Detection of *Campylobacter jejuni* liver dissemination in experimentally colonized turkey poults

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ABSTRACT Consumption of contaminated poultry products, including chicken livers, is the main source of human campylobacteriosis and approximately 90% of human cases are caused by *Campylobacter jejuni* subsp. *jejuni* (*C. jejuni*). Recent culinary trends that favor undercooked chicken livers may be responsible for outbreaks. Turkey is an emerging human protein source, and poultry livers are commonly prepared in popular cuisine such as pâté. The mechanism of how *Campylobacter* disseminates to poultry liver tissue is unknown. We have previously demonstrated that certain strains of *C. jejuni* persistently colonize turkeys with the highest density in the ceca. Whether *C. jejuni* disseminates to the liver of turkeys following intestinal colonization is unknown. In this study, 45 D of hatch turkey poults were co-housed for 30 D. Five poults were euthanized to screen for *Campylobacter* colonization, and were free of detectable *Campylobacter*. The remaining 40 poults were randomly split into 2 rooms, with 20 poults per room. At 35 D of age, poults were inoculated by oral gavage with 1×10^6 cfu of *C. jejuni* isolate NCTC 11168 or mock-inoculated with sterile medium. Ten poults from each room were euthanized at 7 and 14 D post-inoculation (dpi), and cecal contents and livers were cultured and/or enriched for *Campylobacter*. Livers were harvested aseptically. The ceca of *C. jejuni*-inoculated poults were highly colonized at 7 and 14 dpi with approximately 10^8 cfu/mL of cecal contents. At 7 and 14 dpi, 3 and 5 of 10 liver samples were positive for *C. jejuni* culture (8.6×10^3 cfu/g of liver $\pm$ 4.43×10^3 and 5.10×10^3 cfu/g of liver $\pm$ 1.74×10^3), respectively. At 14 dpi, liver samples were cultured by enrichment, and 6 of 10 were positive for *Campylobacter*. Some liver samples may be below the limit of detection for direct plate culturing. These data determined that turkey liver is a potential reservoir of *C. jejuni* following intestinal colonization, and identified a potential food safety consideration when turkey liver is prepared for human or pet food consumption.

Key words: *Meleagris gallopavo* (Turkey), *Campylobacter jejuni*, cecal colonization, liver dissemination, food safety

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INTRODUCTION

Campylobacteriosis is the most prevalent bacterial foodborne disease in humans due to consumption of contaminated poultry, with over 90% of cases caused by *Campylobacter jejuni* subsp. *jejuni* (*C. jejuni*). Food products containing chicken liver, including pâté, parfait, and mousse, were identified as sources of human campylobacter outbreaks (Merritt et al., 2011; Farmer et al., 2012; Parry et al., 2012; Centers for Disease Control and Prevention 2013; Edwards et al., 2014; Scott et al., 2015; Glashower et al., 2017). Of human concern, *C. jejuni* or *Campylobacter coli* have been isolated from chicken livers (Barot et al., 1983; Khalafalla, 1990; Boukraa et al., 1991; Wieliczko, 1994; Baumgartner et al., 1995; Fernandez and Pison, 1996; Cox et al., 2006a, 2007, 2009; Strachan et al., 2012; Harrison et al., 2013; Firlieyanti et al., 2016; McLauchlin et al., 2017; Karki et al., 2018). Recent culinary trends, especially intentional undercooking of liver, may explain the increase of chicken liver products responsible for human campylobacteriosis. Due to undercooking, an estimated 19–52% of chicken livers do not reach an internal temperature of 70°C, and present an increased risk for human transmission (Jones et al., 2016). Techniques such as frying chicken livers at ≥70°C for 2 to 3 min (Whyte et al., 2006), freezing livers (Harrison et al., 2013), or washing with organic acids (Hutchison et al., 2015) are reported to inactivate *Campylobacter*. Surface contamination on the liver capsule is likely due to contamination during slaughter, but *Campylobacter* may be also

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located within the organ's parenchyma. Using an aseptic technique to eliminate capsular contamination from feces or ceca, *Campylobacter* were isolated from liver parenchyma (Boukraa et al., 1991; Cox et al., 2006a,b, 2009; Berrang et al., 2018). Thus, *Campylobacter* on the liver surface are more likely inactivated by proper cooking technique, whereas *Campylobacter* within the liver parenchyma may represent a greater risk for food safety. *Campylobacter* may adapt to liver parenchyma to better survive in refrigerated poultry products, and affect its transmission to humans. Some strains of *C. coli* isolated from chicken livers are hyper-aerotolerant, more so than *C. jejuni* strains isolated from chicken livers (Karki et al., 2018). Incubation of *Campylobacter* with retail chicken liver juice supports biofilm formation in *C. coli* and the survival of *C. jejuni* at 4°C (Karki et al., 2019).

Turkey is an emerging human protein source, and the 2014 per capita consumption of turkey in the United States was 12.4 lbs. (Bentley, 2017). With increased consumption of turkey products, the risk of human campylobacteriosis may increase. In contrast to the link between chicken livers and human disease, whether turkey livers represent a food safety risk for humans is less known. Approximately 10% of turkey offal (e.g., gizzard, liver, and heart) samples were positive for *Campylobacter* contamination (Atanassova et al., 2007), as well as approximately 15% of turkey giblets (Khalafalla, 1990). It is unknown from these studies whether *Campylobacter* were isolated from the surface or parenchyma of these turkey tissues. In the present study, we tested the hypothesis that turkey poults intentionally inoculated with *C. jejuni* lead to liver dissemination. We demonstrated dissemination of *C. jejuni* to the liver parenchyma in a subset of turkey poults experimentally colonized with *C. jejuni*.

MATERIALS AND METHODS

Animal Experimental Design

This animal experiment was conducted according to the regulations established by the National Animal Disease Center Institutional Animal Care and Use Committee. Day of hatch hybrid poults (n = 45) were obtained from a commercial breeder and housed in a single Animal Biosafety Level-2 room. Throughout the study, poults were fed a turkey poult starter ration with water available ad libitum. The *Campylobacter* status of experimental poults was determined at day 30 of age by randomly selecting and humanely euthanizing 5 poults by intravenous barbiturate overdose. Necropsy was performed to harvest cecal contents and 1 g of contents from each poult was cultured by enrichment in 10 mL of Bolton's broth base containing *Campylobacter* selective supplement (Neogen Corporation, Lansing, MI) for 48 h in a microaerophilic environment (85% N_2, 10% CO_2, and 5% O_2) at 42°C (Sylte et al., 2018). As a positive control, a pure culture of *C. jejuni* strain NCTC 11168 was enriched. After incubation, 100 µL of enrichment broth was cultured, in duplicate, for 48 h at 42°C in a microaerophilic environment on Campy Line agar containing 25 µg/mL sulfamethoxazole (CLA-S) (Line et al., 2008). Poults were considered free of *Campylobacter* colonization after enrichment if no colonies resembling a pure culture colony of *C. jejuni* strain NCTC 11168 were recovered (Sylte et al., 2018). After cohousing for 33 D, the 40 remaining poults were distributed equally into 2 Animal Biosafety Level-2 rooms (n = 20 poults/room) for challenge. Room temperature, humidity, and lighting cycle were approximately the same for both challenge rooms. *C. jejuni* strain NCTC 11168 inoculum was prepared, as described previously (Sylte et al., 2018). All poults within a room were individually orally gavaged with 1 mL of Bolton's broth base containing 1×10^6 cfu of *C. jejuni* strain NCTC 11168, or mock-inoculated with 1 mL of sterile Bolton's broth base. At 7 and 14 D post-inoculation (**dpi**), 10 poults each from the *C. jejuni* and mock-inoculated rooms were euthanized, as described above. Necropsy was performed to harvest ceca and liver samples for *C. jejuni* enumeration. In order to limit fecal contamination from the skin or feathers, euthanized poults were soaked in a soapy water bath. Sterile Mayo scissors were used a single time to make an incision in the skin. Skin was reflected and dirty gloves were removed. New gloves were worn and fresh sterile Mayo surgical scissors and thumb forceps were used to make an incision into the abdominal cavity to expose the right liver lobe. Fresh sterile Mayo surgical scissors and thumb forceps were used to aseptically transfer a piece of ventral right liver lobe to a sterile 50 mL conical tube. Cecal contents were collected in a sterile 50 mL conical tube. Liver samples and cecal contents were stored on ice before culture.

Enumeration of C. jejuni From Cecal Contents

Direct plating enumeration and enrichment culture of *Campylobacter* from cecal contents have been described previously (Sylte et al., 2018). Validation of colonies resembling those from pure cultures of *C. jejuni* NCTC 11168 was performed using *C. jejuni hipO* and *Campylobacter* 16S-specific quantitative PCR (**qPCR**), as described previously (Sylte et al., 2018). A colony was validated as *Campylobacter* if it was both *hipO* gene and *Campylobacter* 16S positive. For statistical purposes, if no colonies resembling *C. jejuni* grew from direct plating, the sample was assigned the culture limit of detection value of 10^3 cfu/g of contents. If no colonies grew after enrichment, they were considered negative (zero) for *Campylobacter* colonization. Poults were considered positive for *C. jejuni* colonization if at least one colony grew on CLA-S agar.

Enumeration of C. jejuni From Liver Samples

For each animal, 1 g of liver was placed in 9 mL of sterile PBS in a gentleMACS C tube (Miltenyi Biotec Inc.,

Auburn, CA) and homogenized using a gentleMACS Octo Dissociator (Miltenyi Biotec Inc.) using the gentleMACS program RNA_02. The homogenate was centrifuged in the C tube for 5 min at 2000 × *g* at 4°C, and the supernatant was transferred to a sterile 15 mL conical tube. Liver samples were serially diluted in sterile PBS up to 10^{-4}. Enumeration was performed utilizing the track-plating dilution method (Sylte et al., 2018), and 10 µL of each dilution was plated in duplicate on CLA-S and incubated at 42°C in a microaerophilic environment for 48 h. Colonies resembling pure cultures of *C. jejuni* NCTC 11168 were counted and validated by qPCR, as described above. For statistical purposes, if no colonies resembling *C. jejuni* grew from a sample, the sample was assigned the culture limit of detection value of 10^3 cfu/g of contents. Samples were considered positive for liver dissemination by direct plate culturing if at least one qPCR-validated *Campylobacter* colony grew on CLA-S agar. *Campylobacter* enrichment culture was performed on liver samples harvested 14 dpi. The same liver homogenate supernatant (1 mL) used for direct plating was added to 9 mL of Bolton's broth base containing *Campylobacter* selective supplement. The sample was incubated for 48 h at 42°C in a microaerophilic environment. After enrichment, 100 µL was inoculated, in duplicate, on CLA-S agar and incubated at 42°C in a microaerophilic environment for 48 h. As a control, a pure culture of *C. jejuni* strain NCTC 11168 was used to validate enrichment culture conditions. Colonies were enumerated and validated by qPCR, as described above. Liver samples were considered *Campylobacter* positive by enrichment culture if at least one qPCR-validated *Campylobacter* colony grew on CLA-S agar.

Statistical Analysis

Cecal and liver *C. jejuni* enumeration data were analyzed using an unpaired *t*-test using Prism statistical software v8.1.2 (GraphPad Software Inc., San Diego, CA) to detect a difference among groups. Results were considered significant at values of $P \leq 0.05$.

RESULTS AND DISCUSSION

C. jejuni *Cecal Colonization*

All 20 turkey poults challenged with *C. jejuni* NCTC 11168 were colonized in their ceca at 7 and 14 dpi (Figure 1 and Table 1). No *Campylobacter* was recovered from mock colonized poults (Table 1), demonstrating a lack of detectable *Campylobacter* in these poults. The inoculum dose used in this study (1×10^6 cfu) was >2 $\log_{10}$ less than that used in our previous studies to colonize turkey poults with *C. jejuni* (Sylte et al., 2018; Sylte et al., 2019). A lower inoculum may represent more natural exposure for *C. jejuni* colonization of poults. Poultry are coprophagic, and *Campylobacter* rapidly spreads in situations where sentinels are exposed to seeder chickens (Stern et al., 2001). The inoculum dose used in this study is similar to the amounts of *C. jejuni* shed in chicken feces after exposure (Sahin et al., 2003). Our data demonstrate that a lower dose of *C. jejuni* inoculum can persistently colonize the ceca of turkeys.

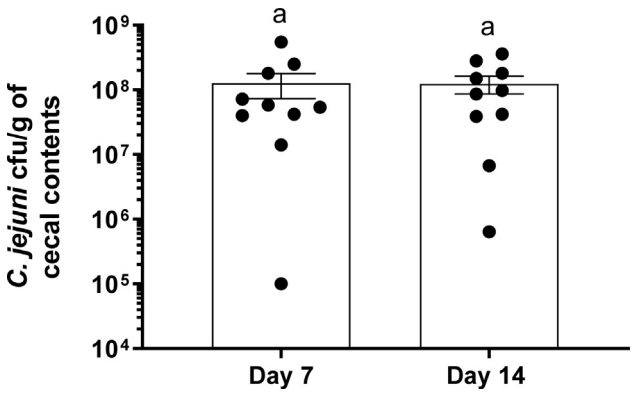

Figure 1. Enumeration of *C. jejuni* strain NCTC 11168 from cecal contents of turkey poults. Data represent the *Campylobacter* cfu/g of cecal contents from each poult (black circles) and the mean (column) ± SEM for days 7 and 14 post-inoculation. Statistical differences in the number of enumerated *Campylobacter* cfu/g of cecal contents were determined using an unpaired *t*-test. Significant differences ($P < 0.05$) between treatments are represented by different letters.

Dissemination of C. jejuni to Turkey Liver Tissue

Whether turkey liver parenchyma harbors *Campylobacter* following intestinal colonization was unknown. Giblets from commercial turkeys, consisting of gizzards, hearts, livers, and spleen, were positive for *C. jejuni* (Khalafalla, 1990). The site of liver contamination (e.g., capsule or parenchyma) is a controversial subject. *Campylobacter* was detected in 56 of 117 retail chicken livers, of which 36 were surface contaminated, 18 were on the surface and in the parenchyma, and 2 in the parenchyma alone (Barot et al., 1983). However, these parenchyma positive samples were disregarded as a result of surface contamination. Parenchymal dissemination was demonstrated using aseptic harvesting of chicken organs. Nineteen of 52 aseptically harvested liver and gallbladders from broilers were positive for *Campylobacter* (Cox et al., 2007), as were livers from broiler hens (Cox et al., 2006b). Likewise, searing the liver surface with a hot spatula and cutting into the parenchyma with a sterile scalpel also recovered *Campylobacter* from liver parenchyma (Boukraa et al., 1991), and differentiated surface and parenchymal *C. jejuni* in retail chicken livers (Berrang et al., 2018).

In the present study, turkey liver samples were harvested aseptically before removing the ceca, the main reservoir of *C. jejuni* in turkeys (Sylte et al., 2018), to reduce the potential of cecal contamination of the abdominal cavity or capsular surface of the liver. Three of 10 (8.6×10^3 cfu/g of liver ± 4.43×10^3) and 5 of 10 (5.10×10^3 cfu/g of liver ± 1.74×10^3) liver samples were positive for *C. jejuni* at 7 and 14 dpi, respectively

Table 1. Summary of *C. jejuni* direct plate enumeration and post-culture validation from cecal samples 7 and 14 D after inoculation.

			Cecal colonization		
Days post-inoculation	Inoculation	*C. jejuni* colonization	Direct plate culture positive	Enrichment culture positive	*Campylobacter* qPCR positive
7	*C. jejuni*	Yes	10/10	10/10	10/10
	Mock	No	0/10[1]	0/10	ND[2]
14	*C. jejuni*	Yes	10/10	10/10	10/10
	Mock	No	0/10[1]	0/10	ND

Abbreviation: qPCR, quantitative PCR.
[1]Below the limit of detection (10^3 cfu/g of contents).
[2]ND: not determined.

(Figure 2). Because liver samples were aseptically harvested before ceca removal, we felt the risk from surface contamination was minimized. Similar to previous studies in chickens (Whyte et al., 2006; Baumgartner and Felleisen, 2011; Firlieyanti et al., 2016), the quantity of *Campylobacter* cultured from turkey liver tissue was less than that from cecal contents (Figure 2), but the quantitative range of *Campylobacter* cultured from turkey livers at 7 and 14 dpi was similar to the range of *Campylobacter* (10^3–10^4 cfu/g) cultured from chicken livers (Baumgartner and Felleisen, 2011). No *Campylobacter* was isolated by direct plate or enrichment culture of liver samples from mock-inoculated poults. Enrichment culture was performed on 14 dpi samples and 6 out of 10 were positive for *C. jejuni* (Table 2), which suggests that some turkey liver samples may be colonized below the limit of quantitative detection (10^3 cfu/g of sample). It is possible that *Campylobacter* liver dissemination is focal and those samples that were negative by direct plating or enrichment may be false negatives due to culturing only 1 g of tissue. Increasing the amount of liver or including tissue from multiple liver lobes may better identify the number of turkeys with *C. jejuni* liver dissemination. Furthermore,

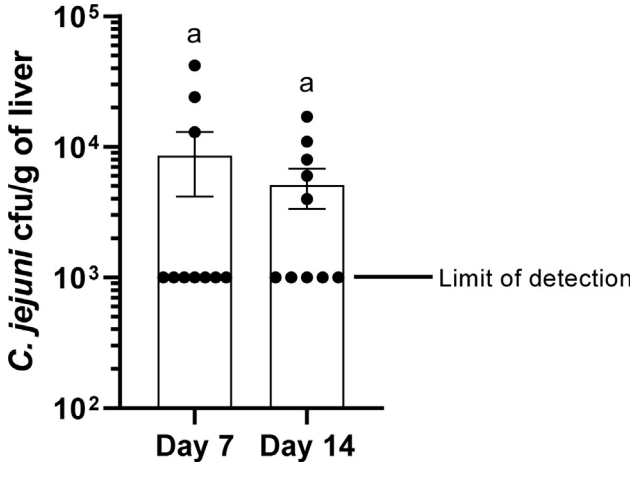

Figure 2. Enumeration of *C. jejuni* strain NCTC 11168 from liver of turkey poults. Data represent the *Campylobacter* cfu/g of liver tissue from each poult (black circles) and the mean (column) ± SEM for days 7 and 14 post-inoculation. Poults with no recovered *C. jejuni* were assigned a value of 10^3 cfu/g of liver, the limit of detection. Statistical differences in the number of enumerated *Campylobacter* cfu/g of liver were determined using an unpaired *t*-test. Significant differences ($P < 0.05$) between treatments are represented by different letters.

the duration of turkey liver colonization by *C. jejuni* is currently undetermined and will impact foodborne risk.

The location of *Campylobacter* within the liver parenchyma of poultry is unknown. We have previously used immunohistochemistry (**IHC**) to detect *Campylobacter* outer membrane protein antigen in cecal tissue of experimentally colonized poults (Sylte et al., 2019). Considering the low abundance of *Campylobacter* recovered in the present study and from chicken livers (Whyte et al., 2006; Baumgartner and Felleisen, 2011; Firlieyanti et al., 2016), a single 5 µm histological section may be insufficient for IHC detection of *Campylobacter* antigen in liver samples. Future studies may focus on *Campylobacter* IHC from multiple liver lobes to enhance its detection from culture and enrichment positive animals. The composition of intestinal microbiome may also impact extraintestinal dissemination by *Campylobacter*. The number of liver and spleen samples positive for *C. jejuni* from germ-free or antibiotic treated chicks was greater when compared to germ-replete chicks (Han et al., 2017). Members of the intestinal microbiome may help control extraintestinal dissemination in poultry. Identifying specific bacterial taxa involved in limiting extraintestinal *Campylobacter* dissemination may help define a probiotic option to promote food safety.

The data presented here extend our knowledge of the presence of *Campylobacter* in poultry liver parenchyma to include experimentally colonized turkey poults. Contamination of turkey liver products used for human or pet food may need to be considered as a source of foodborne pathogens, such as *Campylobacter*.

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Table 2. Summary of *C. jejuni* direct plate enumeration, enrichment, and post-culture validation from liver samples 7 and 14 D after inoculation.

Days post-inoculation	Inoculation	*C. jejuni* colonization	Liver dissemination		
			Direct plate culture positive	Enrichment culture positive	*Campylobacter* qPCR positive
7	*C. jejuni*	Yes	3/10	ND[2]	3/10:ND
	Mock	No	0/10[1]	ND	ND
14	*C. jejuni*	Yes	5/10	6/10	5/10:6/10
	Mock	No	0/10[1]	0/10	ND

Abbreviation: qPCR, quantitative PCR.
[1]Below the limit of detection (10^3 cfu/g of contents).
[2]ND: not determined.

or endorsement by the USDA. USDA is an equal opportunity provider and employer.

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