetic acid, particularly at low temperatures and moderate pH values. However, at a higher temperature, chitosan solution augmented its bactericidal influence on *E. coli* O157, particularly when a high pH environment compromised the effect of the acid solvent. As many previous studies have failed to include the proper control treatments with acetic acid alone, the results from such studies needed to be treated with caution. It could be that (1) their observed antimicrobial effects are due to acetic acid alone, (2) acetic acid is modifying the activity of chitosan in a concentration–dependent manner (e.g. in dose response experiments), or (3) acetic acid is confounding the pH effect of chitosan (e.g. in pH experiments). Further research is therefore required to establish a stronger base for the antimicrobial process of chitosan solution against *E. coli* O157 and for promoting its use as an antimicrobial agent for reducing food-borne illness.

4.6. Acknowledgements

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Abstract

The antimicrobial action of chitosan and functionalized chitosan derivatives is well known; however, limited research has been undertaken on its use in meat environments. In this study the antibacterial activity of a newly developed arginine–functionalized chitosan was tested against pathogenic *Escherichia coli* O157 in chicken juice. The chicken juice was representative of the liquid which accumulates in food packaging and which is frequently implicated in food poisoning incidents. Aliquots of chicken juice (50 ml) were inoculated with a *lux*–marked strain of *E. coli* O157 to approximately 8.75 $\log_{10}$ CFU ml^{-1}. Samples were subsequently mixed with chitosan–arginine of varying concentrations (0–500 mg l^{-1}) and incubated at 4 or 20 °C to mimic refrigeration and room temperatures respectively. Pathogen persistence and metabolic activity (indexed by bioluminescence measurements) were subsequently quantified in the measurements showed that the metabolic activity was subsequently quantified in the liquor at 0 (immediately after mixing), 3, 12, 24, 48, and 72 h post-ncubation. The presence of chitosan-arginine significantly reduced both the numbers and metabolic activity of the pathogen in a dose–dependent manner with greater inhibition seen at higher concentration. In addition, it also suppressed the growth of general food spoilage bacterial, reduced malodor and prevented pathogen re-growth up to 72 h. The results imply that incorporating water soluble chitosan-arginine into packaging may help maintain both product shelf life and freshness as well as minimize the risk of food poisoning in both retail outlets and domestic homes.

Keywords: Antibacterial; Cross contamination; Meat hygiene, Polysaccharide, VTEC, Zoonoses.

5.1. Introduction

With the increase in both intensive poultry production systems and the global demand for poultry meat, the importance of poultry meat hygiene and safety is increasing emphasized (Sofos & Geornaras, 2010). One potentially lethal human pathogen found in chicken is verocytotoxin–producing *Escherichia coli* (VTEC), which constitutes one of the most severe causes of food-borne infections on a global scale, particularly in Europe and the USA (Mead, 2004). *E. coli* O157 was found in 1.5% of poultry carcasses from Wisconsin, USA (Doyle & Schoeni, 1987), and in 9.3% of cloacal swabs taken from poultry at a slaughterhouse in Slovakia (Pilipcinec, Tkacikova, Naas, Cabadaj, & Mikula, 1999). In relation to the subsequent risk for human infection, *E. coli* O157:H7 cells are known to survive well in chicken breasts kept at −20 °C for more than 18 months (Conner & Hall, 1994; Watabe et al., 2008). In addition, *E. coli* O157:H7 has also been isolated from the ceca of chickens from which cross–contamination of the carcass may occur during processing (Doyle & Schoeni, 1987). As a consequence, both pre–packed raw chicken and ready-to-eat foods containing chicken can pose a threat to consumer health, as evidenced by a recent outbreak of VTEC O157 infection associated with processed chicken wraps (Whittaker et al., 2009). Juice seepage from raw, packaged chicken is particularly prone to causing cross–contamination of foods and surfaces in consumers' homes and has led to infection with a range of pathogens (Harrison, Griffith, Tennant, & Peters, 2001).

Various intervention strategies are currently employed to ensure food safety, and health–conscious consumers are increasingly choosing products that contain natural preservatives in place of potentially harmful synthetic additives. Chitosan and its derivatives, which are biodegradable, biocompatible, non-toxic, and bactericidal, are considered to

represent a promising agent for packaging materials and bio–preservatives to maintain food freshness and safety (Kong, Chen, Xing, & Park, 2010). Normally, chitosan is insoluble in aqueous solutions above pH 7, limiting its application (No, Meyers, Prinyawiwatkul, & Xu, 2007). However, the recent synthesis of chitosan derivatives, including acetylthiourea derivatives of chitosan, N–trimethyl chitosan, N–diethyl methyl chitosan and chitosan–N–2–hydroxypropyl trimethyl ammonium chloride, as well as Schiff–base and sulphanilamide derivatives of chitosan, have overcome this solubility problem in addition to enhancing its bactericidal properties (Xiao, Wan, Zhao, Liu, & Zhang, 2011; Zhong et al., 2008).

The antimicrobial activity of chitosan and chitosan derivatives against both Gram–positive and –negative bacteria is well established (Tang et al., 2010). Generally, the cytotoxic effect of chitosan and chitosan derivatives is dependent upon: (1) microbial factors, associated with microorganism type and growth stage; (2) inherent properties of chitosan, including its positive charge density, molecular weight, solution concentration, hydrophilic/hydrophobic characteristic and chelating capacity; (3) their physical properties, e.g. water–solubility; (4) environmental factors, such as medium pH, temperature and ionic strength (Kong et al., 2010). Alongside cost, the varied combination of these different factors within published studies has hampered the adoption of unified guidelines for its use in pathogen control within the food industry.

A novel water soluble chitosan derivative, arginine–functionalized chitosan, is obtained by modification with arginine groups to form stable peptide bonds between the arginine carboxylic acid and the amine on the glucosamine and reaches varying degrees of substitution (Fig. 5.1). With a high pKa of 12.48, the guanidinium side chain of arginine is positively charged in neutral pH environments. The biocidal effect of this newly–developed

chitosan derivative, however, is not well understood, and its application to meat environments under different storage temperatures remains unknown. The aim of this study was to quantify the dose-dependent antibacterial activity of chitosan-arginine in poultry juice contaminated with *E. coli* O157 and general food spoilage bacteria under simulated refrigeration and room temperature storage conditions.

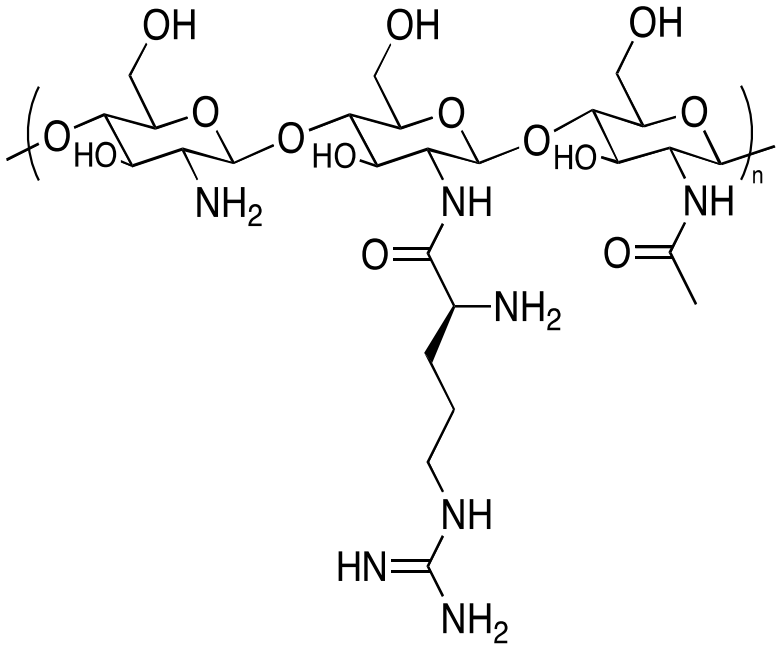

Figure 5.1. Schematic representation of the chitosan–arginine derivative used in this study.

5.2. Materials and methods

5.2.1. Preparation of chitosan–arginine solution

Chitosan–arginine (85% deacetylated with arginine constituting 25% of the total monomers on the polymer backbone; 41 kDa, purity > 99%) was synthesized by Synedgen, Inc. (Claremont, CA, USA). A chitosan–arginine stock solution (1 g l^{-1}) was made in distilled

water and the solution sterilized by passage through a 0.2 μm syringe filter for storage and later use.

5.2.2. Preparation of E. coli *O157inoculum*

A strain of *E. coli* O157 (3704 Tn5 LuxCDABE; Ritchie et al., 2003) was prepared from a fresh overnight LB broth (Difco Ltd., Teddington, Surrey, UK; 37 °C, 18 h, shaken at 150 rev min−1) (Williams, McGregor, Killham, & Jones, 2008). A 1 ml aliquot of the strain was allocated to 100 ml LB broth (Difco Ltd.; 37 °C, 12 h, 150 rev min−1). Cells were washed three times in ¼–strength Ringer's solution and concentrated by centrifugation as described in Avery, Killham, and Jones (2005). The strain has been proven to be non–toxigenic due to the absence of toxin activity, however it still accurately reflects survival patterns of toxigenic strains (Ritchie et al., 2003).

5.2.3. Preparation of chicken juice

A total of six processed intact raw chickens (i.e. de–feathered and eviscerated), were purchased from a commercial supermarket in Bangor, North Wales. Each chicken was placed in a sterile stomacher bag and repeatedly washed with sterile distilled water to obtain a final wash solution volume of 600 ml per chicken. Chicken juice was selected as the testing media because it reflects a high nutrient enrichment environment typical of that in food packaging in which *E. coli* O157 multiplies. It also represents one of the major risk pathways for surface contamination and subsequent cross–contamination in the home. Changes in the chemistry of the chicken juice ± chitosan (500 μg ml^{-1}) were also monitored at both 4 and 20 °C. Samples were chemically characterized in terms of their pH (pH–209 meter; Hanna Instruments Inc., Woonsocket, RI), electrical conductivity (CDM210 meter; Jenway Ltd., Dunmow, UK) and

total organic C and N (TOC–VN analyser; Shimadzu Corp., Kyoto, Japan) at two time points, namely, 0 (immediately after addition) and 72 h post–incubation.

5.2.4. Antibacterial testing

5.2.4.1. Enumeration of E. coli O157*counts and metabolic activity*

Chicken juice (30 ml) was aliquoted into 21 × 50 ml sterilized polypropylene tubes. Overnight cultures of the lux–marked *E. coli* O157 (grown to log phase) were then inoculated into the chicken juice to approximately 8.75 log10 CFU ml^{-1}. Chitosan–arginine was then added to the tubes to obtain a range of final concentrations of 100, 200, 400, 500 μg ml^{-1} and incubated at 4 or 20 °C. Samples were taken for microbial enumeration at 0 (immediately after incubation), 3, 12, 24, 48, 72 h post–incubation and numbers of *E. coli* O157 determined by the drop–plate method. Briefly, 0.1 ml of the samples was spread onto three CT–SMAC plates (Oxoid Ltd., Basingstoke, UK), which were then incubated at 37 °C for 48 h. Presumptive *E. coli* O157 colonies (non sorbitol–fermenting) were confirmed by agglutination with a latex test kit (Oxoid DR0620). Luminescence of *E. coli* O157 was measured at the same sampling points using a Tecan Infinite 200® PRO luminometer (Tecan Austria GmbH, Grödig, Austria) and results displayed in RLU (relative light units). All treatments were preformed in triplicate.

5.2.4.2. Enumeration of coliforms and total viable counts

Total viable counts (TVC) (indicator of a food product's general microbiological load and shelf life determinant; Forsythe, 2000) and coliforms in control chicken juice (not inoculated with *E. coli* O157) ± 500 μg ml^{-1}chitosan–arginine were enumerated in triplicate. Samples (0.1 ml) were diluted 10–fold and 0.1 ml was subsequently spread onto plate count agar (Oxoid, CM813) and incubated at 37 °C for 48 h. Another 0.1 ml of each sample was

diluted in a similar way and spread onto Oxoid Brilliance™ *E. coli*/coliform selective agar (Oxoid CM1046)

5.2.5. Statistical analyses

Data were analyzed using SPSS Statistics (IBM version 16.0 for Windows). All plate count data for *E. coli* O157 were log10 (x + 1) transformed prior to analysis to meet the assumptions of ANOVA. Multivariate analyses were used to analyze the effects of chitosan–arginine concentration on luminescence and transformed cell counts, whereas t–tests were used to analyze the effect of environmental temperature on these two dependent variables. Post-hoc tests using Tukey HSD at $P < 0.05$ were adopted to identify significant differences between each treatment condition. Student t–tests were performed with 2–tailed significance to detect the effects of temperature (4 versus 20 °C) and chitosan–arginine on TVC and coliform numbers.

5.3. Results

5.3.1. Changes in chicken juice chemistry during storage

The chemical characteristics of the chicken juice samples ± chitosan–arginine are shown in Table 5. 1. Overall, sample pH values did not change considerably over the 72 h period post–incubation, although the chicken juice treated with chitosan–arginine exhibited a significantly higher pH ($P < 0.05$). Electrical conductivity remained relatively stable over the incubation period at both temperatures, although there was a reduction in samples held at 20 °C in the presence of chitosan–arginine. Total organic C (TOC) and nitrogen (TON) remained relatively constant over time within samples; with levels of both being greater in chicken juice

amended with chitosan–arginine, though not significantly so ($P > 0.05$). There was, however a temperature effect, where levels were significantly greater in all samples when kept at 20 °C.

Table 5.1. Chemical characterization of chicken juice and chicken juice amended with 500 μg ml^{-1}chitosan-arginine (Ch-arg) and held at two different temperatures. Time refers to hours post–addition with the Ch–arg (0 h refers to immediately after additions). Values represent means ± SEM (n = 3). EC = electrical conductivity.

	Chicken juice		Chicken juice + chitosan–arginine	
	0 h	72 h	0 h	72 h
pH	6.42 ± 0.01	6.39 ± 0.03	6.76 ± 0.01	6.74 ± 0.02
Electrical conductivity (mS cm^{-1})	1.56 ± 0.01	1.67 ± 0.01	0.77 ± 0.01	0.88 ± 0.01
Total organic C (mg C l^{-1})	6.86 ± 0.08	7.19 ± 0.02	4.22 ± 0.05	4.45 ± 0.10
Total organic N (mg N l^{-1})	3.99 ± 0.19	4.08 ± 0.05	1.80 ± 0.13	1.98 ± 0.02

5.3.2. Effects of chitosan–arginine on E. coli *O157cell counts*

The antimicrobial action of chitosan–arginine against *E. coli* O157 in chicken juice is shown in Fig. 5.2. At both 4 and 20 °C, post–hoc LSD pairwise comparisons showed that chicken juice amended with chitosan–arginine caused significant reductions in pathogen cell counts, compared with the control treatment ($P < 0.001$). This antimicrobial effect appeared to be very rapid, as evidenced by the dramatic reduction in *E. coli* cell counts within 3 h. Over the subsequent 72 h period, cell counts continued to progressively decline. The bactericidal effect of chitosan–arginine was clearly concentration–dependent, with higher concentrations (400–500 μg ml^{-1}) causing significantly greater cell count reductions than at lower concentrations (100–200 μg ml^{-1}). Overall, the addition of chitosan–arginine at higher concentrations caused a 4.25 log count reductions at 4 °C and a 7 log count reduction at 20 °C after 72 h.

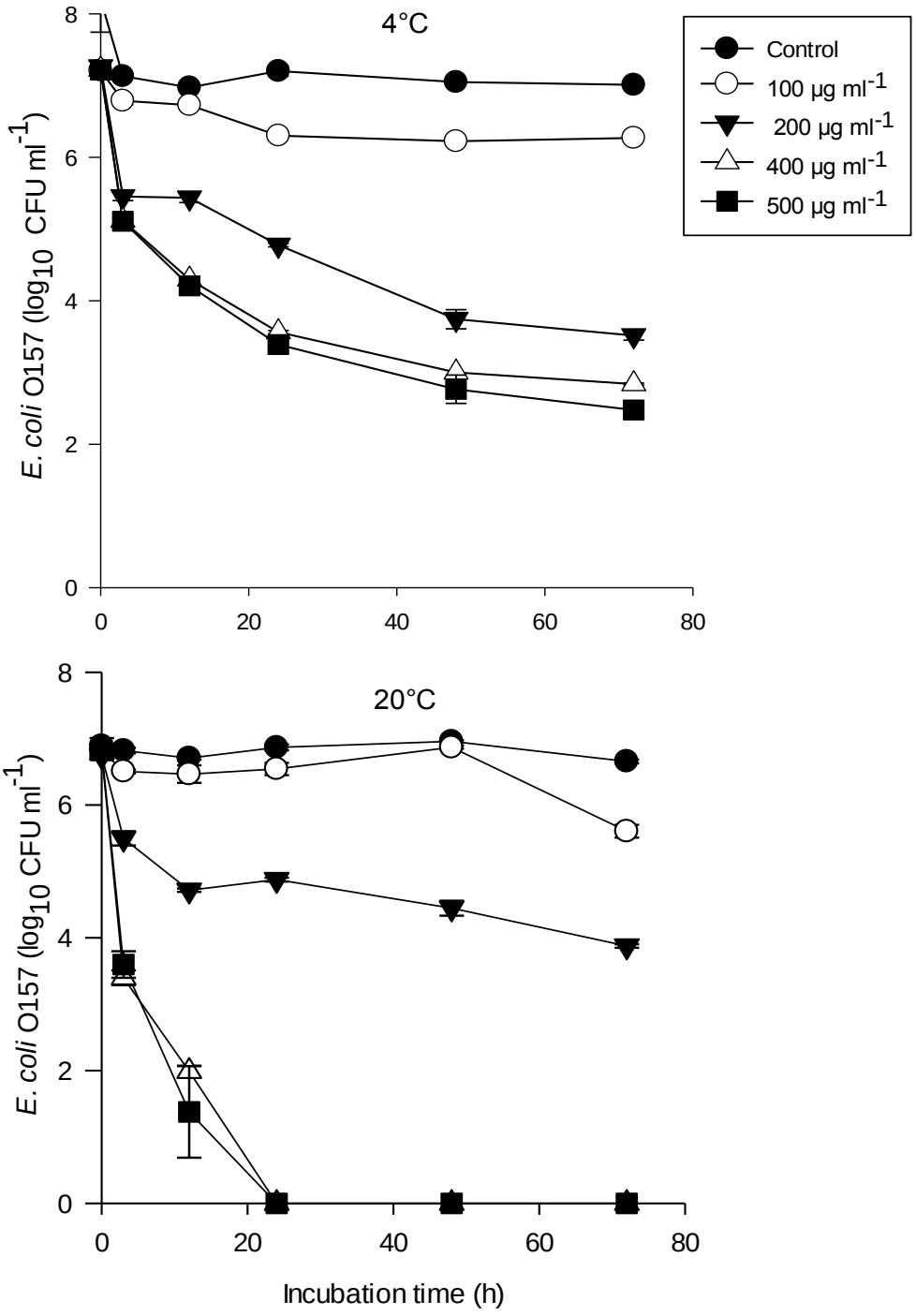

Figure 5.2. Dose–dependent effect of chitosan-arginine (0–500 µg ml^{-1}) on *E. coli* O157 survival in chicken juice samples stored at 4 or 20 °C. Values represent means ± SEM (n = 3).

In contrast, chitosan–arginine added at the lowest concentration (100 μg ml^{-1}) only reduced numbers by only 0.5 log cell count at 4 °C, and 1.5 log cell counts at 20 °C. Statistical analysis revealed that temperature was a significant factor regulating chitosan-arginine's antimicrobial effects (P = 0.003, 2-tailed t–test), with higher temperatures leading to a stronger antimicrobial action.

5.3.3. Effects of chitosan–arginine on E. coli *O157cell activity*

Statistical analysis indicated that chitosan–arginine had a significant inhibitory effect against *E. coli* O157 activity as indexed by the reduction in luminescence (Fig. 5.3). At 4 °C, *E. coli* cell activity was immediately reduced in all treatments within 3 h post–incubation, however, the addition of chitosan-arginine caused a significantly greater reduction ($p < 0.001$), although its action was relatively independent of chitosan–arginine concentration. At 20 °C, *E. coli* O157 activity initially increased in the control and 100 μg ml^{-1} chitosan-arginine treatments, however, after 12 h bacterial cell activity progressively declined. For the other treatment groups with chitosan–arginine concentrations ranging from 200–500 μg ml^{-1}, bacterial luminescence dropped close to zero within 3 h post–incubation. Statistical analysis again indicated that temperature was a significant regulator of the inhibitory action ($p < 0.001$), with higher temperatures increasing chitosan–arginine's inhibitory effect.

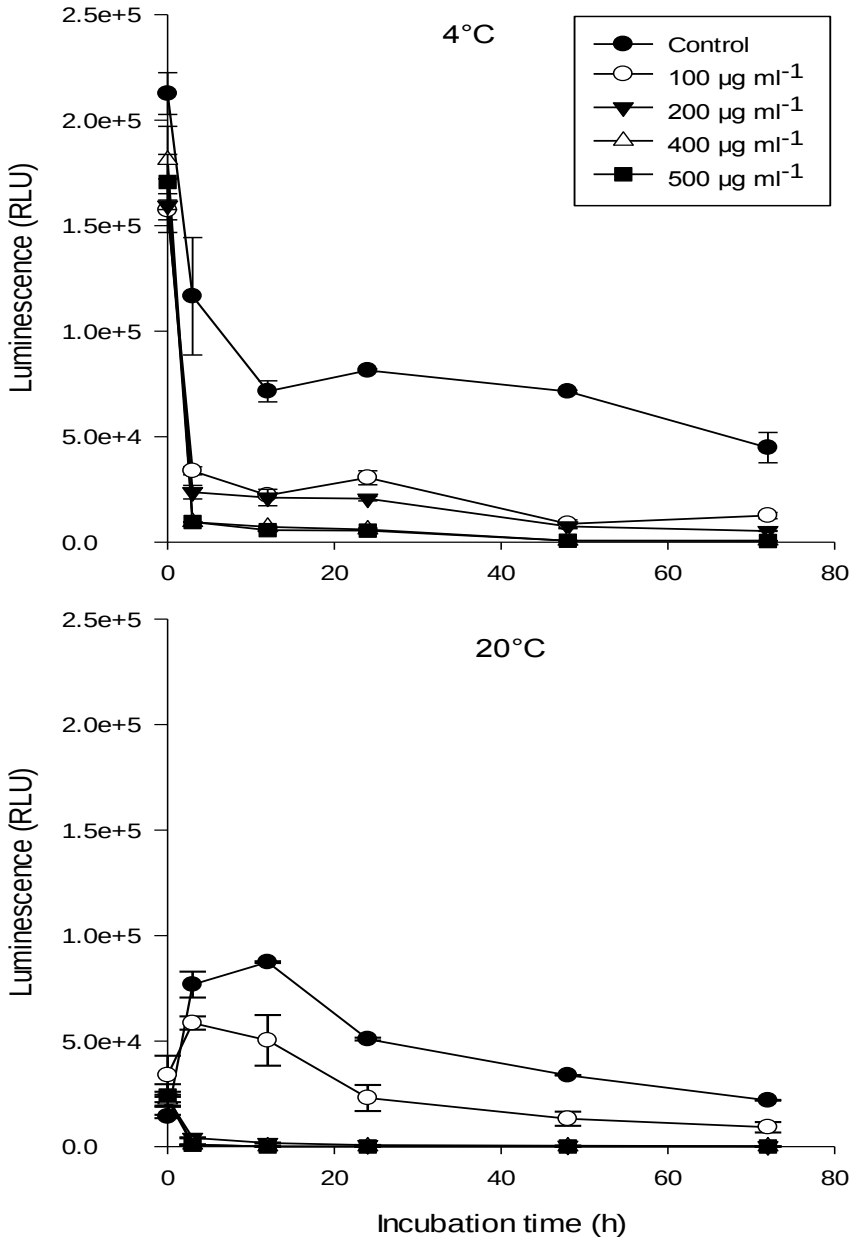

Figure 5.3. Dose-dependent effect of chitosan-arginine (0-500 µg ml^{-1}) on *E. coli* O157cell activity (luminescence) in chicken juice samples stored at 4 or 20 °C. Values represent means ± SEM (n = 3).

5.3.4. Effects of chitosan–arginine on coliforms and total viable counts

Statistical analyses revealed that both TVC and coliform numbers were significantly less in chicken juice samples treated with chitosan–arginine in comparison to the control treatment ($P < 0.001$; Fig. 5.4).

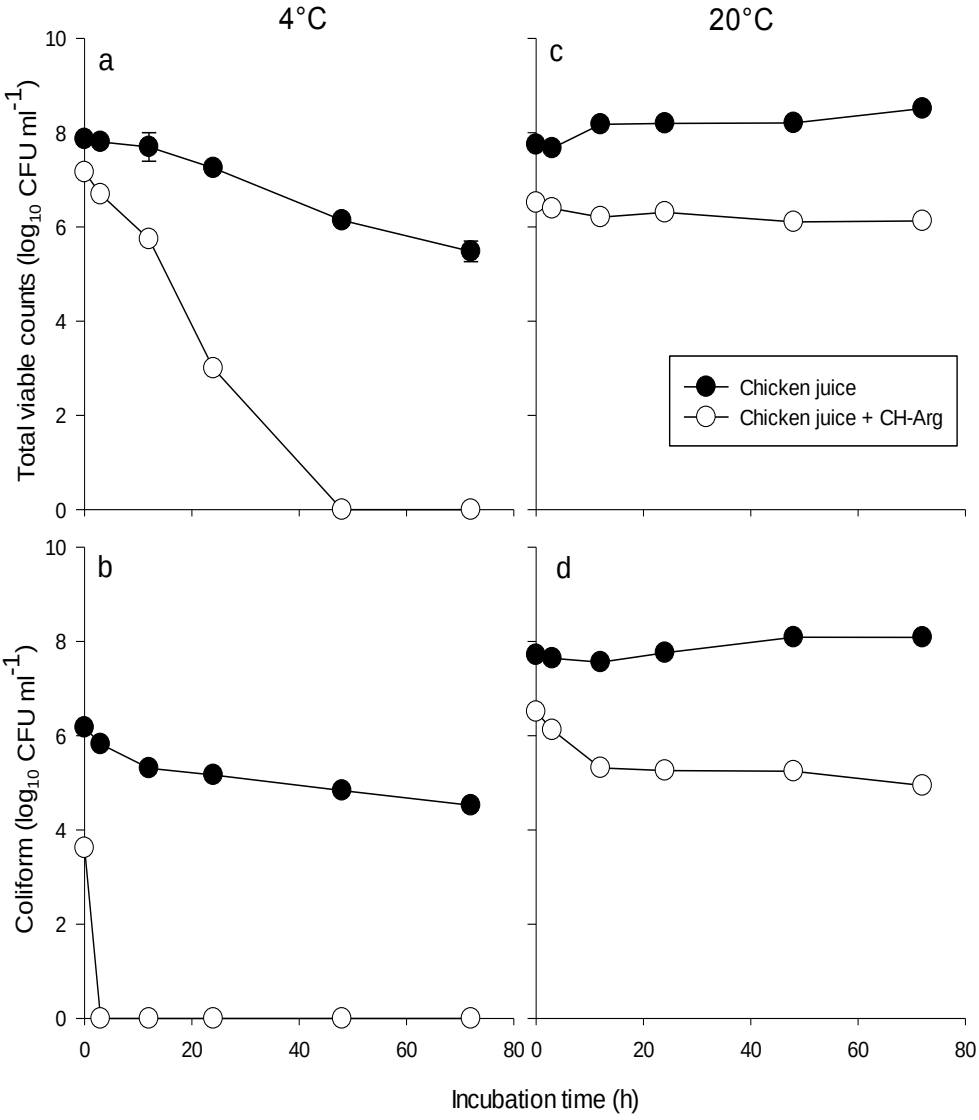

Figure 5.4. Changes in total viable counts (a, c) and coliforms (b, d) over 72 h post-incubation period in chicken juice ± 500 μg ml^{-1} chitosan–arginine (Ch-arg) when incubated at 4°C and 20 °C. Values represent means ± SEM (n = 3).

Importantly, at 4 °C, chitosan–arginine induced a rapid decline in TVC and coliforms (Fig. 5.4a, b); whereas at 20 °C, the presence of chitosan–arginine led to a moderate decline (Fig. 5.4c, d; Figure 5.5). Comparing bacterial results of chicken juice post–treatment with chitosan–arginine with an industry standard (Malpass,M.C. & Williams,A.P. & Jones,D.L. & Omed,H.M. 2010)(Malpass, M.C. & Williams, A.P. & Jones, D.L. & Omed,H.M. 2010) TVC and coliform numbers at both temperatures were sufficiently low to be fit for human consumption.

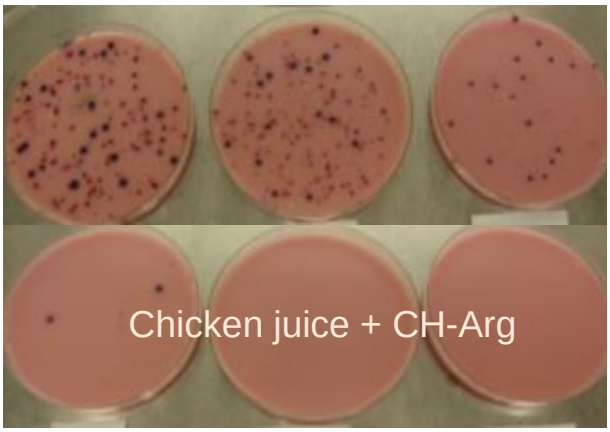

Figure 5.5. Bacterial counts of chicken juice ± Ch–arg

5.4. Discussion

It has been generally reported that the antimicrobial properties of chitosan and its derivatives are concentration–dependent, with greater disruption to microbial growth at higher dose rates (Liu, Du, Wang, & Sun, 2004). In addition, the activity of chitosan and derivatives is notably subject to the influence of environmental growth conditions, such as incubation temperature (Tayel et al., 2010). The present experiment considered the effects of both concentration and environmental temperature upon chitosan-arginine's ability to control and inhibit *E. coli* O157 *in* chicken juice representative of that found in food packaging.

Our microbiological assessments showed that, while bacterial cell populations were sustained in pure chicken juice samples, the introduction of chitosan–arginine at a concentration of 200–500 μg ml^{-1} induced a significant reduction in *E. coli* O157 cell numbers. Variations in the size of cell count reduction were subject to the influence of temperature, consistent with Kong et al. (2010) who argued that environmental factors including temperature would influence the antimicrobial effect of chitosan and its derivatives. Chitosan–arginine appeared to be more effective in reducing spoilage micro-organisms at the lower temperature and *E. coli* O157 *at* the higher temperature. This may be due to spoilage bacteria in the chicken juice being dominated by psychrophiles as the chickens were refrigerated when purchased, whereas *E. coli* O157 *is* a mesophilic organism and was grown at a higher temperature. Our luminescence results imply that chitosan-arginine better targets those cells with high activity; spoilage organisms may therefore be more susceptible to its actions at lower temperatures, and *E. coli* O157 at higher temperatures.

We also showed that the pathogen's metabolic activity was greatly inhibited in most of the amended treatments. Cell inactivation was very rapid (3 h post-incubation) and chitosan was seen to maintain its inhibitory capacity for long time periods (as evidenced by the lack of bacterial re-growth over the 72 h incubation period in any of the chitosan treatments). However, at the lower concentrations of chitosan–arginine, *E. coli* O157 persisted in a state of low metabolic activity; as seen in previous studies in different environments (Williams et al., 2008). Whether *E. coli* O157 cells of low metabolic activity are equally capable as highly active cells in terms of causing infection is still unclear and warrants further investigation.

Although it is difficult to directly compare with unmodified chitosan and other chitosan derivatives, our results concur with the general observation that the antimicrobial action of

chitosan–arginine is dose-dependent and most effective between 200 and 500 μg ml^{-1}. A recent study on another chitosan derivative also showed that it could effectively inhibit *Staphylococcus aureus* and *E. coli* at similar concentrations to that reported here (>150 mg l^{-1}); Xiao et al., 2011). Three of the major problems preventing the adoption of chitosan for pathogen control by the poultry industry have been the cost of chitosan, its mode of application and the choice of dose rate. Obviously, it is economically desirable to use the lowest concentration to achieve effective pathogen control. The consistency of our findings with those of Xiao et al. (2011) suggests that dose rates >100 μg ml^{-1} are required to ensure its effectiveness as an antimicrobial agent.

The mode of action of chitosan–arginine has yet to be clearly identified, probably as a result of its potential to act at a number of different cellular target sites. However, when concentrations are above 200 mg l^{-1}, the positively charged chitosan-arginine can complex negatively charged components of cell walls, inducing membrane depolarization, the free exchange of nutrients and wastes and ultimately cell lysis (Liu et al., 2004; Xiao et al., 2011). The mode of action is therefore believed to be similar to that of antimicrobial peptides (AMPs) although the effective dose for AMPs is generally much lower due to their predominantly low molecular weight nature (<1 kDa). Unlike AMPs, however, which can be readily decontaminated by the peptidases in the target cell, our results suggest that few bacteria present in chicken juice possessed sufficient chitinase or deaminase activity to counteract the effects of the added chitosan. Further, the persistence of the toxic response over 72 h suggests that these enzymes are not readily inducible.

The consumer home represents where the greatest risk of cross-contamination and human infection occurs. Pre-packaged poultry meat and its by–products are growing in popularity

among consumers worldwide; however, to minimize infection risk implies that its packaging should remain intact during both transport and storage (Yashoda, Sachindra, Sakhare, & Rao, 2001). In reality, however, this is not always achievable and cross–contamination of food surfaces with pathogens originating from compromised chicken packaging is well documented (Harrison et al., 2001). Here we show the future potential for the internal coating of poultry packaging with soluble chitosan–arginine to form an effective barrier against pathogen leakage. An external coating may also be useful for non-meat foods which may also be prone to cross–contamination (e.g. salad vegetables). The greater effectiveness at room temperature is particularly pertinent since poor food handling practices at homes often result in meat products not being kept under refrigeration temperatures at all times. Research to determine the re–growth potential of pathogen cells following contact with chitosan-arginine would be of interest to determine whether it provides a lasting protective effect, e.g. following removal of contaminated chicken from packaging that contained the compound.

Our experiments confirmed that the traditional way of preserving food by lowering the temperature to 4 °C is effective in itself for controlling (presumably through extending the lag phase), but not eliminating bacterial growth. However, the treatment of meat juice with chitosan-arginine almost completely eliminated bacterial growth which in theory may increase the shelf life of the product. Our empirical analysis of the odor from the chicken juice treated with chitosan-arginine also confirmed a significant reduction in unpleasant odors emanating from the liquor in comparison to the untreated controls. This increased shelf life through a reduction in product discoloration and odor may help to offset the cost of the antimicrobial packaging; however, a full economic analysis is required to confirm this.

In conclusion, the present study has revealed key factors that directly influence the antimicrobial action of a novel water soluble chitosan derivative, chitosan-arginine, in a chicken juice environment. The study identified critical doses required to prevent pathogen proliferation under both refrigeration and room temperature conditions. Incorporation of chitosan–arginine into packaging may reduce both food spoilage and the risk of pathogen transfer that arises due to liquid seepage. Other potential applications include the incorporation of chitosan-arginine into carcass wash water to create a convenient 'meat detergent' or in situations where synthetic biocides cannot be employed. Chitosan-arginine may also prove useful in the preservation of milk and wet food products (e.g. wet noodles), especially where odor is an issue.

5.5. Acknowledgements

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Abstract

Chitosan-arginine has been proposed as an anti-microbial agent to reduce the proliferation of spoilage and pathogenic bacteria within meat products destined for human consumption. Its effectiveness, however, is likely to be dependent upon the metabolic state of the target bacteria. Within contaminated beef juice held at room temperature (20°C), we evaluated the effect of chitosan-arginine on metabolic activity and cell numbers, at different growth stages of a lux-marked metabolic reporter strain of *Escherichia coli* O157:H7. Results showed that chitosan-arginine was most bioactive against cells in the lag and exponential phases. In comparison, there was a reduced, although still significant, inhibitory effect on *E. coli* O157 viability and metabolic activity in the stationary phase. Chitosan-arginine reduced, but did not eliminate *E. coli* O157 growth in the meat juice over a 48 h period. Our results suggest that chitosan-arginine has the potential to limit the growth and activity of food spoilage bacteria; however, it cannot cause complete elimination of bacterial contaminants originally present. Chitosan-arginine should therefore be viewed as a potential preventative measure rather than a biocidal agent that completely eliminates the risk of pathogen transfer in the food chain.

Keywords: Antibacterial, Chemically modified chitosan, Food safety, Spoilage bacteria, VTEC

6.1. Introduction

With growing demands for restricted use of chemicals in food preparation, in addition to the increasing consumer awareness of food safety (Vapnek & Spreij, 2005), attention has focused on the use of naturally derived preservatives within the food industry (Ruirui et al., 2012). One such candidate is chitosan, a commonly found polysaccharide in shellfish whose use has been proposed as an antimicrobial agent and preservative, including incorporation into foodstuffs, use as a surface coating and use as a component of food packaging (Lahmer et al., 2012; Aider, 2010; Agullo et al., 2003). In previous studies on meat products, chitosan has been shown to enhance the microbiological, chemical, odour and colour qualities of the product (Darmadji & Izumimoto, 1994). Chitosan is a highly cationic polymer which slows putrefaction by reducing lipid oxidation and the growth of spoilage bacteria, whilst also maintaining the sensory quality of meat during storage.

In spite of the antimicrobial properties inherent in unmodified chitosan, its application as a food preservative remains critically dependent on its solubility at neutral pH. As a result, there has been much interest in the formulation of soluble derivatives of chitosan, for example through modification with amino acids, sugars or cyclodextrins (Sashiwa & Aiba, 2004; Mourya & Inamdar, 2008). Derivatization with positively charged functional groups may also increase the surface charge density of the chitosan, enhancing its antimicrobial properties. A number of reports have suggested that chitosan-arginine has a superior antimicrobial effect against Gram-negative bacteria than unmodified chitosan (Xiao et al., 2011; Tang et al, 2010). However, the biocidal effect of chitosan-arginine in meat environments (arguably the most important from a health risk perspective) remains poorly understood.

Factors influencing the antimicrobial effect of chitosan and its derivatives include temperature, pH, target microorganism, chitosan type and concentration, solvent and food chemical and physical properties (Fernandes et al., 2008; Yang et al., 2007; Liu et al., 2006; No et al., 2007). Based on other biocides, we hypothesise that the antimicrobial action of chitosan derivatives will be highly dependent on the growth stages and metabolic state of cells (Chavant et al., 2004). It has been reported that *Staphylococcus aureus* is most susceptible to a lactose-chitosan derivative in late exponential phase. On the other hand, a lower susceptibility of cells was seen at the mid-exponential and late-stationary phase (Chen & Chou, 2005). Yang et al. (2007) suggested that *E. coli* O157:H7 cells appeared to be less susceptible to a maltose-chitosan derivative at stationary phase, whilst Liu et al. (2006) recorded greater antimicrobial activity when adding chitosan at lag phase, in comparison with exponential and stationary phase. Collectively, these studies have yielded inconclusive results, and additionally, the effect of cell age on chitosan effectiveness under realistic food conditions is still to be investigated.

The aim of this study was to determine the effect of cell age on the effectiveness of Ch-arg, a novel chitosan derivative, when used as an antimicrobial agent in a beef juice media containing *E. coli* O157. Beef juice typifies the high-nutrient environment often found in meat packaging, in which *E. coli* O157 can multiply. It has also been identified as a major source of cross-contamination in food processing environments.

6.2. Materials and methods

6.2.1. Preparation of chitosan-arginine solution

Chitosan-arginine deacetylated (85%, 41 kDa, purity > 99%) was synthesized by Synedgen, Inc. (Claremont, CA, USA), with 25% arginine monomers on the polymer chain. A

chitosan-arginine stock solution (1 g l^{-1}) was made in distilled water and the solution sterilized by passage through a 0.2 μm syringe filter for storage and later use.

6.2.2. Preparation of E. coli O157 inoculum

The method applied in this experiment was adapted from Williams et al. (2008) and Avery et al. (2005). A stock of *E. coli* O157 containing a chromosomal inserted metabolic reporter gene (strain 3704 Tn5 LuxCDABE; Ritchie et al., 2003) was prepared from a fresh overnight Luria Bertani (LB) broth (Difco Ltd., Teddington, Surrey, UK), by growing *E. coli* O157 at 20°C for 18 h at 150 rev min^{-1}. The cells were washed with quarter strength Ringer's solution three times and concentrated by centrifugation.

6.2.3. Preparation of beef juice extract

Three bovine meat joints (each ca. 5 kg) were hung in a cold room (4 ± 1°C) in sterile plastic bags for 10-12 days, and beef juice draining from the meat was collected as detailed by Williams et al. (2008). The beef juice was subsequently diluted to a $^{1}/_{10}$-strength solution by adding distilled water to simulate the dilution which frequently occurs in domestic kitchens during the rinsing of meat, its packaging and kitchen surfaces. The juice was then centrifuged for 10 min at 10,000 rev min^{-1} at 10°C before being sterilized by passing through a 0.2 μm syringe filter. The juice was stored at 4°C and used immediately as detailed below.

6.2.4. Antimicrobial testing

Beef juice (19 ml) was allocated into 18 individual sterile 100 ml Erlenmeyer flasks. The flasks were then divided into three groups, one for each bacterial growth phase (lag, exponential and stationary phase). The initial population of *E. coli* O157 was adjusted to approximately 8.11 log_{10} CFU ml^{-1} and 1 ml was added to each flask. Ch-arg was

subsequently added to the flasks containing *E. coli* O157 cells when they were in either lag phase (0 h after inoculation), exponential phase (8 h after inoculation) or stationary phase (30 h after inoculation), to yield a final Ch-arg concentration of 500 µg ml^{-1}. The dose rate was chosen based on previous studies (Lahmer et al., 2012). Three control flasks containing beef juice, *E. coli* O157, but no Ch-arg were also run in parallel. All flasks were incubated in the dark at 20°C and the flasks gently agitated on an orbital shaker (150 rev min^{-1}).

Measurements of *E. coli* O157 numbers in the meat juice (drop-plate method on CT-SMAC; Oxoid CM813), and luminescence measured in relative light units (RLU; Infinite 200 PRO® luminometer; Tecan Austria GmbH, Grödig, Austria) were taken at the following times: 0, 3, 6, 12, 24, 48, 72, 96, 120 and 144 h post-Ch-arg addition, as described in Williams et al. (2008).

6.2.5. Statistical analyses

All experimental treatments were performed in triplicate. SPSS v16.0 for Windows (IBM Ltd., Portsmouth, UK) was used to perform the statistical analysis. The plate count data was first $\log_{10}(x + 1)$ transformed to satisfy the assumptions of ANOVA. 2-way ANOVAs were run, with $p < 0.05$ used as the cut-off for statistical significance.

6.3. Results

6.3.1. Effects of chitosan-arginine on E. coli *O157 numbers at different growth phases*

Changes in the viable population of *E. coli* O157 over the 144 h incubation period are shown in Figure 6.1 for the lag, exponential and stationary phases.

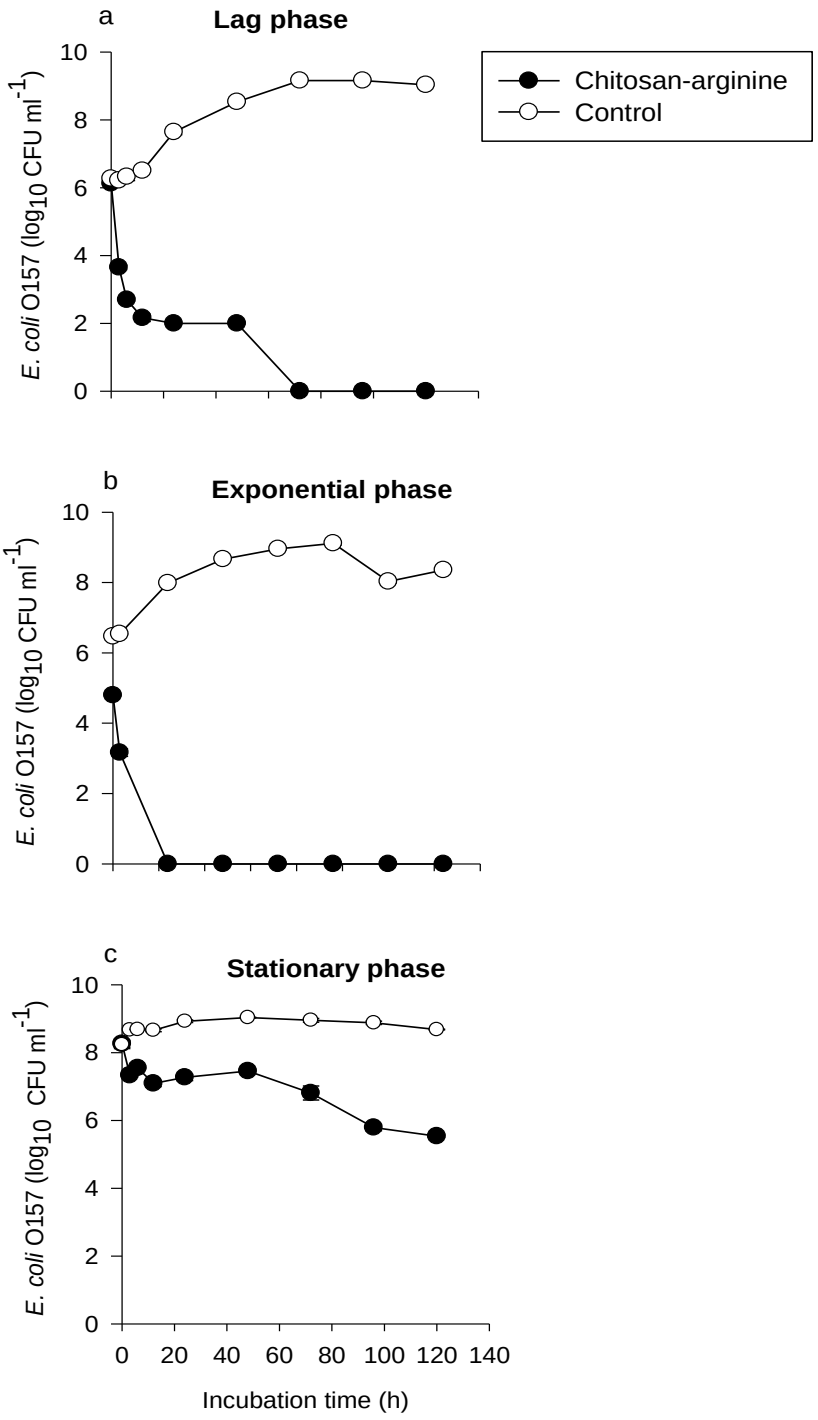

Figure 6.1. Influence of chitosan-arginine and bacterial cell growth phase on temporal changes in the population density of *E. coli* O157 in beef juice cultures incubated at 20°C. Values represent mean ± standard error of the mean (n = 3).

Growth of *E. coli* O157was seen in the control (no Ch-arg) treatment before 72 h incubation, after which cultureable cell counts gradually reduced. Bacterial cell counts showed significantly greater reductions in the samples treated with Ch-arg, particularly when exposed to bacteria in lag and exponential phase. Specifically, in the culture to which Ch-arg was added during the lag phase, viable *E. coli* O157 population reduced from 6.2 to 2.0 $\log_{10}$ CFU ml^{-1} after 12 h of incubation, and this was maintained until 48 h after which there was a sharp reduction to a non-detectable level at 72 h. A 2 (treatment) × 6 (time-point) factorial ANOVA found a significant effect of treatment ($p < 0.001$) and incubation time ($p < 0.001$), and also a significant interaction between the two ($p < 0.001$). At lag phase, *E. coli* O157 cell counts were significantly reduced by amendment with Ch-arg relative to the control treatment (3.11 $\log_{10}$ CFU ml^{-1} to 6.91 $\log_{10}$ CFU $ml^{-1,}$ respectively). During exponential phase, a sharp decline in viable *E. coli* O157 (from 4.4 $\log_{10}$ CFU ml^{-1} to a non-detectable level during the first 24 h) was seen when Ch-arg was added. A 2-way ANOVA (treatment × time-point) revealed a significant effect of treatment (Ch-arg = 0.04 $\log_{10}$ CFU ml^{-1}, Control = 7.0 $\log_{10}$ CFU ml^{-1}, $p < 0.001$), and time ($p < 0.001$) with a significant interaction ($p < 0.001$).

In the culture to which Ch-arg was added during the stationary phase, *E. coli* O157 was reduced from 8.26 to 5.53 $\log_{10}$ CFU ml^{-1} over the 144 h incubation period, but not eliminated. A 2-way ANOVA revealed a significant effect of treatment (Ch-arg treatment = 6.84 $\log_{10}$ CFU ml^{-1}, control = 8.73 $\log_{10}$ CFU ml^{-1}, $p < 0.001$) and time ($p < 0.001$), and also revealed a significant treatment × time interaction ($p < 0.001$).

6.3.2. Effects of chitosan-arginine on E. coli *O157 metabolic activity at different growth phases*

All treatments saw a general trend of reducing luminescence over time. Overall, a lower cell activity was observed in the Ch-arg treatments relative to the control, whether administered during the lag (Ch-arg = 1.91 $\log_{10}$ RLU, control = 3.34 $\log_{10}$ RLU, $p < 0.001$, Fig. 6.2), exponential (Ch-arg = 1.65 $\log_{10}$ RLU, control = 3.76 $\log_{10}$ RLU, $p < 0.001$), or stationary phase (Ch-arg = 3.45 $\log_{10}$ RLU, control = 4.20 $\log_{10}$ RLU, p <0.001). The stationary phase saw a persistent reduction in cell activity under the treatment of Ch-arg, with luminescence falling from 4.87 $\log_{10}$ RLU at time 0 to 1.23 $\log_{10}$ RLU at 144 h. This tendency was more obvious than in the controls, whereby luminescence fell from 4.87 $\log_{10}$ RLU to 3.77 $\log_{10}$ RLU.

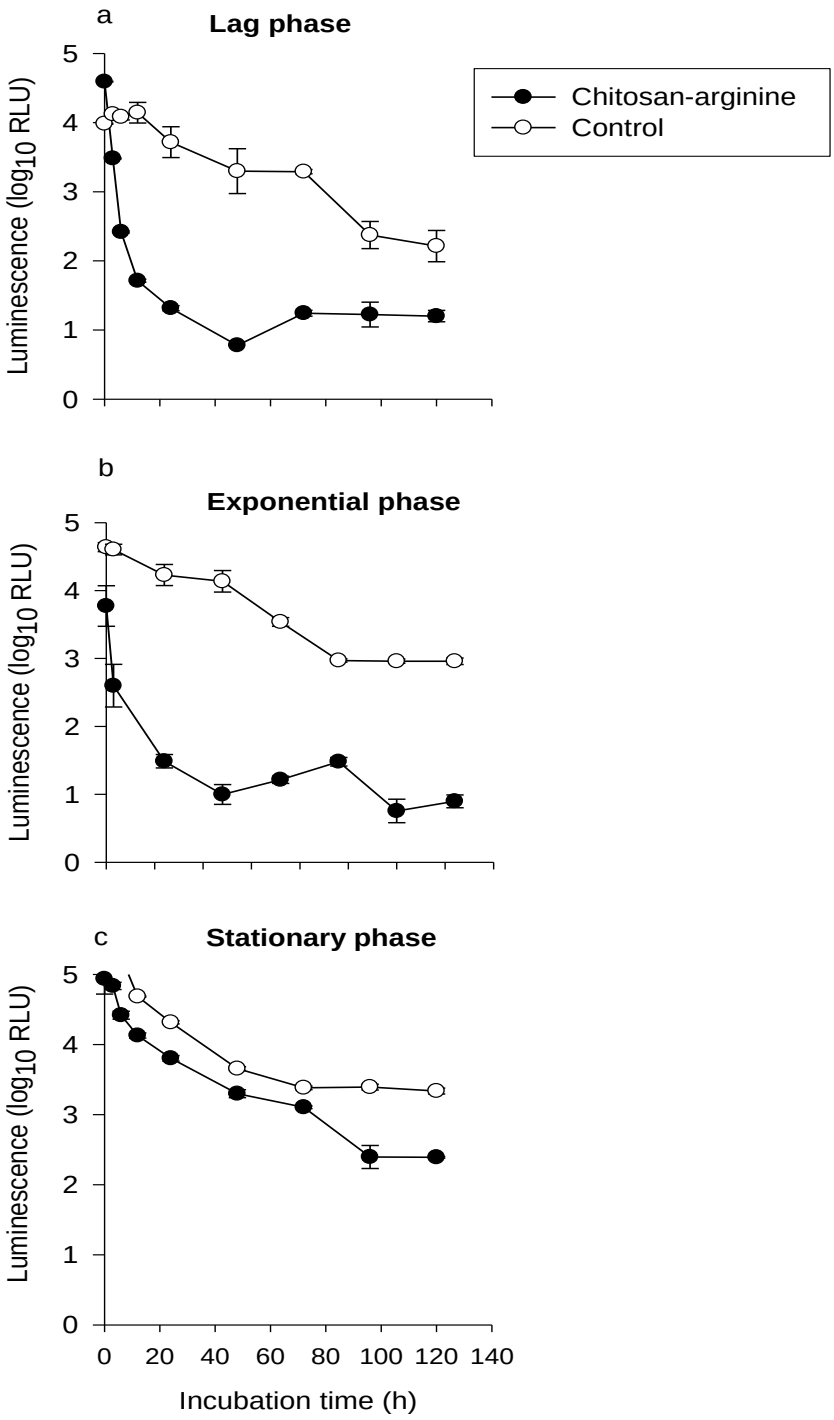

Figure 6.2. Influence of chitosan-arginine and bacterial cell growth phase on temporal changes in the metabolic activity (luminescence) of lux-marked *E. coli* O157 cultures incubated in beef juice at 20°C. Values represent mean ± standard error of the mean (n = 3).

6.4. Discussion

The findings presented here suggest that pathogen cell age represents a critical factor determining the biocidal efficacy of Ch-arg against *E. coli* O157 in an aqueous meat environment. The largest reduction in *E. coli* O157 cell density and metabolic activity (as measured by cell count and luminescence respectively) was observed when Ch-arg was added at the lag or exponential phase. In comparison, non-actively growing cells appeared to be less susceptible to Ch-arg. This supports the findings of Lahmer et al. (2012) which also showed that Ch-arg was less active against non-growing cells at 4°C in comparison to those more metabolically active being held at 20°C.

Our results suggest that chitosan may have targets linked to cell growth rather than general cell metabolism. As the arginine-derivative of chitosan in highly cationic it can be expected to have properties similar to some antimicrobial cationic peptides, many of which are known to act directly on membrane function (Fig 6.3).

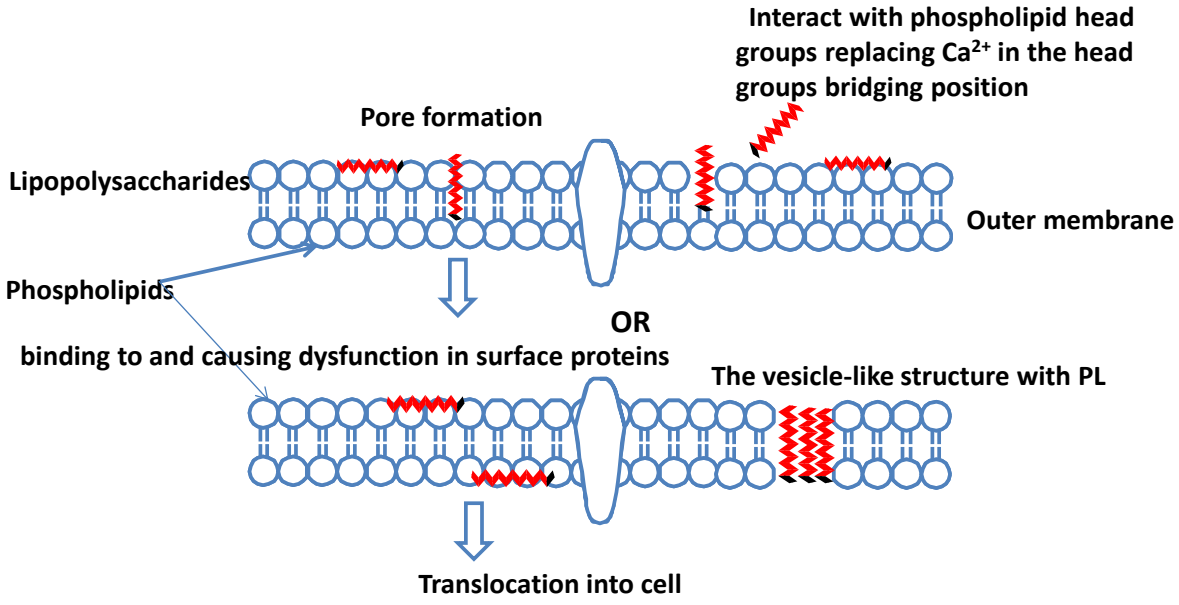

Figure 6.3. Model of potential mechanisms by which cationic peptides exert antimicrobial effects.

In particular, charged amine residues in the peptides are known to (1) readily interact with phospholipid head groups replacing Ca^{2+} in the head groups' bridging position, (2) induce the formation of membrane pores causing unregulated solute loss, and (3) bind to and cause dysfunction in surface proteins which are involved in signalling and cell structure (Herce et al., 2007; Su et al., 2013). Evidence with both pure and chemically modified versions of chitosan also suggests that it similarly rapidly interacts with microbial membranes (Sudarshan et al., 1992; Fang et al., 2001). Due to the profusion of vesicle fusion events occurring during cell growth, any disruption in this process can inadvertently lead to a range of effects ranging from growth inhibition through to cell death. Evidence to support this is firstly provided by Mady et al. (2009) who have demonstrated that chitosan binding to microbial surfaces would induce a shift in membrane zeta potential from slightly negative to positive (Mady et al., 2009), and secondly that chitosan can increase the physical stability of liposomes and reduce their rate of aggregation and fusion which is likely to lead to a cessation in growth (Crommelin, 1984). In terms of the chitosan mode of action, Pavinatto et al. (2009) have shown that chitosan induces order into negatively charged phospholipid layers but that the degree of re-organization also dependent on the presence of other membrane components. This may suggest that different microorganisms may react differentially to chitosan depending upon their membrane structure. However, depending on the composition of the lipid bilayer, chitosan concentration and external pH, chitosan can also lead to spontaneous vesicle fusion or induce vesicle fragmentation (Quemeneurde et al., 2007; Oliveira Tiera et al., 2010; Mertins and Dimova, 2012). Again, both of these would disrupt cell growth, however, this information suggests that the type of derivatization might lead to different membrane targets depending on side chain length, their pKa and charge density, and whether they are amphiphilic or not.

Our results support Liu et al. (2006), who found that the inhibitory effect of chitosan against *E. coli* was greatest during the initial incubation period, and suggested that when chitosan is added at this stage, the lag phase becomes extended. When added at the exponential phase, chitosan led to a marked reduction in optical density, but added at the stationary phase this decline was small (Liu et al. 2006). In a different study, Chen and Chou (2005) found that cells of *Staphylococcus aureus* were most susceptible to water-soluble lactose-based chitosan during the late-exponential phase, followed by the stationary phase, consistent with our results.

To conclude, the effectiveness of Ch-arg appears to be highly dependent upon the metabolic state of the target pathogen. Our results suggest that Ch-arg may prove particularly useful in reducing the growth of food spoilage bacteria at key stages in the food supply chain (e.g. during storage and transportation, prior to consumption). However, unless very high concentrations are used, at which point a bitter taste may be introduced into the food (Devlieghere et al., 2004), chitosan will not completely eliminate pathogenic bacteria within food. Use of high concentrations of chitosan is also unlikely to prove acceptable to industry on economic grounds. Further, chitosan will not prove successful for eliminating pathogens located within the meat itself as chitosan is unlikely to penetrate more than a few mm from the point of administration (due to non-specific binding to meat phospholipids, etc.). Assuming legislative approval for chitosan derivatives, their inclusion within food packaging material or as a surface food coating seems most suited to the prevention of cross-contamination. Specifically, when contaminated juice leaks from the packaging it can be expected to inhibit pathogen growth either before or after contact with external packaging and work surfaces.

Finally, chitosan should be combined with other anti-microbial strategies to provide a multilateral approach to pathogen elimination in meat products.

6.5. Acknowledgements

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Abstract:

Chitosan-arginine has been proposed as an anti-microbial agent to reduce the proliferation of spoilage and pathogenic bacteria within meat products destined for human consumption. In the current experiment its use as an antimicrobial packaging material (cellulose/chitosan-arginine; Ch-arg film) was examined. The film was produced by blending two different concentrations of chitosan-arginine (Ch-arg, 0.5 and 1.5 %) with cellulose previously dehydrated in ethanol and acetone. When placed in contact with chicken and beef juice inoculated with a lux-marked strain of *E. coli* O157, the film incorporating the highest Ch-arg concentration resulted in a significant reduction of *E. coli* O157 in chicken juice; however, there was no effect of the Ch-arg film on *E. coli* O157 in beef juice. The lack of observed effect in the beef juice experiment we ascribe to insufficient surface-to-surface contact between the film and the bacteria in the beef juice and the greater presence of other Ch-arg reactive components in the juice (e.g. fats, blood cells). Results suggest that, in combination with other natural antimicrobials, Chitosan-arginine packaging offers some potential for limiting the growth of pathogenic bacteria in foodstuffs; however, further research is needed to establish a strong evidence base for the antimicrobial process of Ch-arg films.

*Keywords***:** Cross-contamination, foodborne pathogen, polymer film, shelf life

7.1. Introduction

The conflict between consumers' demand of minimally processed fresh food and an adequate shelf-life has slowly been challenged by the emergence of active packaging (Dutta al., 2012). Active packaging incorporates antimicrobial substances and may play an important role in reducing contamination and delaying spoilage, thereby increasing safety of meat products (Restuccia et al., 2010).

However, active substances in contact with food surfaces may be neutralized or diffuse quickly into the product, therefore, the direct surface application may not target food surfaces where most spoilage reactions occur (Restuccia et al., 2010). It may be possible to increase the efficiency of antimicrobial packaging by developing films which contain antimicrobial agents. Previous research suggests that these films either allow the gradual release of active agents (Bastarrachea et al., 2011) or exert their effect when active sites contact bacteria directly (Brody et al., 2001). However, results of studies into the effectiveness of such films have so far been inconclusive (Foster and Butt et al., 2011; Lee et al., 2003).

One inherently promising antimicrobial agent is chitosan, which is biodegradable, biocompatible and nontoxic (Alishahi, & Aider, 2012). Chitosan inhibits the growth of a wide spectrum of bacteria, pathogens and spoilage microorganisms, including *E. coli* O157, the pathogen of interest in this study (Lahmer et al., 2012; Dutta al., 2010). *E. coli* O157 is a frequent contaminant of meat, causing potentially fatal illnesses in humans such as haemorrhagic colitis and haemolytic uraemic syndrome (Soon et al., 2011; Forsythe 2010; Pennington 2010; Rhoades et al; 2009; Conedera et al., 2004). Beef and chicken are high-risk for food contamination, since if not properly sealed, meat juices can contaminate food preparation surfaces or other foodstuffs and hence providing a high-nutrient environment

where pathogens such as *E. coli* O157 can multiply. This poses a major risk for surface contamination and subsequent cross-contamination in the home (Mattick et al., 2003).

Although chitosan-based films have proven effective in food preservation (Dutta et al., 2009), its application as a food preservative is limited by its insolubility at a neutral pH. One functional derivative of chitosan, chitosan-arginine, has a positively charged guanidinium side chain in neutral pH environments, leading to high solubility in water (Tang et al., 2010) and therefore is a good candidate for food preservation. Chitosan-arginine is formed when carboxylic acid in arginine and amine in glucosamine link to form a stable peptide bond. The biocidal effect of this newly-developed chitosan derivative, however, is not well understood (Tang et al., 2010), and its application to meat environments under different storage temperatures remains unknown.

The blending of cellulose and chitosan may be useful for introducing antimicrobial activity into packaging film. Cellulose has a similar molecular structure to that of the chitosan backbone (He et al., 2009; Gindl & Keckes, 2007) and has been used in edible films and coatings since the 1980s due to its suitable physical and chemical properties (Sangsuwan et al., 2008; Li, et al., 2002; Chen et al., 1996).

This present study aimed to assay and characterise the antimicrobial effect of cellulose/chitosan-arginine (Ch-arg) film on *E. coli* O157, in both chicken and beef juice. This research sought to develop a new, more effective film combining cellulose with chitosan arginine.

7.2. Materials and methods

7.2.1. Preparation of chitosan solutions

Chitosan-arginine (85% deacetylated with arginine constituting 25% of the total monomers on the polymer backbone; 41 kDa, purity > 99%) was synthesized by Synedgen, Inc. (Claremont, CA, USA). A chitosan-arginine stock solution (1 g l^{-1}) was made in distilled water and the solution sterilized by passage through a 0.2 μm syringe filter for storage and later use.

7.2.2. Cellulose/Chitosan-arginine film-making

Cellulose film-forming solution was prepared using a procedure by Gindl and Keckes (2007) in which 3 g of microcrystalline cellulose (MCC) was dehydrated in ethanol and acetone, while 8 g of lithium chloride (Aldrich, LiCl, UK) was dissolved in 100 ml N-dimethylacetamide (VWR, Jencons, DMAc). After decanting the ethanol and acetone from the dehydrated cellulose, the LiCl/DMAc solution was slowly added to the cellulose over 5 min, before Ch-arg was drip-fed to the cellulose solution to give final concentrations of either 0.5 (low concentration) or 1.5 % (high concentration), or 0 % (control cellulose-only). The cellulose/Ch-arg mixture was then poured into 250 mm diameter Petri dishes and left under a fume hood for 24 h to solidify.

7.2.3. Preparation of E. coli *O157 inoculum*

A strain of *E. coli* O157 (strain 3704 Tn5 lux CDABE; Ritchie et al., 2003) was prepared from a fresh overnight Loria Bertani (LB) broth (Difco Ltd, Teddington, Surrey, UK; 37°C, 18 h, 150 rev min^{-1}; Williams et al., 2008). The strain has proven to be non-toxigenic, due to the absence of toxin activity by Verocell assay, and toxin genes, but still accurately reflects the survival pattern of a toxigenic strain (Ritchie et al., 2003). Cells were washed and

concentrated by centrifugation as described in Avery et al. (2005). Enumeration of colonies (18 h, 37°C) was determined using the drop-plate method onto cefixime-tellurite-sorbitol-MacConkey agar plates (CT-SMAC; Oxoid, Basingstoke, UK) followed by incubation at 37 °C for 24 h, and showed that the concentration of *E. coli* O157 in the inoculum was 8.93 $\log_{10}$ CFU ml^{-1}

7.2.4. Preparation and characterization of meat juice

A total of six processed intact raw chickens were purchased from a commercial supermarket in Bangor, North Wales. Each chicken was placed in a sterile stomacher bag and thoroughly washed with sterile distilled water to obtain 600 ml of juice per chicken. The juice was then centrifuged for 10 min at 10,000 rev min^{-1} at 10°C, and sterilized by filtering through a 0.2 µm syringe filter.

Approximately 100 ml of beef juice was collected from three bovine meat joints (each ca. 5 kg), hung for 10-12 days in sterile plastic bags in a butcher's cold-room (4 ± 1 °C; Williams et al., 2008). The juice was pooled together, and stored at 4°C for 24 h, before adding distilled water to make $^{1}/_{10}$-strength beef juice. The juice was then centrifuged for 10 min at 10,000 rev min^{-1} at 10°C before being sterilized by passing through a 0.2 µm filter.

The chemistry of both meat juices was also characterized for pH (pH-209 meter; Hanna Instruments Inc., Woonsocket, RI), electrical conductivity (CDM210 meter; Jenway Ltd., Dunmow, UK) and dissolved organic phosphate (DOP). DOP was calculated by subtracting the amount of inorganic phosphate from total phosphate measured using a plasma-atomic emission spectrometer (ICP-AES; Varian Liberty Series, Franklin, MA, USA).

7.2.5. Antibacterial testing

7.2.5.1. Chicken juice

The films containing cellulose alone (control group) or cellulose with 0.5 or 1.5 % chitosan-arginine were produced as previously described. The films were placed on the bottom of glass jars, to which 75 ml of meat juice and 4 ml of *E. coli* O157 inoculum were added, giving a final concentration of 8.93 $\log_{10}$ CFU ml^{-1}. The jars were then covered with metal lids and stored at 20 °C for 120 h (n = 3 for each juice-film combination). At 0, 2, 6, 12 and 24 h post-inoculation, and subsequently every 24 h until 120 h post-inoculation, 2 ml of juice was aseptically removed in triplicate from the bottom of the jars and analysed for *E. coli* O157 as follows: cell count via the drop plate method on CT-SMAC plates (Lahmer et al., 2012); luminescence via a Tecan Infinite 200 PRO luminometer (Tecan Austria GmbH, Grödig, Austria) which displayed results in RLU (relative light units). The Live/Dead BacLight™ Bacterial Viability Kit (BacLight™; Molecular Probes Inc., Eugene, OR, USA) was as used to ascertain the number of live and dead *E. coli* O157 cells. The stain was prepared using a mixture of 1 µl of propidium iodide, 0.7 µl of SYTO 9 and 330 µl of sterile deionized water (Mauriello et al., 2005). A 1 ml sample was taken from each jar at each time point, washed and re-suspended in sterile saline water. Then, 4 µl was taken from the re-suspended sample, and 4 µl of the viable staining solution was applied and left in the dark for 15 min for fluorescence to develop. Samples were viewed using a Zeiss Axioskop fluorescence microscope, with an average count taken over 15 microscope fields.

7.2.5.2. Beef juice

Cell count and metabolic activity of *E. coli* O157 were determined as described for chicken juice. In contrast to chicken juice, the BacLight™ microplate assay was used to determine the percentage of live and dead bacteria using a Varian Cary Eclipse fluorescence microplate reader (Varian Inc., Palo Alto, USA). In order to calibrate the percentage of live *E. coli* O157 in the beef juice, the bacteria was grown until late log phase and then concentrated by centrifugation (10,000 *g*, 10 min). One ml of this suspension was added to a tube containing 20 ml ¼-strength Ringer's solution (live bacteria) and the other 1 ml to a tube containing 20 ml 70% isopropanol alcohol (dead bacteria). Samples were pelleted by centrifugation (10,000 *g*, 10 min) in both tubes and subsequently re-suspended in 20 ml Ringer's Solution. After repeating this process three times, the optical density was measured as 600 nm (approx. 0.6-0.8 OD_{600nm}). Live: dead cells were then mixed in a variety of ratios, to be used for calibration of the BacLight™ method, before *E. coli* O157 was prepared stained, by the manufacture's guidelines (Molecular Probes, 2004). The data was analysed by calculating the % live *E. coli* O157, which was plotted as ratio of live in sample to live in pure *E. coli* O157 (calibration sample) in Sigma Plot (version 19) using an "exponential growth single 2" parameter (equation: f=a*exp (b*x). r= 0.9992; a=0.3566, b= 0.026).

7.2.6. Data analysis

Data were analysed with IBM SPSS Statistics version 19.0 for Windows. A multivariate ANOVA was conducted to examine the effect of treatment (0.5 % Ch-arg, 1.5 % Ch-arg, and cellulose control) on cell count, RLU and percentages of culturable and live *E. coli* O157. Significant effects were identified using post-hoc Bonferroni-adjusted multiple comparisons test at $p < 0.05$, with simple planned contrasts used to examine the main effect of

time. Significant interactions were followed up with repeated measures ANOVAs, with Tukey HSD post-hoc tests at $p < 0.05$. Twelve samples were analysed in total, comprising 4 conditions × 3 replications. Two-tailed independent samples t-tests were used to evaluate the differences in chemical characterization of beef and chicken juices.

7.3. **Results.**

7.3.1. Chemical characterization of meat juice

The chemical characteristics of beef and chicken samples are shown in Table 7.1. In brief, a two-tailed independent samples t-test revealed pH was significantly lower in the beef juice, but that EC, inorganic and organic P of beef juice were significantly greater than that of chicken juice (all $p < 0.003$).

Table. 7.1. Chemical characterization of chicken and beef juice. Values represent the mean ± standard error of the mean (n = 3).

Test	Chicken juice	Beef juice
pH	6.87 ± 0.05	5.34 ± 0.02
Electrical conductivity (EC, mS cm^{-1})	0.56 ± 0.01	1.78 ± 0.04
Total phosphate (P, mg l^{-1})	66.18 ± 0.44	158.97 ± 1.26
Dissolved organic phosphate (DOP, mg l^{-1})	66.09 ± 0.84	157.75 ± 2.64
Inorganic phosphate (PO_4^{3-}, mg l^{-1})	0.07 ± 0.05	1.14 ± 0.20

7.3.2. Effectiveness of C/Ch-arg films on reducing cell counts of E. coli *O157*

Cell counts of *E. coli* O157 in chicken juice treated with low and high concentration Ch-arg containing films are shown in Fig. 7.1 a.

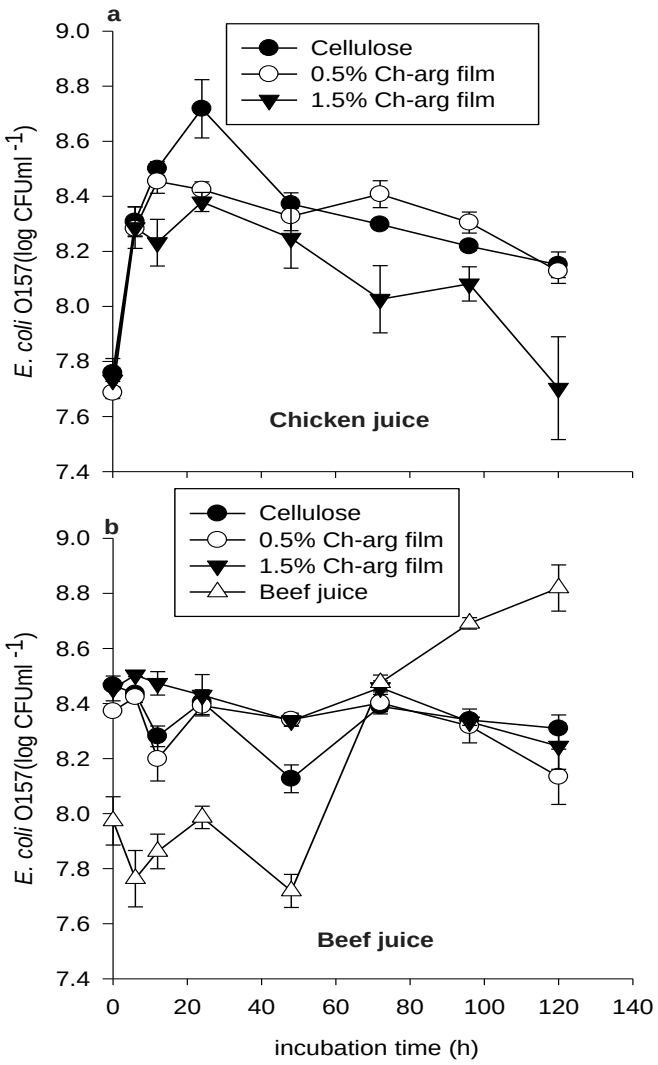

Figure.7.1. Cell counts (CFUml^{-1}), of *E. coli* O157 in chicken juice (a) and beef juice (b) samples stored at 20 °C. Values represent mean ± standard error (n = 3).

Until 6 h post-incubation, *E. coli* O157 in the cellulose control group and the groups treated with Ch-arg maintained similar growth trends, increasing slightly from 7.7 $\log_{10}$ CFU ml^{-1} to 8.3 $\log_{10}$ CFU ml^{-1}. After this, the population of *E. coli* O157 in jars with the high concentration Ch-arg film decreased. For the control group and the group treated with low concentration Ch-arg film, the population of *E. coli* O157 generally continued to grow after 12 h incubation, reaching 8.2 $\log_{10}$ CFU ml^{-1} by the end of the trial. Tukey HSD post-hoc tests

showed no significant difference between the control group and the group treated with low concentration films (p = 0.76), but the difference between the control group and high concentration film group was significant (p = 0.02). Similarly, there was a marginally significant difference between the low and high concentration groups (p = 0.05).

Figure.7.1 b depicts the influence of Ch-arg films on *E. coli* O157 cell counts in beef juice. At time 0, there was a drop in count of 0.5 $\log_{10}$ CFU ml^{-1} within meat juice in the control, relative to the cellulose control condition. Counts in the control then remained comparatively low until 48 h of post- incubation, before increasing to its original level at 72 h followed by a further increase until 120 h. In contrast, counts measured for the two treatment conditions decreased slightly over time, from 8.4 $\log_{10}$ CFU ml^{-1} at time 0 to 8.3 by 120 h incubation time. However, statistical analysis revealed that the difference between cell count at time 0 and time 120 was not significant (p = 0.31). Tukey HSD post-hoc tests revealed that there was a significant difference in count between low and high concentration Ch-arg film (p = 0.038), and between the beef juice control and all treatments (all $p < 0.001$). There was also a significant interaction between treatment and time ($p < 0.001$), with counts in the beef juice control increasing over time, and the other treatments remaining constant. All other comparisons were not significant, including the difference between the cellulose control and low concentration (p = 0.81) and between cellulose and high concentration (p = 0.13).

7.3.3. Effectiveness of cellulose/chitosan-arginine films on cell activity of E. coli *O157*

The effects of Ch-arg on the metabolic activity (as measured using luminescence) of *E. coli* O157 in chicken and beef juice are presented in Figures 7.2 a and b respectively.

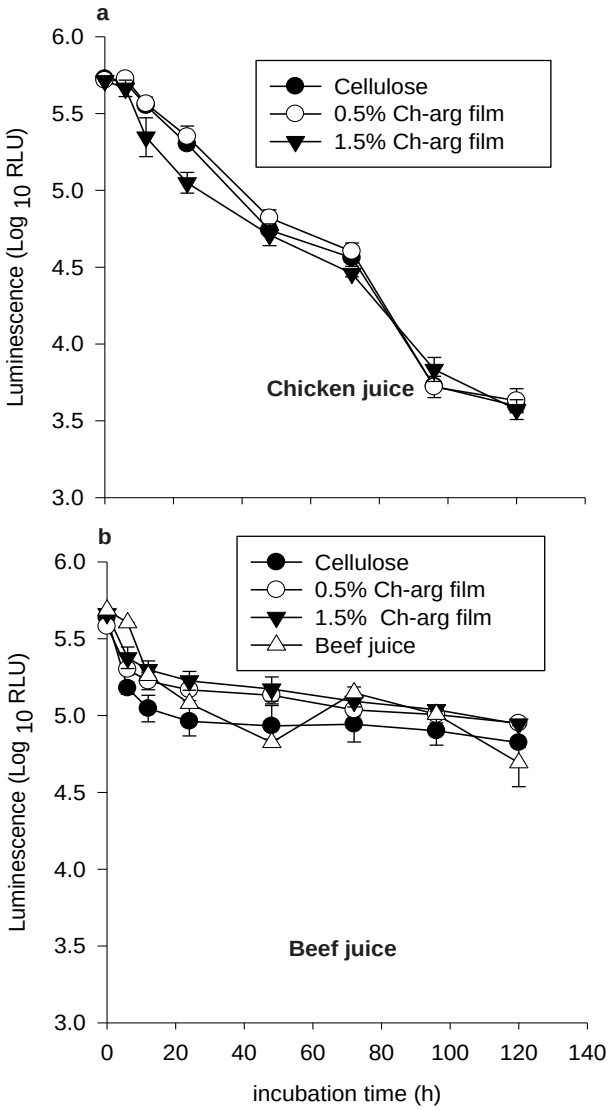

Figure 7.2. Metabolic activity as measured by luminescence (RLU), of *E. coli* O157 in chicken juice (a) and beef juice (b) samples stored at 20 °C. Values represent mean ± standard error (n = 3).

In general, cell activity of *E. coli* O157 in all treatments groups declined during the 120 h incubation. During this time period, there was no significant main effect of treatment condition (p = 0.09 and p = 0.11 for chicken and beef juice, respectively).

7.3.4. Effect of Ch-arg films on the culturability and proportion of live E. coli O157 cells

The percentage of culturable *E. coli* O157 in chicken juice was calculated to ascertain the effect of Ch-arg film on the culturability of *E. coli* O157 cells (Fig. 7.3 a). A multivariate ANOVA revealed no significant difference in percentage of culturable *E. coli* O157 in samples treated with Ch-arg films and cellulose-only film (p = 0.65). Simple contrasts following a repeated measures ANOVA found the culturability of the cells to significantly increase in all treatment groups within 24 h of incubation (p = .004; from 1.1% to an average of 5.7%), before decreasing to a level of less than 2%.

In beef juice, the percentage of live cells generally decreased over time (p <.001, Fig 7.3.b). In the cellulose and Ch-arg treatment conditions, percentage live decreased from 97.23 -69.56 between 0-120 h, whereas the beef juice measure decreased from 88.32 at 0 h to 50.81 at 48 h, before returning to 83.86 by 120 h.

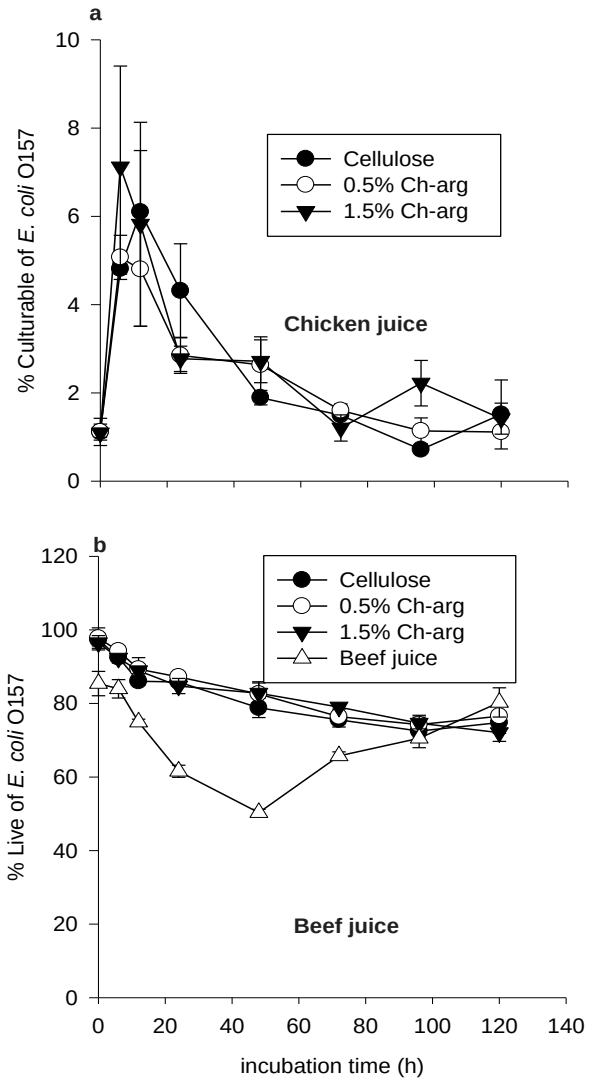

Figure 7.3. Percentage of culturable and live *E. coli* O157 in chicken (a) and beef juice (b) samples stored at 20 °C. Values represent mean ± standard error (n = 3).

7.4. Discussion

In the current study, a higher-concentration of Ch-arg film was seen to exert greatest effect on *E. coli* O157 cell counts. This supports results from previous studies highlighting the concentration dependent property of cellulose/chitosan-arginine film, and finding the number of colonies of *E. coli* to decrease with the increase of chitosan concentration in cellulose/chitosan (C-Ch) blend membranes (Wu et al., 2004). Further, the current study found no significant difference between low concentrations of Ch-arg in films and the cellulose

control in chicken juice, for any of the parameters measured. Neither was any effect seen on the presence of chitosan in beef juice, despite the slight antimicrobial action of Ch-arg solution in beef juice found in previous studies (Lahmer et al., 2012). Results from the chicken juice experiment are consistent with previous research (Xiao et al., 2011; Wu et al., 2004) suggesting a concentration-dependence of the antimicrobial properties of chitosan and its derivatives in solution form, with an increased disruption of microbial cells observed at higher dose rates.

Results from both chicken and beef juice experiments are also consistent with a recent study by Foster and Butt (2011), which found no inhibitory effect of chitosan films on *E. coli* and other bacterial species commonly present in meat during 24 h of incubation. Foster and Butt argued that chitosan chains in the films were unable to interact with the microbial cell walls, impeding antimicrobial effects. However, in the current study the incubation time was longer (120 h), which may explain the antimicrobial effect of high concentration Ch-arg found in chicken juice. This suggests that antimicrobial agents are initially released from Ch-arg film slowly, but under high concentration of antimicrobial agents, later release may be sufficient to reduce the number of bacteria.

In contrast to the percentage of culturable bacteria in chicken juice showed no significant differences between conditions. This suggests that high concentration Ch-arg may be effective in impeding the growth of, but not eliminating *E. coli* O157, with similar numbers of metabolically active *E. coli* O157 detected in each condition. Additionally, the stains used to measure percentage of culturable bacteria differ in spectral characteristics and ability to penetrate viable bacterial cells. SYTO 9 stains all live cells green, while propidium iodide stains dead cells red (Molecular Probes, 2004). This may explain the lack of significance in

the culturable bacteria comparison, since previous research shows some *E. coli* strains to enter a viable but nonculturable (VBNC) state when exposed to stressful environments (Liu et al., 2010). The BacLight method can shed light on this, since although cells in this state do not proliferate in media, they are still metabolically active. As they are still live, they potentially retain virulence (Boulos et al., 1999; Duffy & Sheridan 1998) and so are still hazardous to human meat consumption. Additionally, future work could consider protein concentration, which is also suggested to give a better estimate of pathogens than a viable count method (Mattick et al., 2003).

Results from the current study suggest that Ch-arg chains interact with meat juice cells in addition to microbial cell walls, reducing the chance of antimicrobial action. DOP levels were twice as high in the beef juice as in the chicken juice, suggesting the presence of more blood cells. Therefore, negatively charged phospholipids in blood cells interact with positively charged Ch-arg, preventing surface-surface contact (Fig 7.4), which likely caused the failure of chitosan to exhibit bactericidal properties in the beef juice condition.

In support of this idea, Devlieghere and Debevere (2004) found antimicrobial activity to be inhibited when lipid was added to a chitosan solution. This can be explained by the positioning of chitosan on the outside of the emulsion drops, allowing chitosan to form larger positively charged drops (Jumaa and Müller 1999).

Since Ch-arg lacks the cohesion to make gel alone, it was blended with cellulose. However, this may have reduced potential surface-to-surface contact, thereby reducing the effectiveness of the Ch-arg. Alternatively, cross-linking with other natural compounds may improve the antimicrobial properties of the packaging.

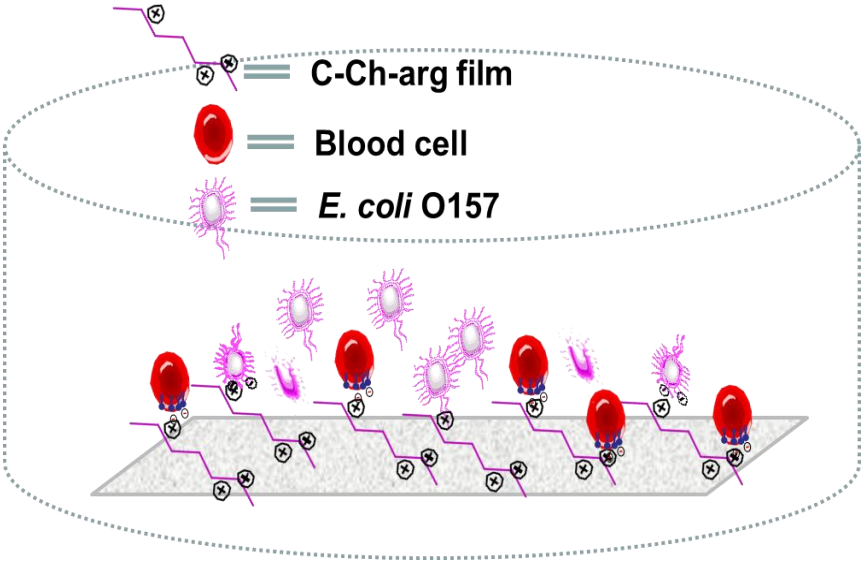

Figure 7.4. Ch–arg chains interact with blood cells in beef juice media.

7.5. Conclusion

To conclude, the study revealed that blending Ch-arg into cellulose-based packaging could potentially reduce the risk of pathogen transfer resulting from meat juice seepage. However, the effect appears to be very much concentration-dependent and it is possible that the concentration of Ch-arg required to be effective against food-borne pathogens may be economically prohibitive to industry. Future studies are needed to gain further insight into the availability of amino group active sites in the Ch-arg film and how to increase the effectiveness of chitosan as an antibacterial agent in food packaging by combining it with other natural antimicrobials.

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8.1. Overall discussions

Finding new mechanisms to enhance food preservation represents an important issue for both manufacturers and health-conscious consumers, as this could lead to significant reductions in food spoilage reducing the risk of pathogen transfer into the human food chain and also reducing the amount of food wasted. The research described in this thesis focuses on new ways of preserving food quality and reducing the risk of pathogen multiplication in meat-based products, using chitosan, a natural polycationic compound. Meat products were selected for study due to their importance in the global good chain and the fact that their improper handling and storage currently represents the biggest risk of food poisoning to consumers.

The aim of this study was to investigate the antimicrobial performance of chitosan and its derivatives, against the pathogen *E. coli* O157 under a range of operating conditions. These included investigations into the effect of chitosan concentration, incubation temperature, pH of the mediums and pathogen metabolic activity. Furthermore, the experiments aimed to investigate the potential applications of incorporating a chitosan derivative into packaging material for fresh meat products. The overall aim was to identify the environmental conditions under which chitosan is most bioactive and to determine the most effective way in which it can be used within the food industry.

The study identified several critical factors that influence the effectiveness of chitosan and its derivatives. Firstly, Experiment 1 found a complex interaction between chitosan concentration and pH and its effect on pathogen survival. The results presented for *E. coli* O157 were consistent with the response of other pathogens suggesting that chitosan may have a broad antimicrobial effect which is desirable trait from a food industry perspective (e.g. No et al., 2002). Lower pH values were associated with a stronger bactericidal effect when

chitosan concentrations were low, whereas at higher chitosan concentrations, a more neutral pH was optimal. However, Fujimoto et al. (2006) suggests that this low pH response may be due to the use of acetic acid as solvent for chitosan confounding the results. Despite the vast amount of work looking at chitosan previously, most previous studies had failed to critically address this issue. Indeed, Experiment 2 suggests that at lower temperature and pH, the bactericidal effect against *E. coli* O157 is predominantly explained by the presence of acetic acid, with chitosan increasing this effect at higher temperature and pH. At low pH acetic acid is generally more cytotoxic as the H.acetate^{0} form can freely diffuse across the membrane and interfere with cell metabolism. In contrast, at higher solution pH values, acetic acid is largely dissociated (p*K*a = 4.76) and the anionic form (acetate^{1-}) cannot pass freely across negatively charged microbial membranes. It also possible that at higher pH values, acetate may complex with positively charged chitosan although the interaction is likely to be weak based on the stability constants for acetate with other cations (Jones, 1998). This suggests that chitosan and acetic acid play different roles in the antimicrobial process, with chitosan tending to be more inhibitory and acetic acid more bactericidal. As lactic acid and nitrite (NO_2^-) are also commonly used as food preservative agents it would also be useful to investigate their interactions with chitosan in a similar way to that undertaken here for acetate.

Results also suggest chitosan to be most effective at relatively high temperatures, when problematic, excessive growth of bacteria is most common. This finding is consistent with Tsai and Su (1999) who found that chitosan incubated in food at 4 °C had a weaker antimicrobial action than when added to food at 25 °C. As the optimal temperature for the growth of most foodborne pathogens is in the region 30-37°C (with little growth seen at low temperatures <8 °C), it suggests that chitosan may only affect actively growing cells, rather

than those residing in a state of dormancy. Although it has been speculated that the bactericidal mode of action of chitosan is via binding to bacterial phospholipid membranes, the findings presented here suggest that it may only be effective against actively dividing membranes. These results suggest that chitosan will be primarily useful during the transportation of meat products, when temperature may not be well controlled (e.g. from the supermarket to the consumer home).

In later experiments, chitosan derivatives were used, which have higher solubility in water, allowing investigation of chitosan in the absence of acetic acid. These derivatives also possess a greater surface charge density which in theory makes them more bioactive than pure chitosan. In Experiment 3, chitosan-arginine was found to significantly reduce the population density and metabolic activity of *E. coli* O157, and number of general food spoilage bacteria. This reduction occurred in a dose-dependent manner, with greater inhibition seen as more chitosan-arginine was added. This is consistent with results of Xiao et al. (2011), who found an antimicrobial effect of chitosan-N-arginine. Critical to adoption by the food industry is minimising the amount of chitosan added to the food product whilst still maintaining a biocidal effect. This is to firstly reduce production costs and secondly to minimise potentially negative effects on product taste (Devlieghere et al., 2004). The results presented here suggest that the concentrations of modified chitosan required to ensure food safety were relatively high and further chemical modification of the chitosan may be needed to increase its biocidal activity (e.g. by increasing charge density or the way the charge groups are arranged along the chitosan backbone).

As hypothesised above, Experiment 4 suggests chitosan-arginine to be most bioactive against cells in the lag and exponential phases, consistent with findings by Yang et al. (2007)

and the suggestion that chitosan is not effective against non-dividing cells. During these phases, chitosan-arginine induced large reductions in cell density and activity, reducing, but not completely eliminating *E. coli* O157 in the meat juice over a 48 h experimental period. This result suggests that like other alternative food preservation techniques (e.g. gamma irradiation), chitosan cannot substitute for the need to maintain good hygiene practices in food handling environments (López-Gonzalez et al., 1999).

The results from these experiments imply that incorporating water soluble chitosan-arginine into packaging may prove useful in the control of food spoilage bacteria. Previous studies (Ouattara et al., 2000) have found chitosan film to be effective in inhibiting bacteria and in Experment 5 chitosan-arginine impregnated film was developed, with potential to be used in this manner. This film was predicted to better inhibit *E. coli* O157 relative to pure chitosan, due to the presence of more amino groups. The film showed potential for inhibiting *E. coli* O157 growth in chicken juice media, although disappointingly in beef juice no evidence of inhibition was found. This is consistent with results from Foster and Butt (2011), and reinforces claims that chitosan effectiveness depends strongly on food components (Devkieghere & Debevere, 2004). As chitosan is relatively non-specific in its interactions with organelles (i.e. does not alter an active site in a key enzyme like some biomolecules), it has the potential to react with many solutes and surfaces outside the target pathogen cells. In the case of beef juice we speculate that this includes cells and solutes released from the meat itself although further work is needed to confirm this.

In general, chitosan derivatives incorporated into food packaging may be well suited to preventing cross-contamination. Chitosan packaging would be expected to inhibit pathogen growth before contaminated juice is able to leak from packaging and cause contamination of

external surfaces. Another topical problem is the use of synthetic materials in packaging, such as polyethylene (PE), polyvinyl chloride (PVC) and polyvinyl alcohol (PVA), which may lead to pollution. Due to its biodegradable properties, its potential for use in packaging of non-food products should also be further explored.

Taken together, these experiments suggest that, combined with other anti-microbial strategies, chitosan and its derivatives have the potential to reduce or eliminate cross-contamination of pathogenic bacteria in poultry and beef meat. This may provide the answer to consumer needs of extended shelf-life and increased overall quality of product. However, further work is required on the development of packaging materials to successfully control pathogens.

The use of high chitosan concentrations in Experiment 1 and many previous studies (Xiao et al. 2011; Liu et al., 2006; Devkieghere & Debevere, 2004) has proven the most successful in reducing bacterial growth. The high concentration used in Experiment 5 was still relatively low, and so increasing this may further increase the usefulness of chitosan impregnated packaging films. However, until more efficient methods of production for chitosan are established (Aider, 2010) this may not be practical on economic grounds.

Comparisons between this and previous studies should be treated with caution, due to the differences in the quality and origin of chitosan, and differing research techniques employed. Future work would benefit from development of standard measurements and methods to quantify the antimicrobial activity of chitosan in a more standardized manner (Kong et al., 2010).

The present studies were carried out under small scale laboratory conditions with a laboratory strain of *E. Coli* 0157 rather than an authentic environmental strain. Specifically, the strain of *E. coli* O157:H7 used in our studies, which is both chromosomally lux-marked (Tn5 luxCDABE) and nontoxigenic, has not been under selective pressures since it was constructed in 2003 (Ritchie et al., 2003). Consequently, it is difficult to know to what extent lux imitates contemporary *E. coli* O157 which has undergone intense selective pressures in the meantime. Therefore, further studies based upon an authentic pathogenic strain and large scale commercial conditions could provide the more realistic and practical information needed for commercialization of food products (No et al., 2007).

Consumer acceptance of chitosan as a food additive is also required. Finally, chitosan and its chemically modified derivatives are currently not on the GRAS (Generally Recognized as Safe) list presented by the US Food and Drug Administration or on the list of EU approved additives supplied by the UK Food Standards Agency. Until chitosan is approved its likely use in food preservation will be severely limited. However, chitosan has been approved as a food additive in Japan and South-Korea suggesting that some markets do currently exist for its use (Mellegård et al., 2011).

8. 2. Future research directions

Further research is required into both theoretical and practical issues regarding the use of chitosan arginine to control *E. coli* and other food spoilage and human pathogenic organisms. Key research needs are:

1. The basic and fundamental question of how a pathogen's cell activity, as measured by bioluminescence, relates with its infectivity to host (human) cells. Specifically, are bacterial cells treated with chitosan entering a viable-but-non-cultureable state and do these cells still

represent an infection risk? It has been suggested that bacterial adherence to host cellular surfaces can be used as an indicator of infectivity. Future work could involve an adherence assay on HeLa or Caco-2 cells which are mixed with *E. coli* O157.

2. More practical ways of producing chitosan-arginine gel, or chitosan-arginine-based biocomposite packaging film are required preferably using other natural compounds which can be co-applied to provide more effective food preservation. Other natural cross-linking compounds which may improve the properties of chitosan arginine include corn fibre gum used for oil-in-water (O-in-W) emulsions, Arabic gum, Gur gum and galactomannan obtained from the seeds of *Cyamopsis tetragonalobus.*

3. Further research into other property enhancing extracts from medical plants, such as *Nigella sativa* and *Gores* in combination with chitosan is also required.

4. To investigate the application of chitosan derivative-based biocomposite packaging for preservation of different food types (e.g vegetables, fruits, processed foods), and against other common food-borne pathogens such as *Listeria* spp. *Campylobacter* spp. and *Salmonella* spp. Its ability to supress fungi involved in food spoilage is also required (e.g. *Penicillium, Nematospora corvli, Fusarium, Pichia kluvveri etc*)

5. Within abattoirs, chitosan-arginine solutions may also be incorporated into carcass wash water to create a convenient 'meat detergent', and may have further application in the preservation of milk and wet food products (e.g. wet noodles). The effectiveness of chitosan to treat dry surfaces is also warranted (e.g. processing equipment).

6. Finally, the cost-effectiveness of chitosan arginine must be determined, in addition to the willingness of manufacturers and consumers to use chitosan and its derivatives. Findings must be disseminated widely so that the technology can be developed and potentially adopted

within the food processing sector. Twinned with this, cost effective ways of producing chitosan derivatives from raw materials is required.

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Abstract

Escherichia coli O157:H7 is a potentially lethal pathogen which has been responsible for several outbreaks of milk-borne illness in recent years. The objective of this study was to evaluate the survival and metabolic activity (indexed by bioluminescence) of a chromosomally *lux*-marked strain of *E. coli* O157:H7 in raw, pasteurized and microfiltered pasteurized milk at 4 and 20°C for up to 14 d. Results showed that the population of *E. coli* O157:H7 and its metabolic activity decreased in all samples during storage at 4°C, with no significant differences in numbers observed between the different milk types; but metabolic activity was significantly higher (P<0·05) in the microfiltered pasteurized milk than that in raw milk. At 20°C, *E. coli* O157:H7 counts and cell activity peaked at day 2, and then declined progressively. At 20°C, survival and metabolic activity were significantly lower in raw milk compared with pasteurized milk. We conclude that storage temperature is more important in regulating the survival of *E. coli* O157:H7 in contaminated milk than its origin/pre–treatment conditions.

Keywords: Cross-contamination, dairy products, food poisoning, hygiene, microbiological quality.

1. Introduction

Due to the high nutrient content of milk, it is an optimal medium for the growth of several microorganisms (Barbano et al. 2006). Consumption of raw milk, if not heat–treated or pasteurized, can be particularly problematic and is responsible for many disease outbreaks worldwide. Outbreaks are also associated with improperly pasteurized milk, and dairy products made from unpasteurized milk (Wang et al. 1997; Vernozy–Rozand et al. 2005).

Escherichia coli O157:H7 was first identified as a human pathogen in 1982 when outbreaks of bloody diarrhea and severe abdominal cramps occurred in the USA (Riley et al. 1983). The majority of affected individuals are children and the elderly, who can develop complications including haemorrhagic diarrhoea, haemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (Griffin, 1995). Though only a small percentage of raw milk samples have been found to be *E. coli* O157:H7 positive (Duncan & Hackney, 1994), contamination with this pathogen has resulted in several milk–borne outbreaks of gastroenteritis (Chapman et al. 1993). Since *E. coli* O157 is an ordinary inhabitant of the bovine intestinal tract, the route of contamination with *E. coli* O157:H7 is through faecal contact with feedstuffs, or during milking without strict hygiene practices (Hussein & Sakuma, 2005).

To date, the milk industry has successfully tackled issues of milk safety through various intervention strategies. Pasteurization has proved to be an effective measure in ensuring the safety of milk and dairy products. While unpasteurized raw milk can pose a public health concern, post–pasteurization contamination with *E. coli* O157 should also be noted. Faulty on–farm pasteurizers have also resulted in an outbreak of *E. coli* O157 (Goh et al. 2002). Incidentally, microbial growth has been shown to be greater in pasteurized samples of whey than its unpasteurized counterpart at a range of storage temperatures (Marek et al.

2004). Therefore, effective pasteurization and avoiding post–pasteurization cross–contamination in the fridge environment are both necessary to ensure the safety of milk and milk products (Heuvelink et al. 1998).

Although human pathogen outbreaks associated with milk are relatively rare, it is important to minimise this threat to maintain consumer confidence in dairy products and to protect the dairy industry. To date, no studies have examined the metabolic activity of *E. coli* O157:H7 in different types of milk during storage, an important evaluator of the pathogen's potential infectivity (Jawhara & Mordon, 2004). The aim of this study was to improve our understanding of the pathogen's behaviour in milk through studying both its survival and metabolic activity in raw and different types of pasteurized milk under ambient (20°C) and refrigeration (4°C) conditions. To do this, a bioluminescent (lux–marked) strain of *E. coli* O157:H7 (strain 3704 Tn5 luxCDABE; Ritchie et al. 2003) was used. Measuring bioluminescence in relative light units (RLU) indicates the degree of cellular metabolic activity (Ritchie et al. 2003), and has proved to be useful in improving our understanding of the pathogen in a range of contrasting environments (e.g. Jawhara & Mordon, 2004; Williams et al. 2008a, b; Thorn et al. 2011).

2. Materials and Methods

2.1. Preparation of milk

Raw milk was collected from the tank of a dairy farm located in Bangor, North Wales. The samples were kept at 4°C in sterile ice bags during transportation. Milk was used within 3 h after arrival at the laboratory. Part of the raw milk remained unpasteurized, whilst part was heat–treated in glass containers to 63·5°C (30 min) to prepare laboratory-pasteurized milk.

Fresh full–fat commercially-pasteurized and full-fat microfiltered pasteurized milk (Cravendale) were purchased from Arla Foods UK Ltd (Leeds, UK).

2.2. Screening milk samples for E. coli *O157:H7*

Milk samples were tested for the presence of *E. coli* O157 before inoculation. Isolation and detection of *E. coli* O157:H7 involved enrichment followed by immunomagnetic separation (IMS). To start with, 5 ml of each milk samples were mixed with 45 ml of modified Tryptone Soy Broth (mTSB) (Oxoid CM 0989; Oxoid Ltd., Basingstoke, UK) and incubated at 37°C for 6 h. Afterwards, 1 ml of the enriched sample was analysed by Dynamag™–2 IMS (Invitrogen Dynal A.S., Oslo, Norway) with 0·02 ml of Captivate® *E. coli* O157 immunomagnetic beads (Lab M Ltd, Bury, UK) and incubated at 25°C for 30 min. After IMS, the beads were washed three times using phosphate buffered saline with 0·05% Tween 20 as wash buffer, and resuspended in 0·1 ml of the same buffer. They were then spread equally on three SMAC plates (sorbitol MacConkey agar plates (SMAC; Oxoid CM813) supplemented with cefixime (0·05 mg l^{-1}) and potassium telluride (2·5 mg l^{-1}) CT–SMAC), and incubated at 37°C for 18 to 24 h.

2.3. Inoculation of milk samples with E. coli *O157:H7*

An inoculum was prepared from a fresh overnight culture (LB broth; Difco Ltd, Teddington, Surrey, UK; 18 h, 37°C, 150 rev./min) of *E. coli* O157:H7 (Ritchie et al. 2003) in stationary growth phase. Cells were washed and concentrated by centrifugation as described in Avery et al. (2005). An inoculum (1 ml) of the mixture at the appropriate dilution was added to 99 ml of each milk type and mixed thoroughly in sterilised screw–cap bottles to obtain the desired final concentration of approximately 10^3 CFU ml^{-1}. All bottles of inoculated milk and uninoculated milk (control) were incubated at 4 and 20°C.

2.4. Survival and metabolic activity of E. coli O157:H7

E. coli O157 cells were enumerated at 0 (immediately after inoculation), 1, 2, 4, 6, 8, 10, 12 and 14 d post–inoculation. Milk samples were serially diluted in Ringer solution (Oxoid), and serial dilutions were plated onto CT–SMAC and incubated at 37°C for 18 to 24 h. Non–sorbitol fermenting *E. coli* O157:H7 colonies were confirmed by agglutination with a latex test kit (Oxoid DR0620).

A parallel experiment was designed to assess variations in the activity of *E. coli* O157 among the different milk types (raw, laboratory–pasteurized, full-fat commercially-pasteurized, and microfiltered pasteurized). Bioluminescence of bacteria in milk was measured at 0 (immediately after inoculation), 1, 2, 4, 6, 8, 10, 12 and 14 d post–inoculation. At each time–point, a 1–ml aliquot from samples used for the enumeration study detailed above was placed into a plastic luminometer cuvette and its luminescence (RLU) was determined using a SystemSURE plus Pi-102 Luminometer (Hygiena International Ltd, UK).

2.5. Aerobic plate counts and pH

Aerobic plate counts (APC) were determined from uninoculated milk samples (control) at 0 (immediately after inoculation), 1, 2, 4, 6, 8, 10, 12 and 14 d. The uninoculated samples were serially diluted in Ringer solution, and serial dilutions (1:10) were plated onto plate count agar (PCA; Oxoid) and incubated at 30°C for 48 h.

Samples' pH values were determined with a standard pH meter (Hanna instruments pH 211). Calibration was performed using two standard buffer solutions at pH 4·0 and 7·0.

2.6. Statistical analysis

Outcomes in the experiment were changes in *E. coli* O157:H7 cell counts and cell activity (bioluminescence), aerobic plate counts, and pH values during the 14 d incubation period. Log (y+1) transformation was performed on *E. coli* O157:H7 cell counts and cell activity, aerobic plate counts, which together with pH data were subjected to ANOVA tests

and Tukey's test with significance at $p<0{\cdot}05$ using SPSS 18·0 software (SSPS Inc, Chicago, Illinois, USA).

3. Results

3. 1. Screening milk samples for E. coli *O157:H7*

No *E. coli* O157:H7 was detected by the IMS method in any of the milk samples before inoculation.

3. 2. Survival and metabolic activity of E. coli *O157:H7*

Survival and metabolic activity *of E. coli* O157:H7 at both 4 and 20°C are shown in Fig. 1. At 4°C, *E. coli* O157:H7 populations declined steadily and continuously by 1·0-1·5 log10 CFU ml^{-1} in all samples over 14 d incubation. While log cell count reduction was greatest in raw milk (1·5 log10 CFU ml^{-1}), between-sample variations in survival of *E. coli* O157:H7 were not significant between all samples at this temperature ($p>0{\cdot}05$). Metabolic activity of *E. coli* O157:H7 continuously and steadily reduced (by 1·3–2·07 log10 RLU) over the 14 d, with activity in raw milk diminishing near to zero. Cell activity in the microfiltered milk was significantly higher than that in raw milk ($p<0{\cdot}05$), while no significant difference was seen among laboratory-pasteurized, commercially-pasteurized and raw milk ($p>0{\cdot}05$).

At 20°C, *E. coli* O157:H7 cell counts in all milk samples showed a dramatic initial increase, peaking at day 2 (2·7-3·6 log10 CFU ml^{-1}), then progressively declined until the end of the 14 d incubation. Cell counts in raw milk samples decreased most (about 2·5-log cell count reduction using day 0 as baseline) and the count reduction was significantly higher ($p<0{\cdot}001$) in raw milk compared with pasteurized samples. Counts did not statistically differ between the different types of pasteurized milk. Further ANOVA tests revealed that temperature was a significant factor moderating survival in all samples, with higher

environmental temperatures leading to higher pathogen counts (p<0·001). Metabolic activity of *E. coli* O157:H7 at 20°C in all milk samples increased significantly on day 1, which continued to rise and peak (2·3–2·75 log10 RLU) at day 2. Cell activity dropped significantly afterwards in all samples, reaching zero in raw milk at day 6 and at day 10 in pasteurized milk samples (Fig. 1).

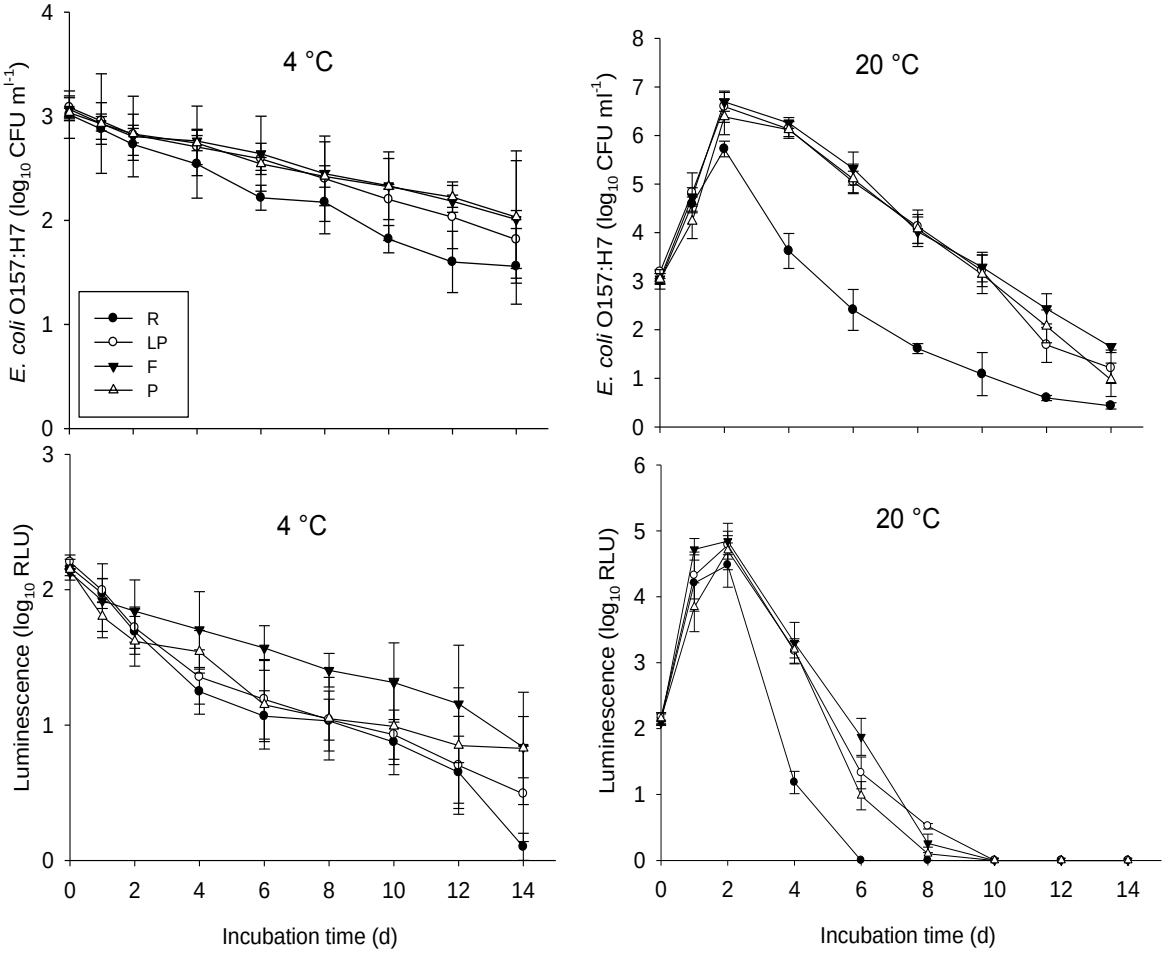

Figure 1. Survival and metabolic activity of *E. coli* O157:H7 in milk samples stored at 4 °C and 20 °C (R = raw milk, LP = laboratory–pasteurized milk, F = microfiltered pasteurized milk and P = commercially–pasteurized milk). Values represent means ± SEM (n = 3).

Cell activity in the three pasteurized milk samples was significantly higher than that in raw milk (p<0·05). Further ANOVA analysis indicated that temperature was an important

influence on *E. coli* O157:H7 cell activity, with the higher temperature inducing a peak at day 2 which was not observed at the low temperature.

3.3. Aerobic plate counts and pH

Changes in APC (measured in log10 CFU ml^{-1}) and pH values are shown in Fig. 2. At 4°C, average APC increased significantly more in raw (around 3·9 log count growth) than in pasteurized milk (<1·35 log count growth) (all p<0·001, mean counts: raw>laboratory–pasteurized>commercially-pasteurized>microfiltered). At 20°C, APC increased approximately 5–6 log10 CFU ml^{-1} in all milk samples after 14 d of incubation. APC in raw milk were significantly higher (p<0·001) than that in the three types of pasteurized milk, with values in microfiltered milk being significantly lower than those in the other pasteurized milk types (p<0·001). In the ANOVA test, temperature was found to be a significant factor in aerobic cell growth in the pasteurized milk samples. The growth rates in pasteurized samples were significantly higher under room temperature than under refrigeration temperature; although growth slowed down from day 8. In all, there was a final increase of around 6 log10 CFU ml^{-1} in APC from day 0 to 14.

At 4°C, no substantial changes in pH were observed in the pasteurized milk samples (Fig. 2), staying between 6·5 and 6·7; however pH values in raw milk exhibited a gradual decrease to 5·7. Over 14 d at 20°C, pH values decreased rapidly in all samples, from an average of 6·7 to 3·3 in raw milk, and to around 4·0–4·2 in pasteurized milk samples. Over the course of the experiment, there were significant differences in pH among the four types of milk (all p<0·001, pH mean: microfiltered>commercially-pasteurized>laboratory–

pasteurized>raw). Changes in pH values were negatively associated with the increase in APC, with higher numbers of aerobic microorganisms leading to lower pH values.

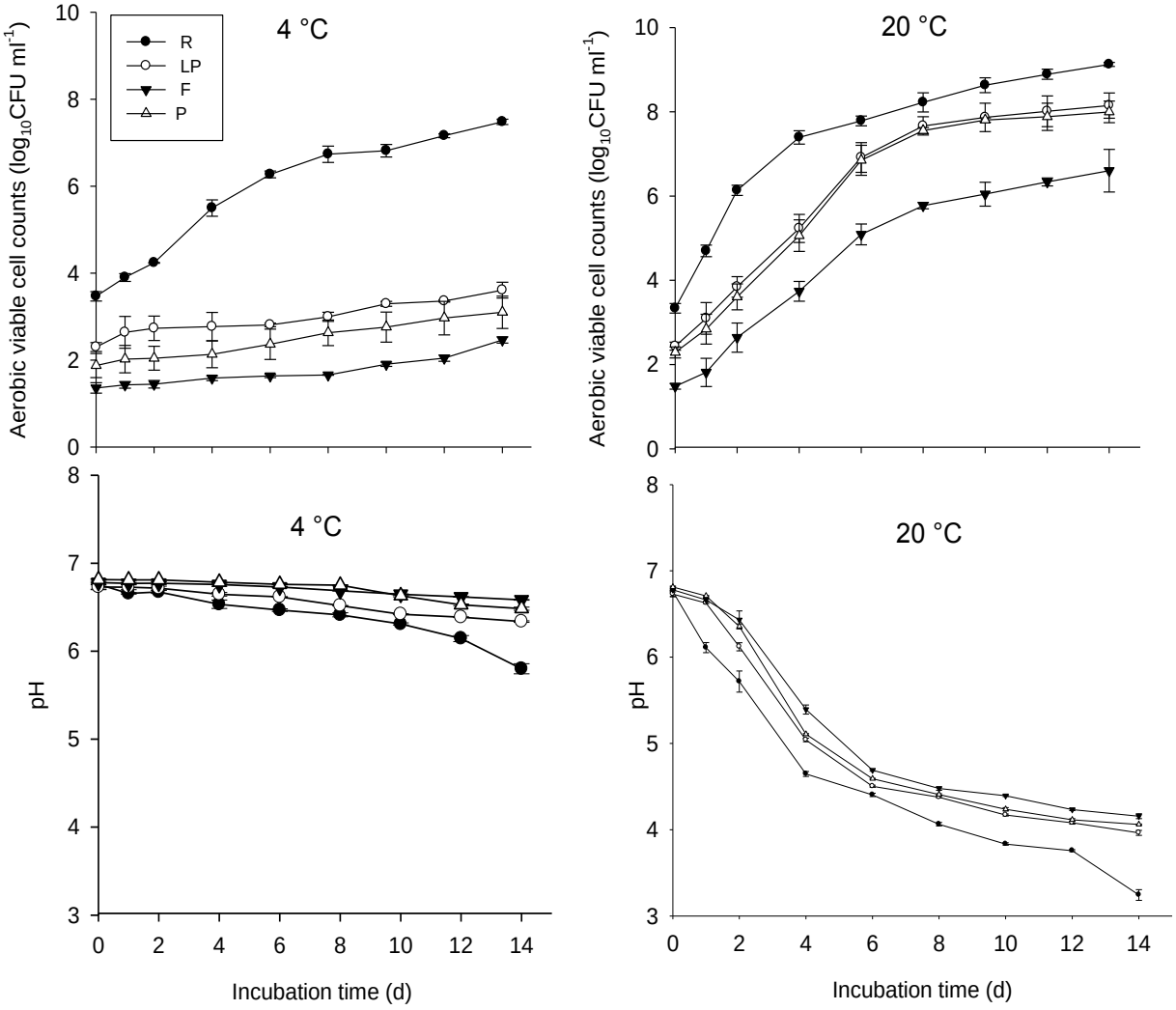

Figure. 2. Changes in aerobic plate counts (measured in $\log_{10}$ CFU ml^{-1}) and pH in milk samples stored at 4 °C and 20 °C (R = raw milk, LP = laboratory–pasteurized milk, F = microfiltered pasteurized milk and P = commercially–pasteurized milk). Values represent means ± SEM (n = 3).

4. Discussion

Pasteurized and unpasteurized milk may be contaminated with *E. coli* O157:H7 when inadequate farm hygiene measures (milking and milk handling) are present or post-pasteurization contamination occurs. Given the low infective dose of *E. coli* O157:H7 (Chart,

2000) and the association of milk with past infections, it is important to understand the behaviour of the organism in dairy products. Whilst others have previously studied changes in numbers of the organism in dairy products (e.g. Wang et al. 1997; Mamani et al. 2003; Marek et al. 2004), this is the first study to concurrently monitor the pathogen's metabolic activity. Given the association between metabolic activity and infectivity, this paper presents novel findings of interest to dairy microbiology and food safety.

The present study confirmed that temperature is an important factor that influences the survival and activity of *E. coli* O157:H7. We observed that *E. coli* O157:H7 could not grow under refrigeration conditions in any type of milk, which was largely consistent with results from previous studies on a limited range of milk types (Wang et al. 1997). Previous studies have recommended that milk be kept at ≤5°C as even at 7°C, *E. coli* O157 can grow at a significant rate (Heuvelink et al. 1998). Whilst other studies have also found the organism to survive and proliferate at room temperatures (Wang et al. 1997; Mamani et al. 2003), this study additionally revealed a corresponding increase in the pathogen's metabolic activity at elevated temperatures.

E. coli O157:H7 numbers and metabolic activity consistently decreased at a greater rate in raw milk than in the three types of pasteurized milk. Greater APC values were recovered from raw milk and this is expected to result in elevated competition with, and/or antagonism against the pathogen, as reported elsewhere (Wang et al. 1997; Elwell & Barbano, 2006). Storage of raw milk at 20°C also reduced pH considerably, most probably due to lactic acid production by the elevated counts of background micro–organisms (Kuippers et al. 2000). Acidic conditions (pH<3·5, Fig. 2) are likely be detrimental to survival of *E. coli* O157:H7; however it should be noted that the pathogen was not found to be eliminated at these low pH

values, consistent with previous studies that show its acid resistance and adaptation in acidic environments (Leyer et al. 1995; Mamani et al. 2003; Carter et al. 2011). In addition, raw milk may also contain several compounds with bioactive components (e.g. lactoferrin, lactoperoxidase and lysozyme) that can reduce or eliminate populations of pathogenic bacteria; however these will be lost during heat treatment (IDF, 1991).

To conclude, examining the role of incubation temperature has practical significance in understanding how *E. coli* O157:H7 and other aerobic cells behave in the food chain, from retailer fridge storage to the consumer home where the greatest risk of human infection occurs. We have shown that allowing contaminated milk to reach room temperature for even a space of 2 h can induce a transient proliferation of *E. coli* O157:H7 numbers and metabolic activity. Microfiltering milk did not have a significant effect on pathogen proliferation in comparison to normal pasteurization procedures and especially in comparison to the importance of storage temperate. Although pasteurization represents an effective measure to reduce pathogenic risks and improves the microbial quality of milk, consistent hygiene quality standards must be observed both pre- and post-pasteurization to guard against any possible pathogen and spoilage microorganisms.

5. Acknowledgements

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1. Introduction

With high water activity and moderate pH (5.5 to 7.0), meat is an ideal substrate for bacterial growth as it contains rich proteins and is low in carbohydrates (Anon, 2012). During the storage of meat, bacterial growth commonly induces undesirable organoleptic changes. If bacterial growth at the meat surface-packaging film interface could be reduced, this should extend product shelf–life (Ouattara et al., 2000). During transport, two factors that may lead to the growth of spoilage organisms are elevated temperatures and moist conditions due to humidity and condensation.

The ability of wool to act as an insulator is accepted and it is often used for such purposes in the construction industry. Due to its complex physical and chemical composition, wool can also help control humidity and reduce condensation (Woolcool® packaging company, 2012). Given these properties, the potential of wool to be used as packaging liners for the transport of meat is of interest. Woolcool® is a eco–friendly type of packaging, made of 100% pure sheep's wool, hygienically sealed in recyclable food–grade wrap[1] (Fig. 1).

Figure 1. Woolcool® packaging liners for boxes[1].

2. Objective

To determine whether meat stored in boxes lined with Woolcool® is of different microbiological quality to meat transported in conventional expanded polystyrene (EPS) boxes.

3. Methods

3.1. Packaging material

Cardboard boxes that were lined with Woolcool®, ± meat, and conventional EPS boxes ± meat were received at Bangor University (n = 1 of each box type). The boxes were received and analysed 40 hours after packing. Those boxes containing meat included pork (sausages, chops, bacon, gammon), beef (rolled brisket, various steaks, stewing beef) and lamb (breast, chops) products; with the same products within both box types. Apart from the sausages, all products were vacuum-packed; although the seal on some products had been broken, particularly those products that contained bone material (lamb chops and lamb breast). Ice packs were included within the meat boxes, although they had completely thawed on arrival to Bangor.

3.2. Microbiological characterisation

3.2.1. Sampling rationale

The microbiological quality of food products can be characterised on the basis of a range of conventional tests. In our experiment, microbiological analyses were conducted by measuring total viable cell counts, Enterobacteriaceae, *E. coli*, *K. pneumonia*, and *P. aeruginosa* and yeasts. Total viable cell counts (TVC) were performed by the plate–spread method. If the TVC of bacteria in fresh meat exceeds a certain number, spoilage of meat will occur. Therefore, TVC gives an indication of the keeping quality of meat. Enterobacteriaceae

refers to a group of Gram–negative bacteria that exist predominantly in animals, including most of the major food–borne pathogens such as *E. coli* O157:H7, *Shigella* and *Salmonella*. It is an indicator of faecal and environmental contamination showed by meat off-odour and off-flavour. The populations of three specific types of Enterobacteriaceae (*E. coli*, *K. pneumonia* and *P. aeruginosa*) were evaluated. Finally, the number of yeasts was monitored as yeasts are a major cause of food spoilage and play a crucial role in the sensory quality of meat (Dillon and Board, 1991).

3.2.2. Analyses

Swabs were taken from a number of areas within the boxes, including the surface of the boxes (at the top, middle and bottom), within the Woolcool® felt fibres, and from condensed liquid found on the surface of meat packs. A lamb breast joint from each box was sampled directly.

Plate Count Agar (Oxoid, product no. CM0463), violet red bile agar (Oxoid, product no. CM0107), malt extra agar (Oxoid, product no. LP0039) and brilliance *E. coli*/coliform agar (Oxoid, product no. CM0956) were used to assay bacteria numbers found on the different surfaces. Swabs were taken from meat at the top, middle, and the bottom of the boxes and then inoculated into 10 ml of ¼–strength Ringer solution (Oxoid, product no. BR002). This was then subject to a ten–fold serial dilution series. For the lamb breast (Fig. 2), a 25 g sub–sample was aseptically removed and mixed with 225 ml of Ringer solutions in a Seward 400 stomacher machine (Seward Ltd., Worthing, UK) at 230 rev min^{-1} for 30 s (Malpass et al., 2010), and 100 µl of the homogenate plated accordingly following serial dilution as described previously. Plates were incubated for 18-24 h at 37°C for bacterial analysis and for 3-4 days at 25°C for yeast.

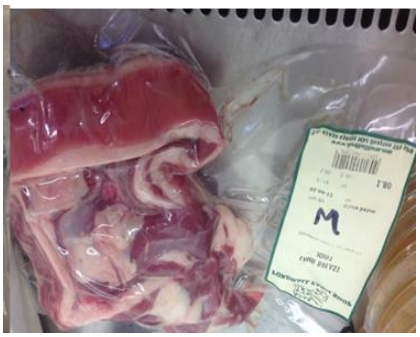

Figure 2. Vacuum-packed lamb breast sample.

4. Results

4.1. General quality

Meat quality is indicated by a list of sensory attributes such as colour, smell, and flavour. These factors are important for consumers to decide their purchase of meat (Maltin et al., 2003). In the experiment, after 40 hours storage of meat in EPS packed boxes and Woolcool® packed boxes, no difference was detected between these two types of packaging in terms of meat colour or smell. No difference in flavour or texture was obvious following tasting of equivalent meat products following identical cooking procedures.

4.2. Microbiological characterisation

Microbiological analyses based on the measures of TVCs, Enterobacteriaceae, *E. coli*, *K. pneumonia*, and *P. aeruginosa* and yeast are presented in Table 1. All samples taken from empty boxes were negative for the microbes tested (data not shown). Likewise, all samples taken from inside the Woolcool® packaging were also negative (data not shown). If a mean was taken of all microbial counts, a simple t–test indicates that there was a marginal but statistically significant difference in counts from swabs taken at the bottom of the boxes, with numbers in the Woolcool®-packaged boxes being lower (p = 0.048, 2-paired). However, such differences were not evident in the means of counts from swabs taken at the middle and top of

boxes ($p > 0.05$). Where microbial populations were analysed independently, only *K. pneumonia* showed any difference between the two box types, being significantly lower in the Woolcool®-packaged boxes ($p < 0.001$; Table 1).

Table 1. Microbial counts of swabs taken from EPS boxes containing meat and Woolcool®-lined boxes containing meat. Following packaging, meat was stored at room temperature for 40 h prior to analysis. Samples were taken from the top, middle and bottom surfaces of boxes; from condensation on meat products; and from a lamb breast joint within each box. P–values refer to significance of difference between adjacent means, where values < 0.05 show statistically significant differences. 'n.d' refer to 'none detected'.

Test	EPS–packed + fresh meat products (CFU ml^{-1})					Woolcool®–packed + fresh meat products (CFU ml^{-1})					
	Top	**Middle**	**Bottom**	**Condensation**	**Meat**	**Top**	**Middle**	**Bottom**	**Condensation**	**Meat**	**P value**
Total viable counts	2.82	3.46	2.91	2.67	4.00	2.02	3.12	2.40	2.60	3.99	$p = 0.36$
Enterobacteriaceae	1.00	n.d	1.52	1.56	3.60	n.d	2.430	1.91	n.d	3.71	$p = 0.13$
E. coli	0.98	1.73	1.42	1.46	3.21	n.d	1.17	0.65	n.d	2.90	$p = 0.16$
K. pneumonia	0.59	1.53	1.39	0.33	3.15	n.d	0.89	0.89	n.d	2.86	$p < .001$
P. aeruginosa	n.d	1.24	1. 47	0.43	3.16	n.d	1.24	1.24	n.d	2.79	$p = .01$
Yeast	2.08	2.74	2.56	2.40	4.00	1.59	2.32	2.59	2.07	2.68	$p = 0.2$

5. Discussion and conclusions

The present study assessed the microbiological quality of meat packaged and stored at room temperature for 40 h in conventional EPS boxes and cardboard boxes lined with Woolcool® using standard, approved culturing techniques. It also sampled empty boxes stored under the same conditions.

There were a number of limitations to the study. Firstly, it should be remembered that the number of replicates was low (effectively $n = 1$ for each box type). Secondly, although the meat types within both boxes were the same, directly comparing samples should be done with caution as bacterial contamination of meat can be very localised hence bacterial numbers can vary considerably between samples. However, under the circumstances, the best scientific methodology was practised throughout and the results are of value.

Whilst these results cannot indicate whether one box type is superior to the other in terms of maintaining the microbiological quality of meat, the study does indicate that Woolcool® packaging is at least as good as EPS–packaging for this purpose. This may signify that the product has potential market value for the purpose of liners for transporting meat, and possibly other food products. However, this should be validated against other measures of its suitability (e.g. costs; results from temperature loggers) and ideally from a larger microbiological study.

6. References

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1. Introduction

Meat spoilage is caused by biological deterioration of a product, which is potentially hazardous to health (Haque et al., 2008) and considered unacceptable by the consumer due to defects such as off–flavours, off-odour, sour taste, discoloration and slime formation (Nychas et al., 2008; Maltin et al., 2003). Poor operational techniques during the slaughter of animals and the subsequent stages of processing and storage of the meat may lead to elevated microbial counts and hence reduce shelf life and quality (Dave and Ghaly, 2011; FAO, 2007). Packaging is important in maintaining the quality and safety of meat and the type of packaging can influence the microbial flora of meat (Olaoye and Ntuen, 2011). It can also affect the relative humidity of the meat environment, with lower humidity associated with lower microbial counts.

Central to the above factors is the control of temperature; with meat needing to be stored at refrigeration temperatures (typically 1-4°C) to restrict microbial growth. Packaging that can maintain such temperatures during transportation aids in the delay of spoilage micro–organisms (Renerre and Labadie, 1993).

Wool is often used as an insulator in the construction industry due to its complex physical and chemical composition, which helps control humidity and reduce condensation (Woolcool® Packaging Company, 2012). Woolcool® packaging, made of 100% pure sheep's wool, hygienically sealed in recyclable food-grade wrap, may therefore have potential as a packaging liner for the transport of meat.

2. Objective

This study was conducted to investigate whether raw meat stored in boxes with lined or unlined Woolcool®, is of different microbiological quality to meat transported in conventional expanded polystyrene (EPS) boxes.

3. Material and methods

3.1. Sample collection

Three cardboard boxes were prepared: one containing lined Woolcool® (WC), one unlined Woolcool® (WCUN) and one EPS. A 10 kg variety of fresh meat products (beef, pork, lamb joints) were packed into each box (Figure 1), and left unrefrigerated for 72 hours. The boxes were then opened, and swabs taken from the top, middle and bottom surface of each box and from the condensed liquid found on the surface of meat packs. Samples were also taken from the lamb shoulder joint from each box. They were then analysed for microbiological contamination as described below.

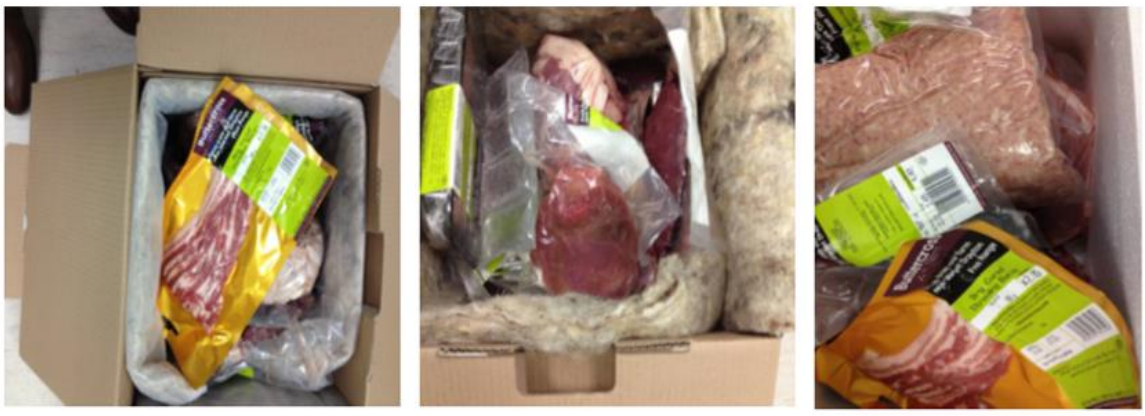

Figure 1. Sample boxes with meat (left-right: Woolcool® lined, Woolcool® unlined, expanded polystyrene).

3.2. Microbiological characterization

The following media were used to assay bacteria counts on meat and box surfaces: Plate Count Agar (Oxoid, product no CM0463) for total viable counts (TVC), Malt Extra Agar (Oxoid, product no LP0039) for fungi and Brilliance *E. coli*/coliform agar (Oxoid, product no CM0956) for *E. coli* and coliforms; as described in Lahmer et al. (2012). The swabs were inoculated into 10 ml of ¼-strength Ringer solution (Oxoid, product no. BR002), which was then subject to a ten–fold serial dilution series. A 25 g sub-sample was aseptically removed from the lamb shoulder joint, and mixed with 225 ml of Ringer solutions in a Seward 400 stomacher machine (Seward Ltd., Worthing, UK) at 230 rev min^{-1} for 30 s (Malpass et al., 2010). One ml of the homogenate was then plated following the serial dilution described previously. Plates were incubated for 48 h at 37°C for TVC, 18-24 h at 37°C for *E. coli* and for 3-4 days at 25°C for fungi. Colonies were counted manually.

4. Results

4.1. Sensory qualities

After 72 hours of storage in EPS or Woolcool® packed boxes, the sensory quality of each lamb shoulder joint was compared qualitatively (subjectively), using sensory attributes such as colour and smell. No difference was detected between meat kept in the two Woolcool® boxes (lined and unlined), but meat in the EPS boxes showed some signs of the early stages of spoilage, presumably due to the breakdown of fat, protein and carbohydrates.

4.2. Microbiological characterization

The results of the microbiological analysis based on the measures of TVC, *E. coli*, other coliforms and fungi are presented in Table 1 and Figure 2.

Table 1. Microbial counts of swabs taken from EPS boxes containing meat and Woolcool®-lined unlined boxes (WCUN, WC) containing meat. Following packaging, meat was stored at room temperature for 72 h prior to analysis. Samples were taken from the top (T), middle (M) and bottom (B) surfaces of boxes; from condensation (C) on meat products; and from a lamb shoulder joint within each box. 'n.d' refers to 'none detected'.

Test	EPS–packed + fresh meat products (CFU ml^{-1})					WCUN–packed + fresh meat products (CFU ml^{-1})					WC–packed + fresh meat products(CFU ml^{-1})				
	T	**M**	**B**	**C**	**Meat**	**T**	**M**	**B**	**C**	**Meat**	**T**	**M**	**B**	**C**	**Meat**
Total viable counts	n.d	n.d	0.77	2.26	7.00	n.d	n.d	2.55	1.43	5.23	n.d	n.d	1.69	0.97	6.00
E. coli	n.d	n.d	n.d	n.d	5.64	n.d	n.d	1.33	n.d	2.39	n.d	n.d	n.d	n.d	4.20
Coliform	n.d	n.d	n.d	n.d	5.34	n.d	n.d	n.d	n.d	3.27	n.d	n.d	n.d	n.d	4.85
Fungi	n.d	n.d	n.d	n.d	6.53	n.d	n.d	n.d	n.d	4.88	n.d	n.d	1.67	n.d	5.16

Swab samples taken from the middle and top were negative for the microbes tested in all box types (data not shown). Post-hoc analyses were run using Tukey HSD statistic, unless homogeneity of variance could not be assumed, in which case Games–Howell was used. For TVC, post-hoc analyses (Games-Howell) found significant differences between EPS and WCUN ($p < .001$), between EPS and WC ($p = .006$) and between WC and WCUN ($p = .014$). For *E. coli* (Tukey HSD), there was a significant difference between EPS and WC ($p = .003$), between EPS and WCUN ($p < .001$) and between WC and WCUN ($p = .001$). For coliforms, post-hoc analyses (Tukey HSD) found a significant difference between EPS and WCUN ($p < .001$) and between WC and WCUN ($p < .001$), but no significant difference between EPS and WC ($p = .069$). For fungi (Games-Howell) the EPS and WCUN comparison was significant ($p = .009$), as was EPS and WC, $p = .001$ but there was no significant difference between WC and WCUN, $p = .259$.

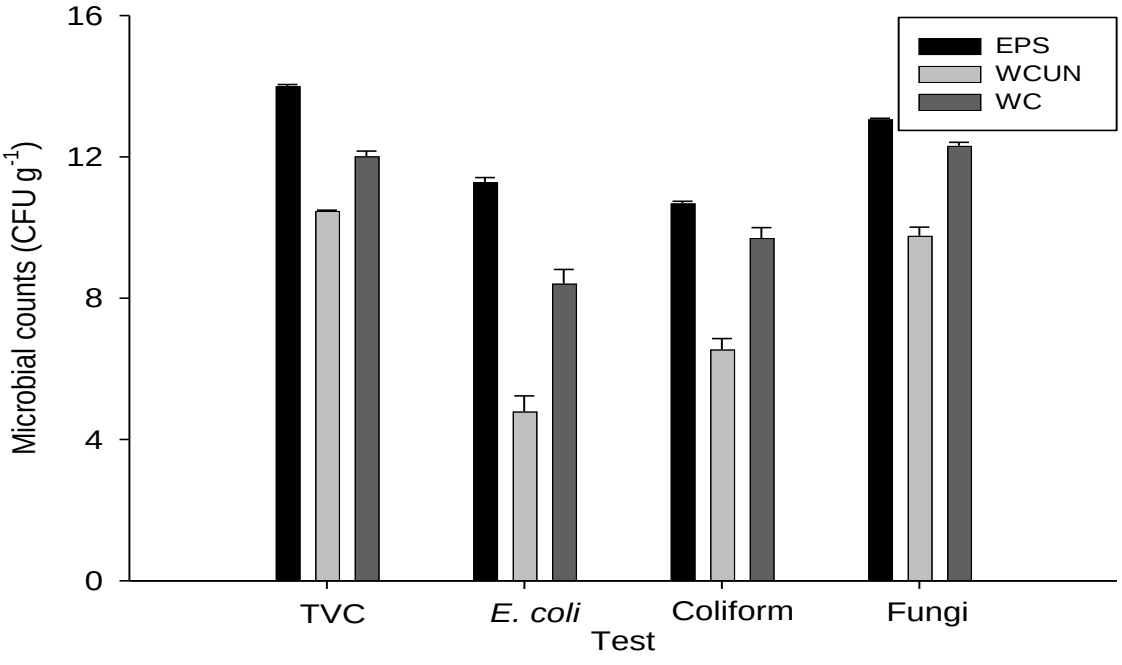

Figure 2. Microbial load analysis in meat (log $CFUg^{-1}$).

5. Discussion and conclusions

In the present study, a variety of meat was stored at room temperature for 72 h in either conventional EPS boxes or cardboard boxes lined or unlined with Woolcool®, before being assessed for microbiological quality. For all microbial measurements, EPS revealed the highest count, with this being significantly higher than WC and WCUN in many cases (with the exception of coliform). In general, WCUN revealed significantly lower counts than WC (except for measurements of fungi).

Although the best scientific methodology was practiced throughout, the study has several limitations. Firstly, the number of replicates was low, with each box type tested only once. Secondly, localised bacterial contamination of meat may result in considerable variation of bacteria count between samples. Therefore, directly comparing samples should be done with caution, although the meat types contained within all boxes were the same and the methods used were consistent throughout.

Although based on a limited sample set, these results suggest that Woolcool® may be superior to EPS in maintaining the microbiological quality of the meat. The findings support those of Lamher et al. (2012) and suggest that the product may have potential market value as packaging liners for transporting meat, and possibly other food products. It should be noted that the study was carried out under small scale laboratory conditions. Further research is needed to allow better generalisation to real-world conditions, and understanding of how these packaging liners could maintain food quality on a larger scale.

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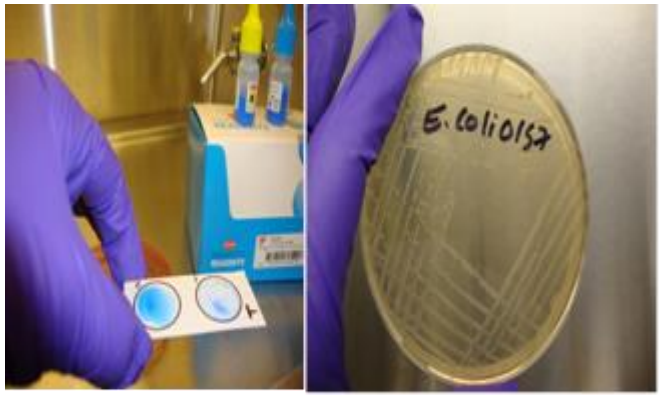

Figure1. Identification of *E. coli* O157

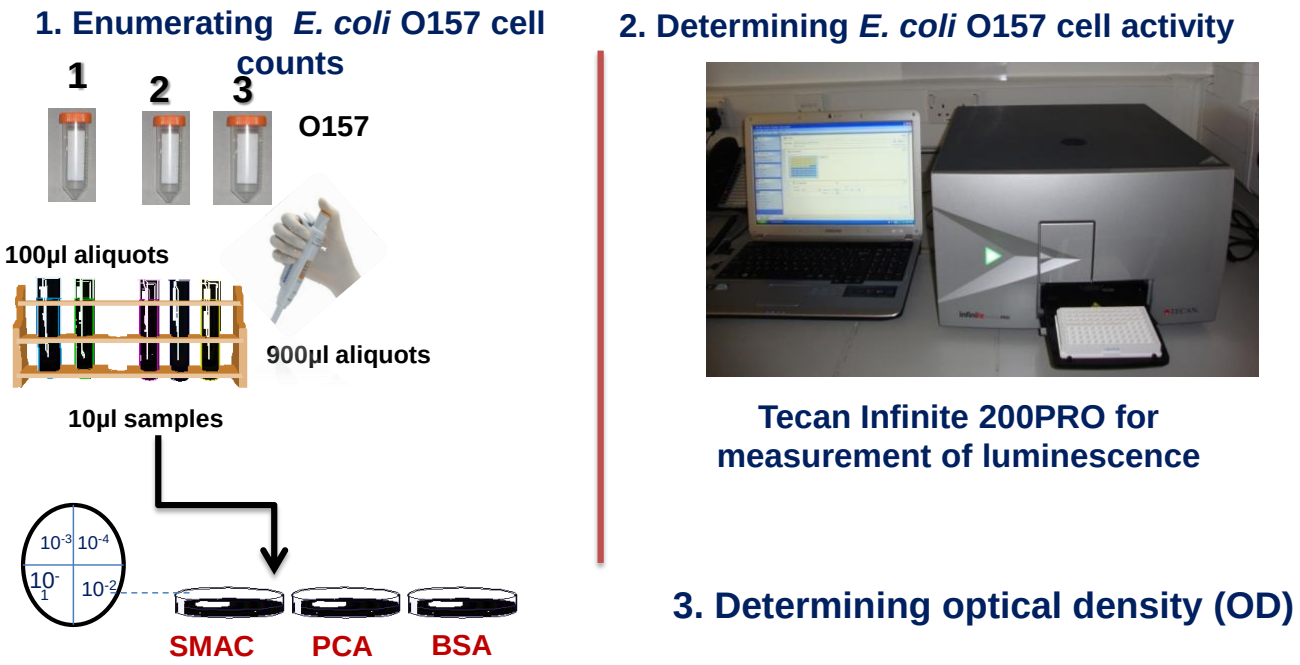

Figure 2. Procedure of microbial assessments of Ch-arg

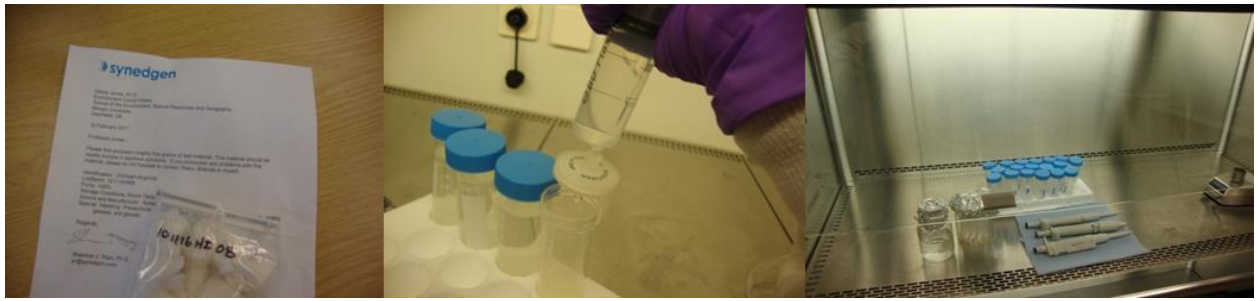

Figure 3. Preparation of Ch-arg solution

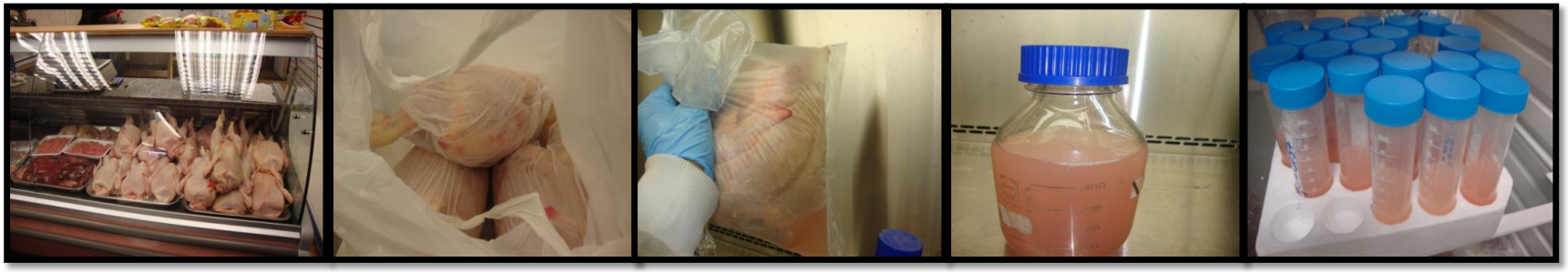

Figure 4. Preparation of chicken juice

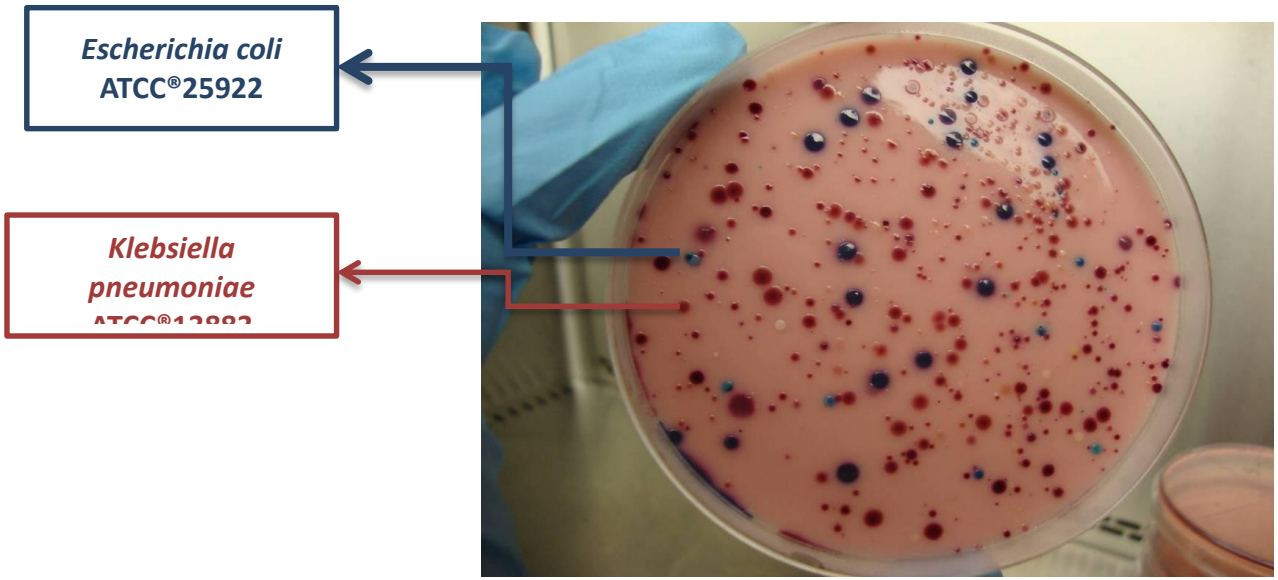

Figure 5. *E. coli*/coliform isolated from chicken juice

Figure 6. Preparation of beef juice

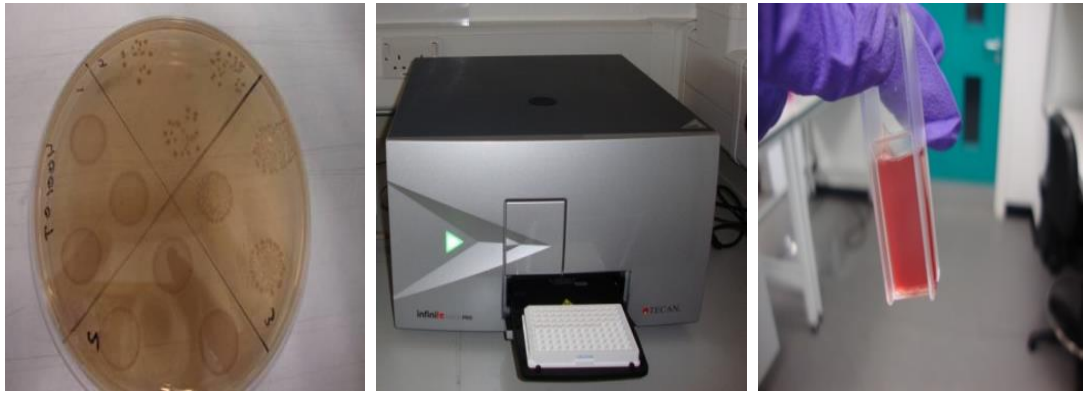

Figure 7. Procedure of microbial assessments of Ch-arg; A: cell count, B: luminescence, C: OD

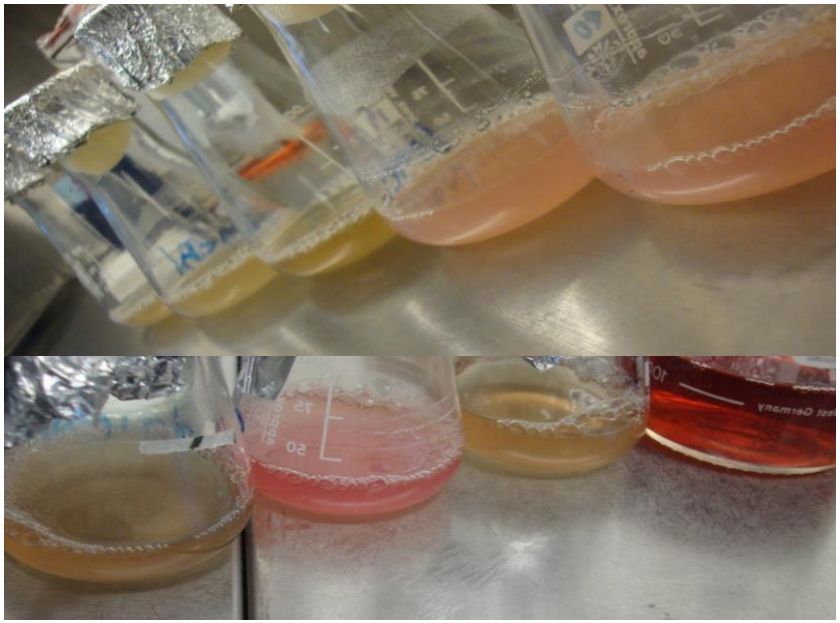

Figure 8. Change of colour over time of beef juice media: Cell age experiment

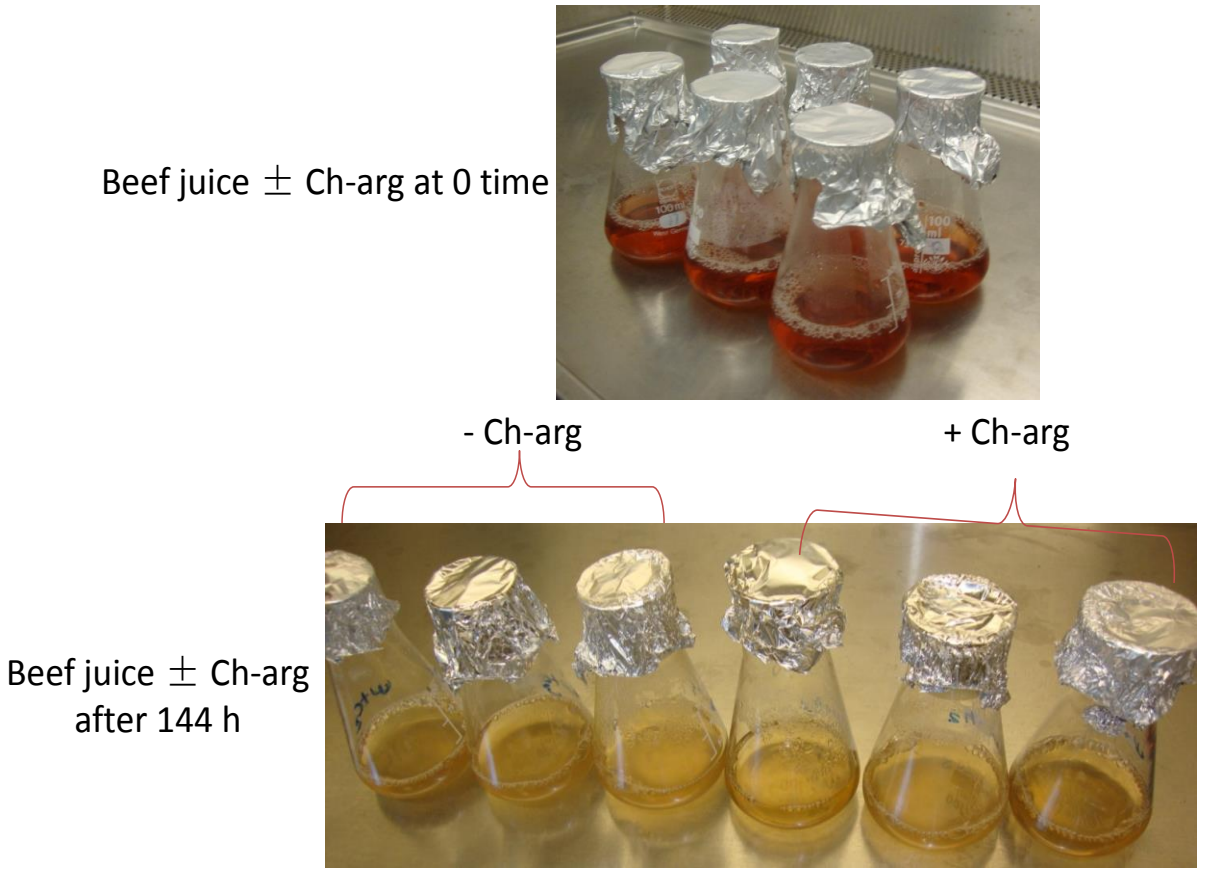

Figure 9. Testing Susceptibility of *E. coli* O157 to chitosan-arginine in beef juice

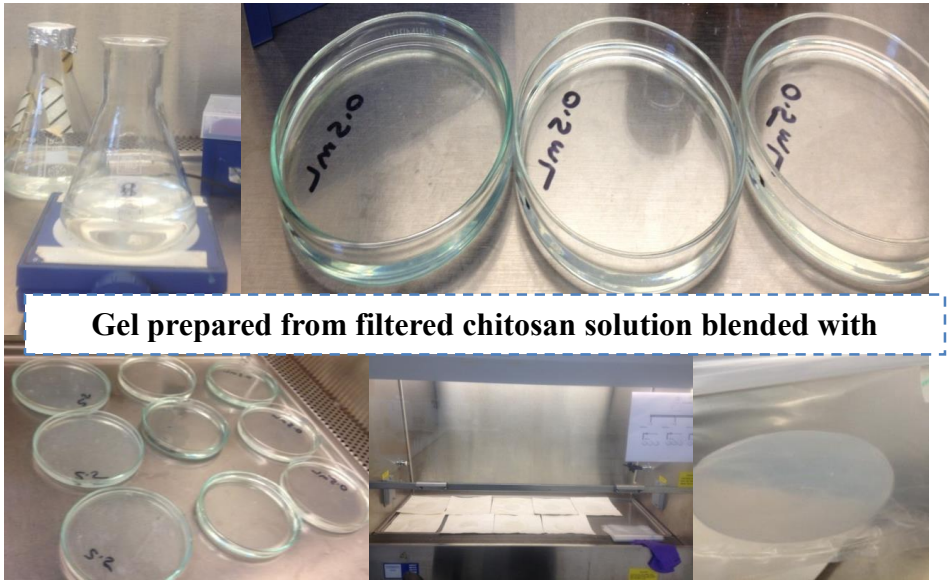

Figure 10. Making Ch-arg film

Figure 11. Chitosan-arginine film

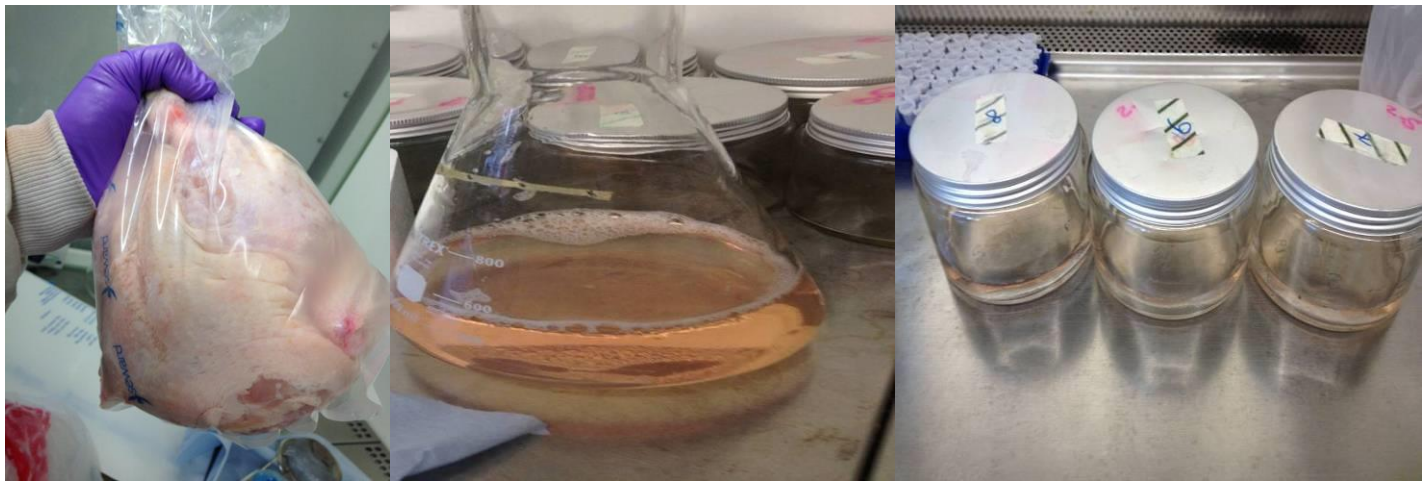

Figure 12. Preparing Chicken meat juice samples

Figure 13. Preparing Chicken meat juice samples

Figure 14. Ch-arg antimicrobial packaging, tested in beef juice

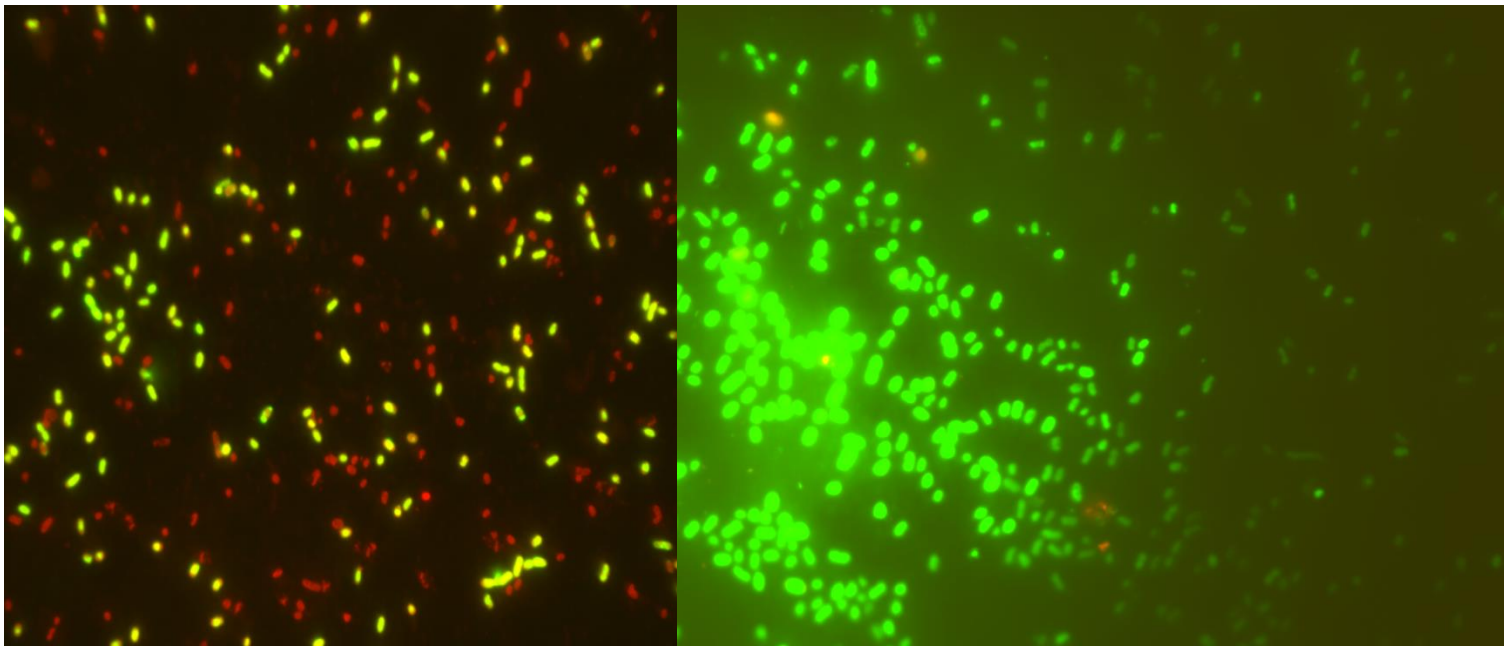

Figure 15. *E. coli* O157 viewed using BacLight™ method

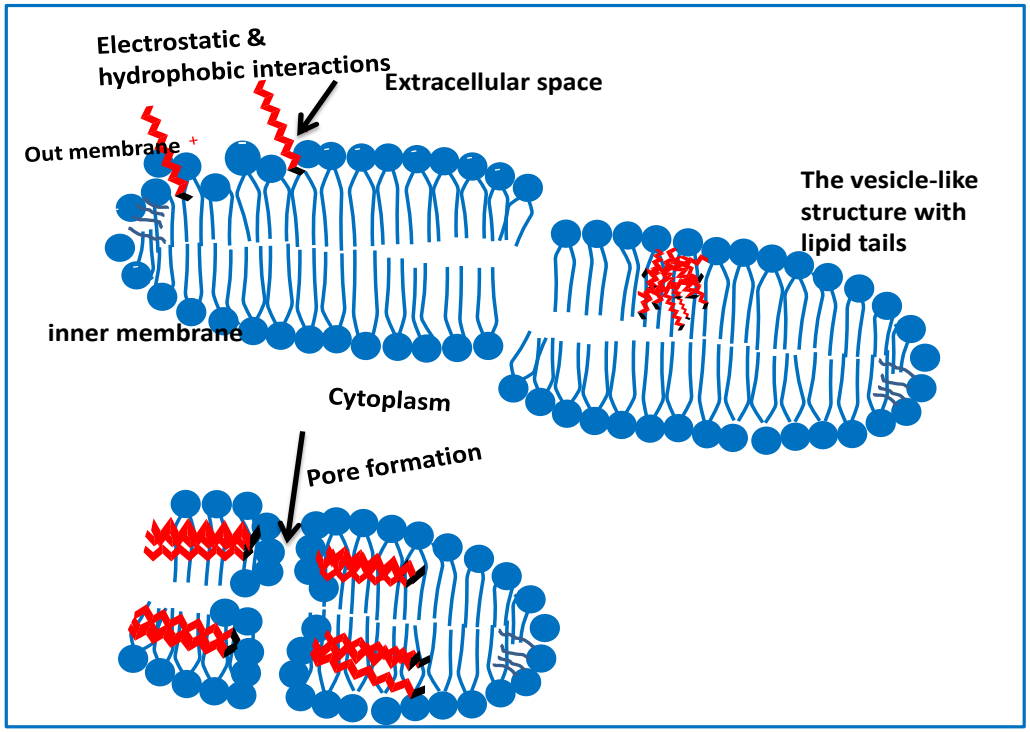

Figure 16. Model of potential mechanisms by which cationic peptides exert antimicrobial effects.