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THE EFFECT OF HOST-SPECIFIC BACTERIOPHAGES TO REDUCE THE PRESENCE OF *Campylobacter* AND *E. coli* O157:H7 FOOD-BORNE PATHOGENS

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ABSTRACT

The severity of illness caused by both *Campylobacter* and *E. coli* infection brings these organisms to the forefront of food safety concerns and encourages research to investigate food processing strategies that reduce this risk. This study aimed to investigate the ability of host-specific phage to reduce the presence of *Campylobacter* and *E. coli* which are not actively replicating on both chickens and ground beef at 4°C and −20°C. The application of bacteriophage to the inoculated chicken skin and ground beef with *Campylobacter* and *E. coli* O157:H7 respectively successfully reduced the number of viable cells and this reduction was quite constant over the period of the experiment. There were significant reductions ($P < 0.05$) in the numbers of *Campylobacter* recovered from fresh or frozen chicken skin compared to those of the control, falling by $\log_{10}$ 0.3 to $\log_{10}$ 1.3 CFU (*C. jejuni* 11168H infected with phage CP8) and $\log_{10}$ 0.4 to $\log_{10}$ 1.6 (*C. coli* OR12 infected with phage CP220). Furthermore, significant reductions ($P < 0.05$) in the numbers of *E. coli* in both fresh and frozen ground beef were observed, falling by $\log_{10}$ 0.3 to $\log_{10}$ 1.1 CFU (*E. coli* 12900 infected with phage CBA120) and $\log_{10}$ 0.4 to $\log_{10}$ 1.2 (*E. coli* 12900 infected with phage CEV2). There was no increase in phages titers over the 10-day period of the experiment and effective cocktails of broad-host-range bacteriophages would be a good choice to reduce the presence of these food-borne pathogens.

INTRODUCTION

Poultry and especially under-cooked chicken are considered major reservoirs of *Campylobacter* infection (Jacobs-Reitisma, 2000; Shane, 2000). Different epidemiological studies have revealed the significant relationship between sporadic human *Campylobacter* infection and the handling, preparation of chicken or consumption of undercooked chicken (Park *et al*., 1991).

Campylobacters can frequently be isolated from the feathers and skin of broiler chickens (Berrang *et al*., 2000). Cross-contamination of kitchen surfaces or utensils or direct hand to mouth contact after handling raw chicken can happen easily in the domestic setting (Jacobs-Reitisma *et al*., 2008). The infectious dose of *Campylobacter* (500 CFU) is relatively low, so contaminated chicken meat is a source of human infection if it is not properly handled or not sufficiently cooked to kill the campylobacters (Friedman *et al*., 2000; Jacobs-Reitisma *et al*., 2008).

Previous studies have shown that freezing to −20°C and cooling to 4°C reduced viable counts of campylobacters (Lee *et al*., 1998; El-Shibiny *et al*., 2009). However, it could be ineffective in substantially reducing the numbers of human pathogens, including *Campylobacter*, associated with the

chicken skin (Whyte *et al*., 2001; El-Shibiny *et al*., 2009). Increasing the concentration of hyperchlorite may decrease the numbers of pathogens but it will reduce the quality of the end product, which is unacceptable to consumers. Interactions may occur between strains in mixed populations that co-colonise chickens, which can result in shifts in the proportion of each strain during the rearing of poultry (El-Shibiny *et al*., 2007).

In 1993, an *E. coli* O157:H7 outbreak of infection involving 700 illnesses and 4 deaths was associated with eating fast-food at a national restaurant chain in USA (Bell *et al.*, 1994). Afterward, 25 million pounds of ground beef were recalled because of *Escherichia coli* O157: H7 contamination (Mead and Griffin, 1998). Bovine foods, such as ground beef is consider a vehicle for *Escherichia coli* O157:H7 outbreaks, which causes bloody diarrhea, hemorrhagic colitis, and, in certain cases, hemolytic uremic syndrome (Kaper *et al*., 2004). The contamination of beef always occurs during slaughter when meat is contaminated by fecal material since *E. coli* O157:H7 is prevalent among cattle entering the slaughterhouse and cattle carcasses at all stages of the slaughter process (Elder *et al*., 2000).

Different studies have revealed the ability of *E. coli* to survive in different environmental conditions (An-Hung *et al*., 1995, Arias *et al*., 2000). Decontamination of meat has been successfully applied commercially in U.S. to diminish the prevalence of *Escherichia coli* O157:H7 from carcasses (Bacon *et al*., 2000). However, *E. coli* may survive *in vitro* exposure to acidic meat decontamination and become acid adapted (Samelis *et al*., 2005) which may increase its ability to cause foodborne illness (Zhao *et al*., 1993; Skandamis *et al*., 2009).

Host-specific bacteriophage have previously been used in the treatment of *E. coli* infections in piglets (Smith and Huggins, 1983), *Campylobacter* infection in chicken skins (Atterbury *et al*., 2003) and *Pseudomonas* infection on beef (Greer, 1986).

There are two types of phage replication: the temperate and lytic phases. The temprate phase which is also known as the prophage infects susceptible bacteria and the phage genome is merged with the bacterial DNA without bacterial lysis until the lystic cycle is activated by external factors such as ultraviolet light (Campbell, 2003). The lytic cycle, the preferred for the biocntrol of pathogenic bacteria, causes cellular lysis, releasing numerous phage progeny (Abedon *et al*., 2001).

The aim of this study was to study the ability of host-specific phage to reduce the presence of a food-borne pathogen which is not actively replicating on both chickens and ground beef at 4°C and −20°C.

MATERIALS AND METHODS

Bacterial strains

C. coli OR12 was isolated from a UK organic chicken farm (El-Shibiny *et al*., 2005), *C. jejuni* 81116H was obtained from I. Connerton, Food Sciences Department, University of Nottingham, UK and *E. coli* O157:H7 NCTC 12900 was obtained from E. Kutter, Phage Lab, The Evergreen State

College, USA. The *Campylobacter* strains were routinely cultured on horse blood agar (BA), which was composed of blood agar base No. 2 (CM0271, Oxoid, Basingstoke, UK) supplemented with 5% defibrinated horse blood (TCS, Buckingham, UK), under a microaerobic atmosphere containing 5% O_2, 5% H_2, 10% CO_2, 80% N_2 at 42 °C for 24 h. the *E. coli* strain was cultured on Tryptic Soy Agar (TSA) (Difco Laboratories, Detroit, MI) at 37 °C for 24 h.

Bacteriophage strains and propagation:

Bacteriophages CP8 and CP220 were obtained from I. Connerton, Food Sciences Department, University of Nottingham, UK while CBA120 and CEV2 were obtained from E. Kutter, Phage Lab, The Evergreen State College, USA. Propagation of high-titer stocks of phages CP8 and CP220 was carried out as previously described (Atterbury *et al*., 2003a) using the plate lysis method (Frost *et al*., 1999) with *C. jejuni* NCTC 12662 as host. Bacteriophages CBA120 and CEV2 were propagated in *E. coli* O157:H7 NCTC 12900 previously described (Raya *et al*., 2006) using liquid amplification method.

Preparation of chicken skin and inoculum:

Chickens were sacrificed when aged 30 days. Caecal contents were collected after slaughter and examined for *Campylobacter* colonisation by direct plating of a 10% (w/v) suspension in maximum recovery diluent (MRD;CM0733, Oxoid) on modified Cefoperazone Charcoal Desoxycholate Agar (mCCDA; CM7309, Oxoid) supplemented with cefoperazone (Pro-Lab Diagnostics). Plates were incubated under microaerobic conditions at 42 °C for 48 h before examination for typical *Campylobacter* colonies. The chicken carcasses were manually plucked and the skin removed. The breast skin from each carcass was cut into 2 cm^2 sections and transferred to 24-well plates (Sterilin, Staffordshire, UK) until inoculation with *Campylobacter* and bacteriophages. Random skin samples from each carcass were confirmed as being *Campylobacter* negative by homogenising the excised sections at 1 g per 10 ml of MRD and direct plating on mCCDA. *Campylobacter* strains were grown in nutrient broth No 2 (CM 67, Oxoid) for 24 h at 42 °C under microaerobic conditions and then diluted 1:10 in fresh nutrient broth No 2. For mixed inoculums the suspensions were diluted 1:5 then combined in equal volumes. Skin samples were inoculated with *Campylobacter* and bacteriophage suspensions onto the 2 cm^2 area of skin and stored in sterile 24-well plates (Sterlin) at 4 °C or at −20 °C for up to 9 days. Sections of skin from each storage condition were removed for the recovery of both *Campylobacter* and bacteriophage, initially 1 h after inoculation (day 1) and then at 24-h intervals thereafter for a period of 10 days.

Preparation of ground beef and inoculum:

Ground beef was purchased from a local grocery and tested for intrinsic *E. coli O157:H7*. Ground beef was divided aseptically into 25-g portions in sterile bags. Beef samples were artificially contaminated with *E. coli* O157:H7 cells and treated with bacteriphages. Viable cell counts were confirmed by retrospective spread-plating 10-fold serial dilution onto tryptic soy agar (TSA) plates. Artificially contaminated beef samples were stored at 4 °C or at −20 °C for up to 9 days. Replicate samples were removed from

storage each day for 9 days and the surviving *E. coli* and bacteriophage enumerated (Raya *et al*., 2006; El-Shibiny *et al*., 2009).

Enumeration of *Campylobacter* and *E. coli*:

The samples inoculated with *Campylobacter* were aseptically transferred into sterile bags containing 10 ml of MRD and mixed together for 5 min as described before (El-Shibiny *et al*., 2009). The suspension was serially ten fold diluted to 10^{-6} using MRD. One hundred microlitres of each dilution were spread onto plates of mCCDA supplemented with agar (L13, Oxoid) to obtain an agar concentration of 2% (w/v) to reduce swarming. The plates were incubated at 42 °C under microaerobic conditions and the colonies counted at 24 h and at 48 h. On the other hand, the samples inoculated with *E. coli* were aseptically transferred into sterile bags containing 10 ml of TSB and mixed together for 5 min. The suspension was serially ten fold diluted to 10^{-6} using TSB. One hundred microlitres of each dilution were spread onto plates of TSA. The plates were incubated at 42 °C under aerobic conditions and the colonies counted at 24 h (Raya *et al*., 2006).

Enumeration of Bacteriophage:

Bacteriophage were recovered by stomaching the skin sections in a sterile bag as described before. An aliquot of the stomachate (1 ml) was centrifuged at 13,000 x *g* for 3 min in a Centaur microcentrifuge to remove bacterial cells. Serial dilutions of the supernatant were used to inoculate a lawn of susceptible host cells (*C. jejuni* NCTC 12662 for *Campylobacter* bacterophage and *E. coli* O157:H7 NCTC 12900 for *E. coli* bacteriophage) using a modification of the surface droplet technique (Salama *et al*., 1990) with enumeration performed according to the method of Miles and Misra, 1938. Bacterial lawns were prepared as described before (El-Shibiny *et al*., 2005; Raya *et al*., 2006). Plates were left to set for 20 min prior to the spotting of phage suspensions onto the surface. After inoculation of the phage the plates were left to dry for 20 min before incubation (in microaerobic conditions at 42°C for *Campylobacter* bacterophage and in aerobic conditions at 37°C for *E. coli* bacteriophage) for 24 h and then were examined for the presence of plaques.

Statistical analysis:

Three independent experiments were carried out in triplicate and statistical analysis was performed on $\log_{10}$ transformed values using the means of triplicate viable count values, for each data point obtained. The data were analysed using the Microsoft Excel software package (MicroSoft Corporation, Redmond, Washington, USA).

RESULTS AND DISCUSSION

The viability of *C. jejuni* 11168H and *C. coli* OR12 from the surface of chicken skin is presented in (Fig. 1). The viability of both *C. jejuni* 11168H and *C. coli* OR12 at the initial time point was identical ($\log_{10}$ 7 CFU and $\log_{10}$ 6.3 CFU respectively) for skin samples stored at 4 and -20°C. However, recovery from the fresh samples continued to gradually decline to reach a base of $\log_{10}$ 4.3 CFU for *C. jejuni* 11168H and $\log_{10}$ 4.0 CFU for *C. coli*

OR12 at the end of the experiment. In contrast, the freeze-thaw treatment of the skin resulted in a fall in *Campylobacter* recovery to $\log_{10}$ 4.0 CFU for both *C. jejuni* 11168H and *C. coli* OR12. The recovery of *Campylobacter* at 4 and -20°C was quite sustained at approximately the same level for the rest of the experiment. Considering these data, it is possible that chicken skin has a protective effect on the *Campylobacter* cells. This protective effect may be due to the presence of feather follicles and folds on the skin surface, in conjunction with the presented oils and fats which may offer some protection from ice crystal formation that may cause a major damage to cells throughout freezing process (Atterbury *et al*., 2003b).

Our previous investigations into the survival of *Campylobacter* isolates stored at 4 and -20°C demonstrated the survival of *Campylobacter* and indicated that survival is actually likely to be enhanced by super-chill treatments particularly if poultry is to be stored at 4°C (El-Shibiny *et al*., 2009). Previous studies have reported that chicken carcasses are contaminated with up to 10^9 CFU of *Campylobacter* (Jorgensen *et al*., 2002). Certainly, this will increase the level of *Campylobacter* contamination and the concomitant risk of food poisoning to the consumer since the infective dose of *Campylobacter* for humans is thought to be about 500 cells (Black *et al*., 1988), and these results were in general agreement with previous studies (for example Chan *et al*., 2001; Solow *et al*., 2003).

The recovery of bacteriophages CP8 and CP220 from chicken skin stored at 4°C remained moderately stable at ~ $\log_{10}$ 4.0 PFU and ~ $\log_{10}$ 4.5 PFU respectively of the initial inoculated titer (~ $\log_{10}$ 5.0 PFU) for the period of the experiment, falling slightly to $\log_{10}$ 3.0 PFU with phage CP8 at day 3. The recovery of phage CP8 inoculated on chicken skin stored at -20°C was quite similar to that recovered from skin stored at 4°C compare to initial inoculated titer ($\log_{10}$ 4.7 PFU) for the period of the experiment, falling slightly to $\log_{10}$ 3.7 PFU with phage CP8 at day 5. Alternatively, it remained stable with phage CP220 at ~ $\log_{10}$ 4.3 PFU of the initial inoculated titer (~ $\log_{10}$ 5.5 PFU) for the period of the experiment. There was no increase in both phages titers over the 10-day period of the experiment.

In order to study the effect of phage application on the viability of *Campylobacter* at 4 and -20°C, sections of chicken skin (2 cm^2) were inoculated with combinations of different bacteriophages and host titers at Multiplicity of Infection (MOI) ~ 0.01 as described in materials and methods. There were significant reductions ($P < 0.05$) in the numbers of *Campylobacter* recovered from fresh or frozen chicken skin compared to those of the control, falling by $\log_{10}$ 0.3 to $\log_{10}$ 1.3 CFU (*C. jejuni* 11168H infected with phage CP8) and $\log_{10}$ 0.4 to $\log_{10}$ 1.6 (*C. coli* OR12 infected with phage CP220) (Fig. 1).

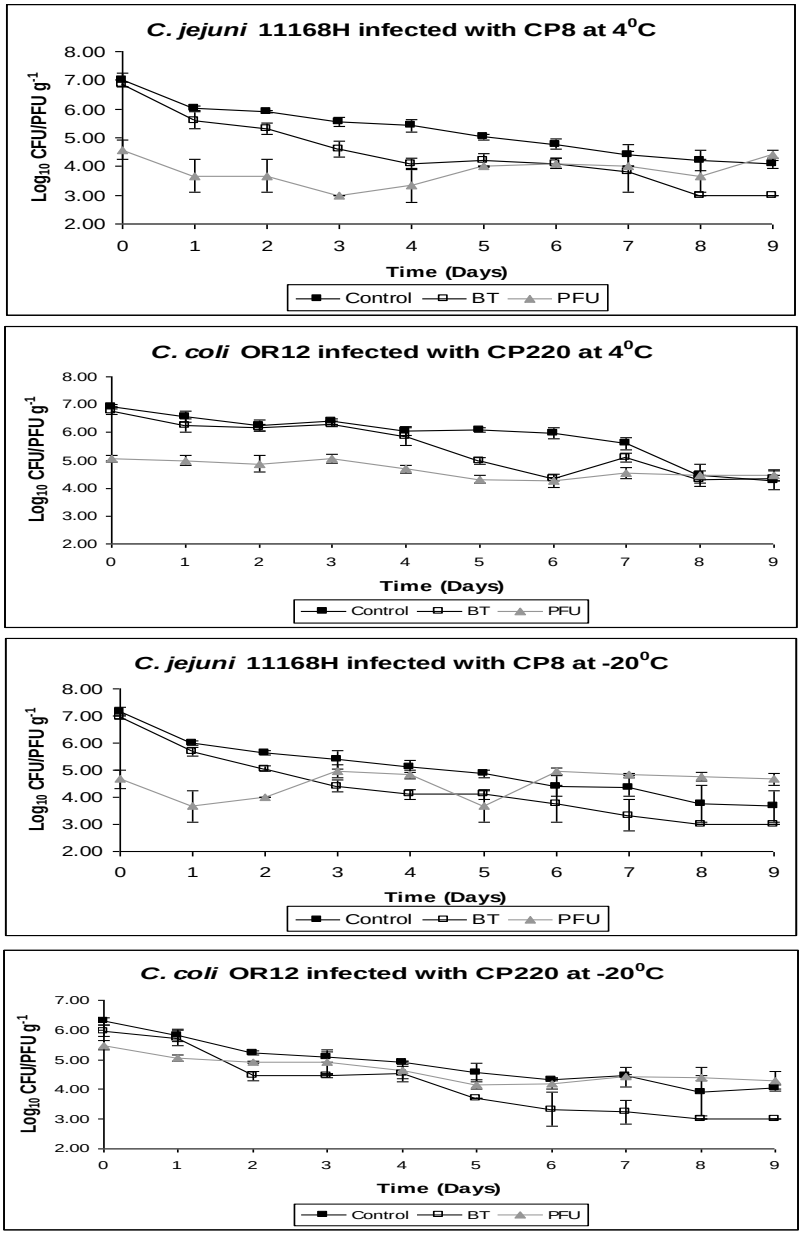

Figure 1: *Campylobacter* strains infected with bacteriophages at 4°C and -20°C. BT; bacterial titers, PFU; plaque forming units.

Changes in the viability mean counts of *E. coli* 12900 strain from ground beef for the two freezing and cooling treatments over the 10 days period of this study are presented in (Fig. 2). The viability of *E. coli* 12900 at the initial time point was identical (log_{10} 8.1 CFU) for ground beef samples stored at 4 and -20°C. The recovery from fresh samples continued to gradually decreased to reach a base of log_{10} 7.2 CFU for *E. coli* 12900 at the end of the experiment. On the other hand, the freeze-thaw treatment of the ground beef resulted in a fall in *E. coli* recovery to log_{10} 7.0 CFU. The recovery of *E. coli* 12900 at 4 and -20°C was quite constant at approximately the same level for the whole experiment. Therefore, in most cases, cooling and frozen storage resulted in little death of inoculated *E. coli* O157:H7 12900 and cannot be relied on to eliminate *E. coli* O157:H7 from naturally contaminated products by that organism especially that the infective dose of *E. coli* is very low of about 10 to 100 organisms (Garbutt, 1997) and some consumers handle/eat foods without adequate refrigeration or heating leading to increased chances of infection.

The recovery of bacteriophages CBA120 and CEV2 from ground beef stored at 4°C remained moderately stable at ~ log_{10} 6.7 PFU and ~ log_{10} 8.0 PFU respectively of the initial inoculated titer, log_{10} 7.5 PFU for CBA120 phage and log_{10} 8.2 PFU for CEV2 phage for the period of the experiment, falling slightly to log_{10} 7.3 PFU with phage CEV2 at the end of the experiment. The recovery of phage CBA120 inoculated on ground beef stored at -20°C was quite consistent at log_{10} 6.8 PFU compare to initial inoculated titer (log_{10} 7.3 PFU) for the period of the experiment. Alternatively, phage CEV2 slightly decreased to log_{10} 6.8 PFU by the end of the experiment compare to the initial inoculated titer (log_{10} 8.0 PFU). There was no increase in both phages titers over the 10-day period of the experiment.

Combinations of different bacteriophages and host titers at MOI ~ 1.0 were applied to study the effect of phage application on the viability of *E. coli* 12900. There were significant reductions ($P < 0.05$) in the numbers of *E. coli* recovered from fresh or frozen ground beef compared to those of the control, falling by log_{10} 0.3 to log_{10} 1.1 CFU (*E. coli* 12900 infected with phage CBA120) and log_{10} 0.4 to log_{10} 1.2 (*E. coli* 12900 infected with phage CEV2) (Fig. 2).

The application of *Campylobacter*-specific bacteriophage to the inoculated chicken skin with *Campylobacter* successfully reduced the number of viable cells and this reduction was quite constant over the period of the experiment. This reduction in *Campylobacter* prevalence may be improved with the combination of freezing and phage treatment. Campylobacters do not replicate when incubated at 4°C and the data support this, since the recovered number of campylobacters from the surface of the skin fell over time. Additionally, bacteriophage numbers also did not increase (replicate) on chicken skin samples inoculated with *Campylobacter* and they slightly fell over time but not significantly from the initial titers. Since the *Campylobacter* umbers have decreased and phage did not replicate this may be de to the successful phage adsorption to the surface of the bacteria upon inoculation without replication until the bacterium itself increases its metabolic activity.

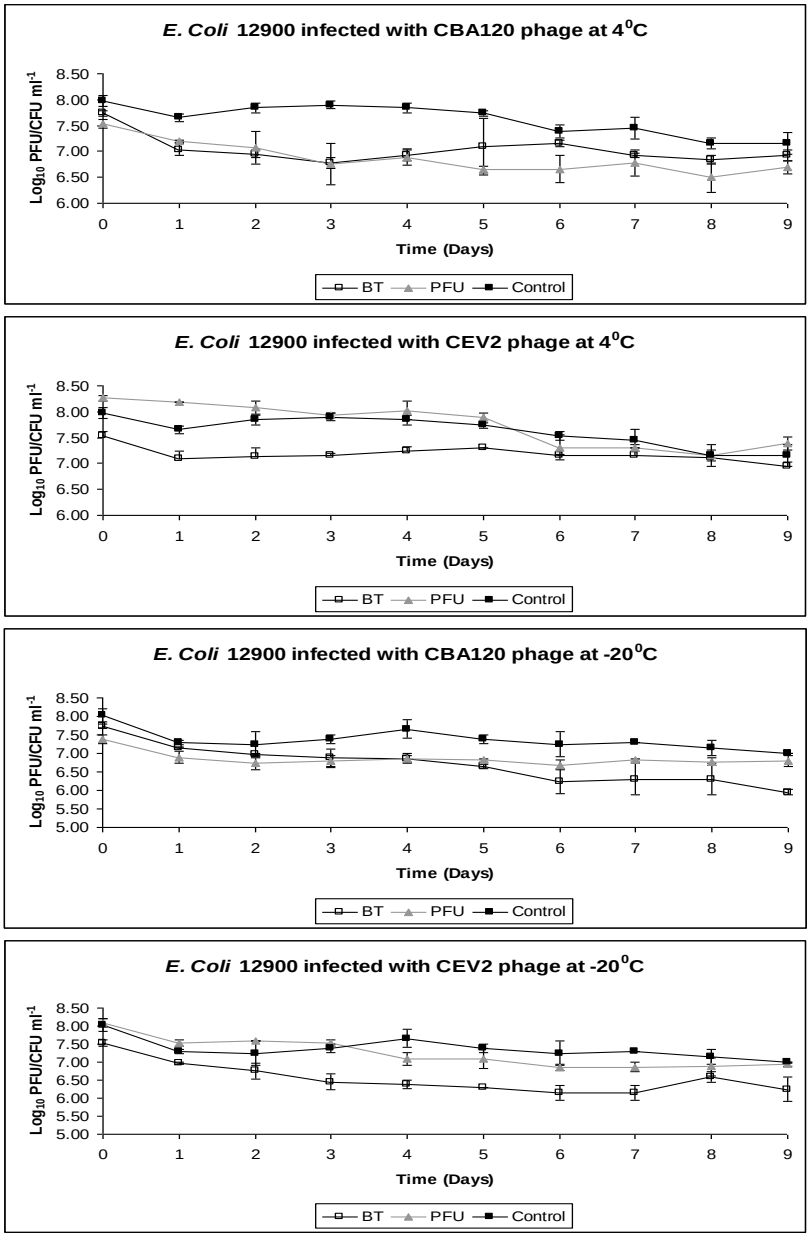

Figure 2: *E. coli* strains infected with bacteriophages at 4°C and -20°C. BT; bacterial titers, PFU; plaque forming units.

Many factors contribute to the increase of the bacterial loads in meat such as; the way the animal is slaughtered and eviscerated, the hygienic conditions in the slaughterhouses and the way by which the meat is generally handled and stored in terms of time and temperature. Early results indicated

that *Escherichia coli* O157:H7 can survive with little decrease in numbers in frozen ground beef patties for up to 9 months (Doyle and Schoeni, 1984). Freezing may play an important safety role to ground beef contaminated with *E. coli* O157:H7, and as a result it considered an unreliable method for product decontamination (Dykes, 2000). Consequently, beef contamination by the *Escherichia coli* O157:H7 causes significant risks to the consumers.

In conclusion, we have demonstrated the ability of *Escherichia coli* O157:H7 and *Campylobacter* strains and their bacteriophage predators to survive independently on ground beef and chicken skin respectively. Phage inoculated exhibit a control effect even in the absence of host growth. Further development of this study could lead to the use of bacteriophage as decontaminating agents in connection with other measures to control chickens and ground beef contaminated with those pathogens. Successful cocktails of broad-host-range bacteriophages would be more effective.

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تاثير استخدام البكتريوفاج للحد من بكتريا *Campylobacter* و *E. coli* O157:H7 المسببة لتلوث الاغذية

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ان خطورة الامراض الناتجة عن الاصابة بكل من بكتريا *Campylobacter* وبكتريا *E. coli* ادي الي الاهتمام الكبير من جانب الباحثين للبحث عن استراتيجيات مناسبة لتقليل خطورتها المتعلقة بسلامة الغذاء. وقد هدف هذا البحث الي دراسة تاثير استخدام البكتريوفاج للحد من هذه البكتريا الغير قادرة علي النمو سواء علي جلد الدواجن (*Campylobacter*) او في لحوم الابقار المفرومة (*E. coli*) علي درجات حرارة التبريد (٤ درجة مئوي) او درجات حرارة التجميد(-٢٠ درجة مئوية). ان استخدام البكتريوفاج لمعالجة جلد الدواجن واللحم المفروم الملوث بتلك البكتريا ادي الي تقليل اعدادها مقارنة باعداد البكتريا الغير معالجة بالبكتريوفاج وكان هذا الانخفاض في اعداد البكتريا تدريجيا خلال مدة اجراء التجربة. بالنسبة لاعداد بكتريا *Campylobacter* فقد لوحظ ان هناك انخفاض معنوي لاعدادها وصل الي 1.3 log10 CFU مع بكتريا *C. jejuni* 11168H باستخدام البكتريوفاج CP8 بينما انخفض العدد معنويا الي 1.6 log10 CFU مع بكتريا *C. coli OR12* باستخدام البكتريوفاج CP220 مقارنة بالبكتريا الغير معاملة. علاوة علي ذلك فقد لوحظ ايضا ان معاملة بكتريا *E. coli* الموجودة في اللحم المفروم سواء علي درجة حرارة التبريد او التجميد ادي الي انخفاض اعدادها الي 1.1 log10 CFUفي حالة معالجتها بالبكتريوفاج CBA120 بينما انخفضت اعدادها الي 1.2 log10 CFU عند معالجتها بالبكتريوفاج CEV2 مقارنة بالبكتريا الغير معاملة. هذا وقد لوحظ ان اعداد البكتريوفاج لم تزيد خلال فترة التجربة وانه من المتوقع ان تكون النتائج اكثر فاعلية في حالة استخدام خليط من اكثر من بكتريوفاج قادر علي قتل تلك البكتريا المسببة للتسمم الغذائي.

قام بتحكيم البحث

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