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Campylobacters and their bacteriophages from chicken liver: The prospect for phage biocontrol

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ABSTRACT

Consumption of foods containing chicken liver has been associated with *Campylobacter* enteritis. *Campylobacters* can contaminate the surface of livers post-mortem but can also arise through systemic infection of colonising bacteria in live birds. The use of bacteriophage to reduce levels of *Campylobacter* entering the food chain is a promising intervention approach but most phages have been isolated from chicken excreta. This study examined the incidence and contamination levels of *Campylobacter* and their bacteriophage in UK retail chicken liver. Using enrichment procedures, 87% of 109 chicken livers were surface contaminated with *Campylobacter* and 83% contaminated within internal tissues. Direct plating on selective agar allowed enumeration of viable bacteria from 43% of liver samples with counts ranging from 1.8–>3.8 $\log_{10}$ CFU/cm^2 for surface samples, and 3.0–>3.8 $\log_{10}$ CFU/g for internal tissue samples. Three *C. jejuni* isolates recovered from internal liver tissues were assessed for their ability to colonise the intestines and extra-intestinal organs of broiler chickens following oral infection. All isolates efficiently colonised the chicken intestines but were variable in their abilities to colonise extra-intestinal organs. One isolate, CLB104, could be recovered by enrichment from the livers and kidneys of three of seven chickens. *Campylobacter* isolates remained viable within fresh livers stored at 4 °C over 72 h and frozen livers stored at −20 °C over 7 days in atmospheric oxygen, and therefore constitute a risk to human health. Only three *Campylobacter*-specific bacteriophages were isolated, and these exhibited a limited host range against the *Camplylobacter* chicken liver isolates. All were identified as group III virulent bacteriophage based on their genome size of 140 kb. The application of broad host range group II virulent phages (8 $\log_{10}$ PFU/g) to liver homogenates containing *C. jejuni* strains of diverse origin at 4 °C resulted in modest but significant reductions in the viable counts ranging from 0.2 to 0.7 $\log_{10}$ CFU/g.

1. Introduction

Following emergence as an enteric pathogen in 1970s (Skirrow, 1977), *Campylobacter* has been a major concern worldwide. In the UK, *Campylobacter* is the most common bacterial cause of gastrointestinal infection recorded in the last two decades (Adak et al., 2005; Food Standards Agency, 2013). The total number of cases of *Campylobacter* infection during 2000–2012 was 781,581, from 1,052,581 laboratory confirmed cases of foodborne disease (Food Standards Agency, 2013). Campylobacteriosis is the most frequently reported foodborne disease but these figures belie actual unreported caseloads that are estimated to be 9 million and 1.3 million cases per year within the EU and USA, respectively (Centers for Disease Control and Prevention, CDC, 2014; EFSA, 2015).

The primary source of the major pathogenic species, *C. jejuni* and *C. coli*, are contaminated chicken and cattle meat (Adak et al., 2005; Suzuki and Yamamoto, 2009; Wilson et al., 2008), whereas less frequently they arise from wildlife (Hughes et al., 2009; Sippy et al., 2012), water, sewage and the environment (Jones, 2001; Waage et al., 1999). These bacteria are prevalent in offal, and in particular chicken liver (Cornelius et al., 2005, Kenar et al., 2009; Noormohamed and Fakhr, 2012; Noormohamed and Fakhr, 2013; Strachan et al., 2012; Vashin et al., 2009; Whyte et al., 2006). Dishes such as liver paté and liver parfait have been reported as potential transmission vehicles for outbreaks of foodborne disease (Centers for Disease Control and Prevention, CDC, 2013; Edwards et al., 2014; Hope et al., 2014; Inns et al., 2010; O'Leary et al., 2009; Wensley and Coole, 2013) and the number of cases is increasing (Little et al., 2010). Moreover, their presence could pose a risk to animal welfare as *Campylobacter* species have been associated with a disease affecting poultry liver termed vibrionic hepatitis (Crawshaw et al., 2015; Jennings et al., 2011; Stephens et al., 1998).

In some cases, the occurrence of *Campylobacter* in liver may be the result of contamination from the intestinal contents during processing (Barot et al., 1983). Nonetheless, isolation from the internal tissue of

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liver samples indicated that *Campylobacters* can be present in these organs (Cox et al., 2007). It has been recognised that bacteria can cross the intestinal barrier of animals and humans, a process known as bacterial translocation. In general, the lymphatic path is perceived as the more convincing primary route of the translocation as compared with the venous system (Balzan et al., 2007). In vitro studies have demonstrated that *Campylobacters* can translocate using either transcellular passage through the enterocytes or paracellular routes via the tight junctions (Backert et al., 2013). Specific translocation mechanisms have been elucidated for enteric pathogens such as *Salmonella*, which uses several routes to pass through the intestinal barrier to inhabit systemic organs (Watson and Holden, 2010). However, further studies are required to obtain evidence of the translocation mechanisms operating for *Campylobacters* in humans and animals (Backert et al., 2013). For example, the capacity of *C. jejuni* to colonise particular tissues is affected by the organism's ability to utilise specific nutrients - asparagine utilisation has been reported to improve the ability of the pathogen to colonise liver (Hofreuter et al., 2008).

Thorough cooking is the key to eliminating the risk of *Campylobacter* enteritis from poultry dishes. However, recipes for meals such as liver paté indicate minimal cooking to preserve the sensory properties and retain a pink appearance inside. To safely cook such dishes, critical core temperatures of 68–70 °C must be reached and held for periods as long as 45 min (Hutchison et al., 2015), which can result in unacceptable sensory characteristics (Whyte et al., 2006). Pre-cooking treatments could be applied to lower the initial contamination level, for instance by freezing and washing of the liver using organic acid (Harrison et al., 2013; Hutchison et al., 2015). However, the use of organic acid was found to cause a colour change or bleaching of the liver surface, and may not be effective for *Campylobacter* naturally present within the internal structures of the liver.

Bacteriophages have gained recognition as therapeutic agents to control pathogens in livestock and poultry (reviewed by Johnson et al., 2008), and represent a potential approach to control *Campylobacters* in livers**.** *Campylobacter* bacteriophages can be isolated from chicken meat and chicken excreta (Atterbury et al., 2003, 2005; El-Shibiny et al., 2005; Loc Carrillo et al., 2007) but to date attempts to isolate *Campylobacter* phages from chicken liver have not been reported. The application of a single dose or mixtures of *Campylobacter* phages have been reported to be effective in reducing the intestinal colonisation of chickens by *C. jejuni* and *C. coli* (El-Shibiny et al., 2009; Kittler et al., 2013; Loc Carrillo et al., 2005). The efficacy of the treatment varies depending on the phage type and dose, the phage-sensitivity of the host, the time interval post administration (Loc Carrillo et al., 2005) and the route of administration, i.e. by oral gavage or via chicken feed (Carvalho et al., 2010). Phage resistant *Campylobacter* have been reported post-treatment at relatively low frequencies of 2–4% (El-Shibiny et al., 2009; Hammerl et al., 2014; Loc Carrillo et al., 2005).

In this study, *Campylobacter* and their phages were isolated from retail chicken liver. *Campylobacter* isolates were tested for their ability to re-colonise extra-intestinal organs of chickens in order to identify *Campylobacter* isolates able to inhabit the liver of broiler chickens. Finally, virulent bacteriophages were applied to *Campylobacter* contaminated chicken liver homogenates to provide proof of principle that bacteriophages can reduce *Campylobacter* contamination within the liver matrix.

2. Material and methods

2.1. Bacterial strains and bacteriophage

Campylobacter jejuni PT14 (Brathwaite et al., 2013) was used as a reference strain and also for phage isolation and propagation. *Campylobacter jejuni* HPC5 (Loc Carrillo et al., 2005) and *C. jejuni* 81–176 (Korlath et al., 1985) were used as controls in the chicken colonisation experiments and the phage treatments of contaminated chicken livers. All *Campylobacter* isolates were cultured on blood agar base no. 2 CM0271 (Oxoid, Basingstoke, United Kingdom) supplemented with 5% defibrinated horse blood (TCS, Buckingham, United Kingdom) under microaerobic conditions (5% O2, 5% H2, 10% CO2, 80% N2) at 42 °C for 18–24 h. *Campylobacter* phages CP30A (GenBank accession number JX569801) and CPX (GenBank accession number JN132397) were propagated on *C. jejuni* PT14 or a contemporary *Campylobacter* isolate using the soft agar overlay method (Atterbury et al., 2003). Phages from the UK typing scheme (ϕ1 to ϕ16) were propagated as described by Frost et al. (1999). In order to obtain high titre stocks of bacteriophage, 30 ml volumes of plate lysates were centrifuged at 40,000*g* for 2 h at 4 °C. The pellets obtained were re-suspended in 1 ml of SM buffer (50 mM Tris·HCl (pH 7.5), 100 mM NaCl, 8 mM MgSO4, 0.01% Gelatin) to give a phage suspension containing approximately 10 $\log_{10}$ PFU/ml.

2.2. Preparation of chicken liver

Chicken liver samples were purchased from local supermarkets in Nottingham and Loughborough in the UK. Samples were kept at 4 °C and analysed before their expiry date as stated on the packaging. Each package contained 5–9 livers which were divided into two halves. Half of the liver was transferred into a stomacher bag (Seward Ltd., Worthing, UK) and 10 ml of Maximum Recovery Diluent CM733 (MRD; Oxoid; Basingstoke, UK) was added. The liver was gently massaged to re-suspend *Campylobacter* on the liver surface. To recover *Campylobacter* from internal tissues, the other half of liver was sterilised by dipping the liver into boiling water for 20–30 s (Whyte et al., 2006) and then tissue was excised with hot scalpel before being stomached with the addition of MRD (1:1 dilution ratio).

2.3. Isolation of Campylobacter from chicken liver

A 4 ml aliquot of suspension from the liver surface sample or the stomached internal tissue was transferred into 4 ml of enrichment media. This consisted of 2× *Campylobacter* Enrichment Broth Lab135 (Lab M, Heywood, UK) made up with the addition of: 10% lysed horse blood (TCS), 0.25 g/l each of sodium pyruvate, sodium metabisulphite and ferrous sulphate (each from Sigma Aldrich, Poole, UK) and *Campylobacter* Enrichment Selectavial SV59 (Mast, Bootle UK), in a bijoux bottle. The total volume of 8 ml resulted in limited airspace in the bottle, hence maintaining microaerobic conditions during incubation at 37 °C for 48 h. Five 10 μl aliquots from each bijoux were dispensed onto mCCDA CM739 agar (Oxoid) prepared with the addition of *Campylobacter* selective supplement code (SR155, Oxoid) and additional Agar No. 1 (Oxoid) added to give 2% and then incubated at 42 °C for 48 h under microaerobic conditions. *Campylobacter* were confirmed after subculture, using microscopic observation of Gram stained cells, together with catalase and oxidase tests.

2.4. Enumeration of Campylobacter

Campylobacter was enumerated using the Miles and Misra technique, with serial dilutions prepared in MRD and 10 μl aliquots spotted in quintuplicate on 2% mCCDA before incubating under microaerobic conditions at 42 °C for 48 h. Typical *Campylobacter* colonies were counted and the total number calculated as either $\log_{10}$ CFU/g for internal tissue samples or $\log_{10}$ CFU/cm^2 for surface liver samples.

2.5. Species identification and Fla-typing using PCR methodologies

Campylobacter DNA was isolated using the GenElute™ Bacterial Genomic DNA Kit according to manufacturer's instructions for Gram negative bacteria (Sigma-Aldrich, UK). The PCR methodology was based on conditions previously described by Linton et al. (1997) for species identification and by Elvers et al. (2008) for FlaA SVR-typing. The oligonucleotides were purchased from Eurofins (Ebersberg, Germany) and consisted