Evaluation of different *Campylobacter jejuni* isolates to colonize the intestinal tract of commercial turkey poults and selective media for enumeration

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ABSTRACT Consumption of contaminated poultry products is the main source of human campylobacteriosis, for which *Campylobacter jejuni* is responsible for 90% of human cases. Although chickens are believed to be a main source of human exposure to *C. jejuni*, turkeys also contribute to cases of human infection. Little is known about the kinetics of *C. jejuni* intestinal colonization in turkeys, or best selective media for their recovery. Enumeration of *C. jejuni* from intestinal samples can be challenging because most selective *Campylobacter* media support the growth of non-*Campylobacter* organisms. In this study, we sought to compare a) *C. jejuni* isolates that persistently colonize different compartments of the poult intestinal tract, and b) selective media to enumerate *C. jejuni* from turkey intestinal samples. Three-week-old poults were orally colonized with *C. jejuni* isolates NCTC 11168 or NADC 20827 (isolated from a turkey flock). Mock-colonized poults were orally gavaged with uninoculated media. Poults were euthanized at d 3, 7, and 21 post colonization and direct plated on different selective *Campylobacter* media [Campy Line agar with sulfamethoxazole (CLA-S), CHROMagar *Campylobacter* (CAC) and Campy Cefex] for enumeration. Isolates NCTC 11168 and NADC 20827 poorly colonized the distal ileum. Both isolates colonized the colon, but the number of NADC 20827 significantly decreased at d 21. Isolates NCTC 11168 and NADC 20827 persistently colonized the cecum for up to 21 days. There was no significant difference in the *Campylobacter* amount recovered on CLA-S and CAC. Campy Cefex failed to prevent growth of background microbes to enumerate *C. jejuni* from turkey samples. Two independent PCR assays (multiplex PCR and qPCR) confirmed that colonies grown on CLA-S or CAC were *C. jejuni*. Data from this study demonstrated that isolates NCTC 11168 and NADC 20827 persistently colonized the cecum, and CLA-S or CAC were successful to enumerate *Campylobacter* from intestinal samples. These findings will be useful to evaluate the host response by *C. jejuni* in turkeys, and test pre-harvest strategies to reduce its colonization and promote food safety.

Key words: *Campylobacter jejuni*, turkey, intestinal colonization, CHROMagar *Campylobacter*, Campy Line agar with sulfamethoxazole

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INTRODUCTION

Campylobacteriosis is the most prevalent bacterial foodborne disease in humans worldwide, with over 90% of cases caused by *Campylobacter jejuni* subsp. *jejuni* (***C. jejuni***). Thermophillic Campylobacters (e.g., *C. jejuni, C. coli*, and others) are intestinal commensals that persistently colonize the cecum of chickens at levels approaching 10^9 colony forming units (**cfu**)/g of cecal contents (Wyszynska et al., 2004; Buckley et al., 2010; Kobierecka et al., 2016; Nothaft et al., 2016). Consumption of contaminated poultry products is a main source of human campylobacteriosis. Little is known about the role of *Campylobacter* transmission from retail turkey products. There have been few studies describing the kinetics of intestinal colonization and enumeration of *Campylobacter* from different intestinal compartments in turkeys. Previous experimental colonization of young turkey poults with a turkey-origin *C. jejuni* isolate produced morbidity (e.g., 20% weight loss), but no mortalities (Lam et al., 1992), and the cecum of turkeys harbored the greatest number of *C. jejuni*, which could be recovered for several wk after experimental colonization (Wallace et al., 1998).

Enumeration of *C. jejuni* using direct plating on routine selective *Campylobacter* media (e.g., modified

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charcoal cefoperazone deoxycholate agar (**mCCDA**), Karmali agar or Campy Cefex agar, or others) is challenging because they often support the growth of non-*Campylobacter* organisms and may confound accurate *Campylobacter* enumeration from intestinal samples. The appropriate selective *Campylobacter* media to enumerate *Campylobacter* from turkey intestinal contents is not known, but different selective *Campylobacter* media have been evaluated for direct culture from chicken cecal contents (Stern et al., 2005; Neal-McKinney et al., 2014; Schroeder et al., 2014; Gonsalves et al., 2016), litter (Kiess et al., 2010), and carcass washes (Oyarzabal et al., 2005; Line et al., 2008). Based on these findings, we elected to use Campy Line agar containing sulfamethoxazole (**CLA-S**) (Line et al., 2008), CHROMagar *Campylobacter* (**CAC**), and Campy Cefex agar to evaluate enumeration from intestinal samples of *C. jejuni* colonized poults. In this study, we sought to determine whether *C. jejuni* isolates NCTC 11,168 or NADC 20,827 (isolated from a turkey flock) could persistently colonize different compartments of the poult intestinal tract and identify selective *Campylobacter* media to enumerate cfu/g of from intestinal samples.

MATERIALS AND METHODS

Maintenance of C. jejuni *isolates, motility, and growth curves*

The wild-type *C. jejuni* isolate NCTC 11,168, provided by Dr. Qijing Zhang at Iowa State University, was originally isolated by Martin Skirrow from the feces of a human with diarrhea in 1977. Wild-type *C. jejuni* isolates NADC 20,826, 20,827, 20,830, 20,831, 20,832, and 20,833 were isolated at the USDA National Animal Disease Center (**NADC**) in Ames, Iowa, in 2005 from turkey feces collected at a commercial turkey flock in Iowa, where no clinical disease was noted. The different *C. jejuni* isolates were grown in pure culture on Muller Hinton (**MH**) agar plates (Neogen Corporation, Lansing, MI) in a microaerophilic environment (containing 5% O_2, 10% CO_2, and 85% N_2 gas) at 42°C. Cultures were cryopreserved at −80°C in MH broth containing 10% glycerol (v/v). The motility of different *C. jejuni* isolates used in these studies was assessed each time broth cultures were prepared. Cryopreserved *C. jejuni* were inoculated onto a MH plate and cultured in a microaerophilic incubator at 42°C for 18 hours. Up to 5 cfu of each isolate were used to inoculate Bolton's broth base (Neogen Corporation), incubating at 42°C for 18 h, shaking (100 rpm) in a microaerophilic environment. Ten mL of each broth culture was cover slipped on a clean microscope slide and visualized at 400X magnification using a Nikon Eclipse Ni dark-field microscope (Nikon Instruments Inc., Melville, NY). Motility was assessed and documented by acquiring videos using NIS-Elements Basic Research software v4.13 (Nikon Instruments Inc.). Isolates were considered motile if they had a positive motility agar test or had least 90% of the organisms actively moving when compared to amotile *C. jejuni* isolate NADC 14,103. If no motility was seen on the agar stab, or <90% were motile, the inoculum was not used.

The growth curve inocula were prepared by static culture in biphasic Bolton's broth base and agar (1.5% w/v) at 1:1 ratio (20 mL of broth and 20 mL of agar) in a sterile vented 75cm^2 tissue culture flask at 42°C in a microaerophilic gas environment. The optical density 600 nm (**OD_{600}**) of the broth phase was adjusted to 0.4 and diluted 1:2500 to begin the growth curves, as described previously (Davis and DiRita, 2008). Growth curves were performed in octuplet, using HONEYCOMB Bioscreen C plates in a Bioscreen C plate reader (Growth Curves USA, Piscataway, NJ), with each isolate cultured in a total volume of 400 mL Bolton's broth base. The OD_{600} was measured every 2 h for 48 h, in a microaerophilic environment at 42°C, with uninoculated media serving as a control to subtract OD_{600} background. Growth curves were repeated for a total of 2 replicates.

Animal experimental design

This animal experiment was conducted according to the regulations established by the NADC Animal Care and Use Committee (ACUP# ARS-2016–567). Day of hatch Hybrid poults (n = 71) were obtained from a commercial breeder and co-housed in a single ABSL-2 room. In order to normalize microbial development in the intestinal tract of the poults, clean pine shavings were seeded with approximately 2 kg of conditioned litter from an on-site flock of adult small Beltsville-white turkeys. Previously, we were unable to detect *Campylobacter* by direct culture or enrichment of feces and litter from this flock. Throughout the study, poults were fed a turkey poult starter ration and had water available ad libitum. The *Campylobacter* status of experimental poults was determined at 15 d of age by randomly selecting and euthanizing 5 poults. Necropsy was performed to harvest cecal contents and 1 g of cecal contents was cultured by enrichment in 10 mL of Bolton's broth base containing *Campylobacter* selection supplement (Neogen Corporation) for 48 h in a microaerophilic environment at 42°C (Bolton and Robertson, 1982; Hunt et al., 1998). Afterward, 100 mL were cultured, in duplicate, for 48 h at 42°C in a microaerophilic environment on Campy Line agar (Line, 2001) containing 2.5 mg/mL sulfamethoxazole (CLA-S) (Line et al., 2008). Poults were considered free of *Campylobacter* colonization if no colonies resembling pure cultures of *C. jejuni* or *C. coli* were recovered after enrichment. After co-housing for 20 d with the conditioned litter, poults were distributed into 3 ABSL-2 rooms for challenge. As an attempt to keep the same environmental microbes present in the challenge rooms, approximately 2 kg of conditioned litter from the initial

room where poults were co-housed were used to seed clean pine shaving litter in each of the 3 ABSL-2 rooms. Room temperature, humidity, and lighting cycle were approximately the same for the challenge rooms. *C. jejuni* inocula were prepared by harvesting at least 5 colonies from a pure culture on MH agar of NCTC 11,168 or NADC 20,827 into the broth phase of a biphasic Bolton's broth base and agar (1:1 ratio) in a 75cm^2 vented tissue culture flask. Flasks were cultured statically at 42°C in a microaerophilic environment for 18 hours. On the d of challenge, the broth phase of the bi-phasic culture was harvested, vortexed, and adjusted to an OD_{600} value of 0.4 in sterile Bolton's broth base. The cfu/mL of each inocula was enumerated using serial dilation on CLA-S, and motility was assessed using dark-field microscopy, as described above. When poults were 21 d old, all poults within a room were individually orally gavaged with 1 mL of Bolton's broth base containing approximately 5×10^8 cfu of NCTC 11,168 or 4×10^8 cfu of NADC 20,827. Mock-inoculated poults were orally gavaged with 1 mL of sterile Bolton's broth base. At d 3, 7, and 21 post colonization, at least 8 poults from the NCTC 11,168 or NADC 20,827 inoculated rooms were euthanized. Five mock-inoculated poults were euthanized at each time point. Necropsy was performed to aseptically harvest distal ileal (approximately10 cm proximal to ileocecal junction), colon, and cecal contents for enumeration of *C. jejuni* from each animal.

Enumeration of C. jejuni *from intestinal samples*

Intestinal contents were stored on ice and transported to the laboratory for culture. One gram of distal ileal, colon, or cecal contents was diluted in 9 mL of sterile PBS, vortexed for 5 s, and serially diluted up to 10^{-6}. Enumeration was performed utilizing the track-plating dilution method (Jett et al., 1997), in which 10 mL of each dilution were plated in duplicate on CLA-S, CAC (CHROMagar, Paris, France), and Campy Cefex plates (Remel, Lenexa, KS). The CLA-S plates were prepared as described previously (Line et al., 2008), and CAC plates were prepared per the manufacturer's instructions. The CLA-S plates were incubated at 42°C in a microaerophilic environment for 48 h, and cfu resembling those from pure cultures of *C. jejuni* NCTC 11,168 or NADC 20,827 were enumerated. CAC plates were incubated at 42°C in a microaerophilic environment for 36 h, and cfu resembling those from pure cultures of *C. jejuni* NCTC 11,168 or NADC 20,827, purple, were enumerated. The selective and differential nature of CAC was demonstrated by lack of growth by *Salmonella typhimurium* (ATCC 68,169/UK-1), and differential growth of small blue colonies when inoculated with *E. coli* isolate NADC 123 (Moon et al., 1968). Campy Cefex plates were incubated at 42°C in a microaerophilic environment for 48 h, and cfu resembling those from pure cultures of *C. jejuni* NCTC 11,168 or NADC 20,827, brown, were enumerated. Enrichment for *C. jejuni* was not performed. For statistical purposes, if no colonies resembling *Campylobacter* grew from a sample on either medium, the sample was assigned the culture limit of detection value of 10^3 cfu/g of contents. Poults were considered positive for *Campylobacter* colonization if at least one colony was cultured on the selective media.

Post-culture validation using Campylobacter*-specific multiplex and qPCR*

After enumeration, plates containing colonies were stored at 4°C prior to testing their identity with PCR. First, multiplex PCR was used to determine whether recovered colonies were *C. jejuni* or *C. coli*. Briefly, 3 colonies per CLA-S or CAC plate from each d and each intestinal sample or pure cultures were pooled in 500 mL of molecular grade water (IDT DNA technologies, Coraville, IA) and boiled for 5 minutes. One microliter of the released DNA was used as the reaction template, and PCR reactions were set up as described previously (Cloak and Fratamico, 2002). A non-template control (**NTC**) was included to detect non-specific amplification. DNA from *C. coli* isolates ATCC 80–102 and NADC 5406 and *C. jejuni* isolates NCTC 11,168 and NADC 20,827 grown on CLA-S or CAC plates were used as positive controls for the multiplex PCR reactions. Samples were initially denatured at 94°C for 4 min, followed by 30 amplification cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min and a final extension step of 72°C for 5 min using a C1000 Model thermal cycler (BioRad; Hercules, CA). Ten microliters of PCR products were separated by electrophoresis on 1.5% agarose gels containing SYBR-Safe dye (Life Technologies, Carlsbad, CA), and visualized using a ChemiDoc Touch imaging system (Bio-Rad). Colonies were considered to be positive for *C. jejuni* if both 100 bp (C-1) and 400 bp (*cadF*) amplicons were present, and *C. coli* if the 400 bp (*cadF*) and 894 bp (*ceuE*) amplicons were present. Second, DNA from the same experimental and pure culture samples used for multiplex PCR were additionally tested using qPCR specific for *Campylobacter* 16S and *C. jejuni*-specific *hipO* DNA (de Boer et al., 2015), with the following modifications. DNA from *C. jejuni* isolates NCTC 11,168 and NADC 20,827 were used as positive controls for the qPCR reactions. The qPCR reactions were performed using a BioRad CFX96 deep well instrument and SSO Advanced SYBR green master mix (BioRad). Each reaction was performed at a volume of 20 mL, containing 10 mL of SSO Advanced SYBR green master mix, 1 mL of F primer (10 pmol), 1 mL of R primer (10 pmol), 7 mL of nuclease free water and 1 mL of DNA template. For *Campylobacter* 16S qPCR reactions, the forward primer sequence was CACGTGCTACAATGGCATATACAA (5' to 3'), and the reverse was

CCGAACTGGGACATATTTTATAGATTT (5' to 3'). For *C. jejuni hipO* qPCR reactions, the forward primer sequence was ATGAAGCTGTGGATTTTGCTAGTG (5' to 3'), and the reverse was AAATCCAAAATCCTCACTTGCCA (5' to 3'). TaqMan probes were not used for either reaction. qPCR reactions were performed in triplicate. A NTC control was used to monitor contamination and primer-dimer formation that could produce false positive results. Amplification and detection of specific products were performed using the BioRad CFX96 deep-well system with the following cycle profile: one cycle of 95°C for 3 min and 40 cycles of 95°C for 5 s and 60°C for 30 s, measuring SYBR green fluorescence after each cycle. Melting curve analysis was performed. Quantification cycle (**Cq**) expression values and melting curve data were acquired using the CFX manager software v3.1 (BioRad). Samples were considered positive for *C. jejuni hipO* if the mean Cq value was <37 or significantly lower than the mean value of the NTC reaction. Samples were considered positive for *Campylobacter* 16S if the mean Cq value was significantly less than the mean value for the NTC reaction.

Statistical analysis

Data for growth curves, enumerating *Campylobacter*, and qPCR were analyzed using Prism 7.03 statistical software (Graph Pad Software Inc., San Diego, CA) to perform one- or 2-way ANOVA followed by a post-hoc multiple comparisons test (Tukey) to detect differences among groups. The Fisher Exact test (Taillard et al., 2008) was used for pair-wise comparison of the number of poults positive and poults negative for *Campylobacter* colonization. Results were considered significant at values of $P \leq 0.05$.

RESULTS AND DISCUSSION

In vitro growth and motility of C. jejuni *isolates*

Different turkey origin isolates of *C. jejuni* were evaluated for growth in Bolton's broth base. Isolate NADC 20,826 grew to a much higher terminal OD_{600} value, with a significantly different ($P < 0.001$) growth rate than the rest of the isolates (Figure 1a). Isolates NADC 20,827 and 20,830 had significantly different ($P < 0.01$) growth rates than the remaining isolates (Figure 1a), and isolate 20,827 was selected to tests its colonization of turkey poults. Isolate NCTC 11,168 was selected because it colonizes the intestinal tract of chickens (Ahmed et al., 2002; Seal et al., 2007; Hiett et al., 2008; Wong et al., 2015), and its genome is sequenced (Gaynor et al., 2004). The genome of NADC 20,827 has not yet been sequenced. Isolate NCTC 11,168 was highly adapted to growth in Bolton's broth base (Figure 1b). In contrast, isolate NADC 20,827 had a significantly different growth rate and lower terminal OD_{600} value ($P < 0.01$). In order to achieve the OD_{600} value of 0.4 needed to inoculate poults, biphasic Bolton's broth base was used to culture both NCTC 11,168 and NADC 20,827. Motility is essential for colonization in different animal models of campylobacteriosis (Guerry, 2007). After culture in biphasic Bolton's broth base, both isolates were ≥90% motile, demonstrating their suitability to colonize poults. Poults used in this study were free of *Campylobacter* prior to experimental colonization, based on lack of *Campylobacter* recovery after enrichment of cecal contents, and remained free after mock colonization (Table 1).

Use of selective Campylobacter *media to enumerate* C. jejuni *isolates from different intestinal compartments*

It is important before making a final decision on a selective medium to consider the medium's ability to suppress background growth and grow *Campylobacter (*Kiess et al., 2010*)*. In the present study, selective *Campylobacter* agars CLA-S and Campy Cefex, and the selective and differential agar CAC, were used to enumerate the number of *C. jejuni* cfu/g of intestinal contents using track dilution, a validated method to enumerate bacteria/mL (Siragusa, 1999). Direct plating of intestinal samples was used to enumerate *Campylobacter* cfu/g of intestinal contents, and was superior to enrichment to isolate *Campylobacter* from chicken litter (Kiess et al., 2010). CLA with sulfamethoxazole was used in the present study because of its ability to block growth of non-*Campylobacter* organisms from chicken samples and the ease of counting colonies from a transparent agar. The use of CLA prevented growth of *Acinetobacter baumannii* from chicken carcass washes (Oyarzabal et al., 2005) and was superior to Campy Cefex to suppress background growth and enumerate *Campylobacter* from chicken litter (Kiess et al., 2010). The use of CLA-S also enhanced *Campylobacter* selection and reduced background growth from chicken carcass washes (Line et al, 2008), when compared to other selective *Campylobacter* media. However, other studies demonstrate that Campy Cefex and modified Campy Cefex agars were superior to CLA to directly culture *Campylobacter* from chicken carcass washes (Oyarzabal et al., 2005). The use of CLA-S, rather than CLA, may have increased their sensitivity to enumerate *Campylobacter* from carcass washes. In previous attempts to directly culture *Campylobacter* from turkey feces, the use of CLA-S (Line, 2001) significantly decreased the background growth of non-*Campylobacter* organisms, when compared to CLA. In a separate study, CLA-S was superior to Campy Cefex to enumerate *Campylobacter* from chicken carcass washes (Line et al., 2008), and Campy Cefex was less successful than CLA to suppress background growth from chicken litter samples (Kiess et al., 2010). We found that *Campylobacter* colonies

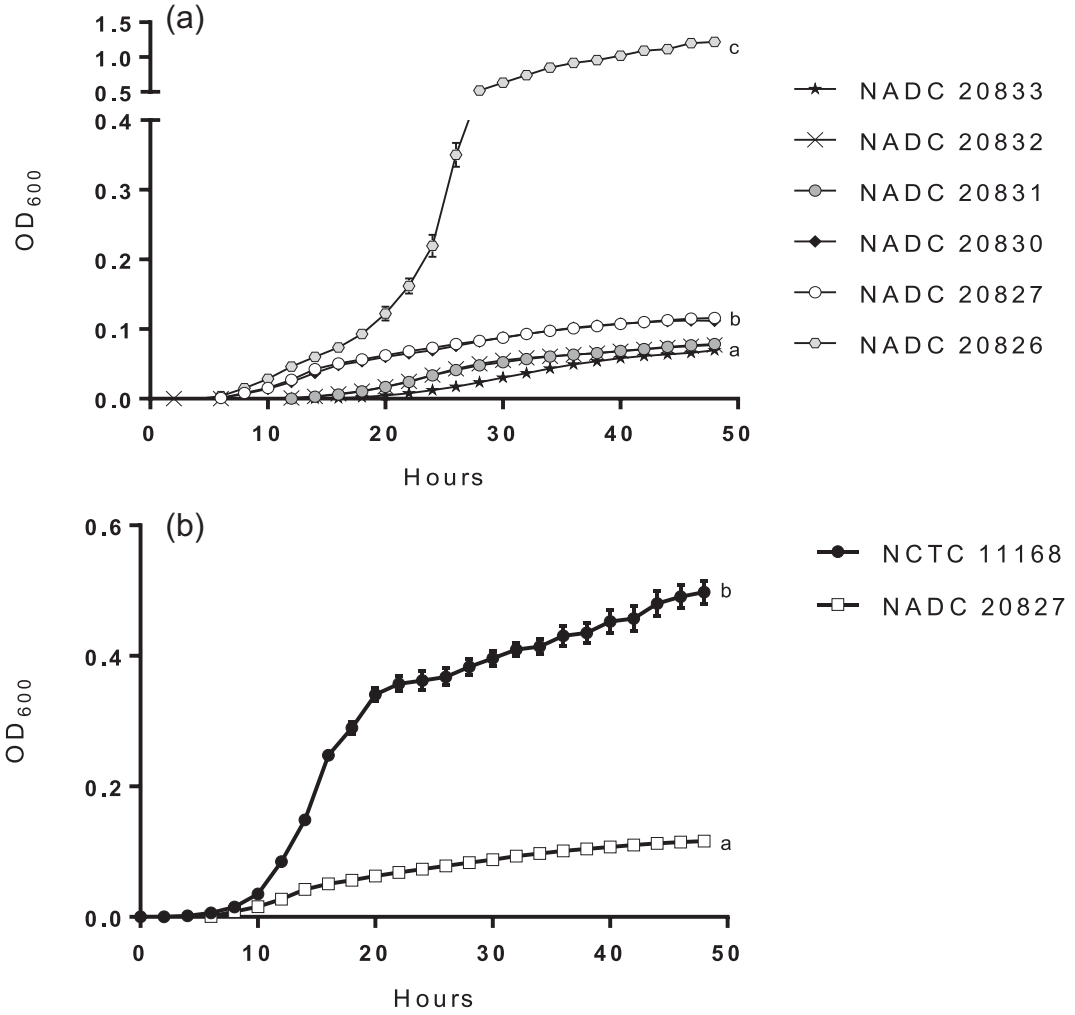

Figure 1. Growth curves of *C. jejuni* isolates. Data represent the mean ± SEM OD_{600} of 8 cultures in Bolton's broth base measured every 2 h for a total of 48 h at 42°C in a microaerophilic environment with the background value of unincolated media subtracted. (a) *C. jejuni* NADC 20,826, 20,827, 20,830, 20,832, and 20,833 were isolated from commercial turkeys and (b) *C. jejuni* NCTC 11,168 and NADC 20,827. Data were analyzed using one-way ANOVA followed by a post-hoc multiple comparisons test (Tukey). Significant differences ($P < 0.05$) in growth rate between isolates are represented by different letters.

on CLA-S could be easily enumerated after 48 h of incubation without risk of overgrowth, and growth of background colonies was heavily suppressed. Occasional non-*Campylobacter* colonies were present from intestinal samples beyond the 10^{-2} dilution, but were at low enough levels to identify and enumerate purple *Campylobacter* colonies.

Campy Cefex agar has been extensively used to enumerate *Campylobacter* cfu in cecal contents from chickens (Stern et al., 2005; Neal-McKinney et al, 2014; Schroeder et al., 2014; Gonsalves et al., 2016). Some selective *Campylobacter* media (e.g., mCCDA, Karmali, Campy Cefex, and other agars) are not transparent because they contain blood or charcoal. As a result, enumeration of *Campylobacter* cfu from animal or environmental samples can be difficult using non-transparent media. In the present study, colonies resembling pure cultures of *C. jejuni* were detected on Campy Cefex agar, but were unable to be accurately enumerated because of heavy overgrowth by non-*Campylobacter* organisms. As a result, we discontinued the use of Campy Cefex after poor results obtained after culturing d 3 samples.

The CAC media is both selective and differential for *Campylobacter*, but has not yet been reported to enumerate *Campylobacter* cfu/g from poultry intestinal contents. Similar to CLA-S, CAC is a blood-free transparent agar, which facilitates visualizing and enumerating *Campylobacter* cfu on these plates, with cleavage of a chromogen to produce purple colonies. To our knowledge, this is the first publication evaluating CAC to enumerate *Campylobacter* from intestinal contents from an animal species. The optimal time to count *Campylobacter* colonies from turkey intestinal samples on CAC was approximately 24 to 36 h after incubation. According to the manufacturer, most non-*Campylobacter* organisms do not grow on CAC, but those that do grow produce blue colonies. When culturing turkey intestinal contents, some samples contained blue colonies in the 10^{-1} dilution, but were not present in subsequent

Table 1. Summary of the number of animals with *C. jejuni* by direct culture on selective *Campylobacter* media and post-culture validation using multiplex *C. jejuni* and *C. coli* PCR.

			Number of poults positive by direct culture		Multiplex *C. jejuni/C. coli* PCR	
Challenge isolate	Sample	Days post colonization	CLA-S	CHROMagar *Campylobacter*	CLA-S	CHROMagar *Campylobacter*
Mock	Cecum	3	0 of 5	0 of 5		
		7	0 of 5	0 of 5	ND†	ND†
		21	0 of 5	0 of 5		
NCTC 11,168	Ileum	3	8 of 8	8 of 8		
		7	8 of 8	7 of 8		
		21	2 of 8*	1 of 8*		
	Colon	3	8 of 8	8 of 8		
		7	8 of 8	8 of 8	Pos	Pos
		21	8 of 8	8 of 8		
	Cecum	3	8 of 8	8 of 8		
		7	8 of 8	8 of 8		
		21	8 of 8	8 of 8		
20,827	Ileum	3	8 of 8	8 of 8		
		7	7 of 8	7 of 8		
		21	1 of 11*	1 of 11*		
	Colon	3	8 of 8	8 of 8		
		7	8 of 8	8 of 8	Pos	Pos
		21	10 of 11	6 of 11*		
	Cecum	3	8 of 8	8 of 8		
		7	8 of 8	8 of 8		
		21	11 of 11	11 of 11		

† = Not done—*C. jejuni* were not cultured from these samples.
Pos = Positive for *C. jejuni* multiplex PCR and negative for *C. coli*.
* = $P < 0.05$ (Fisher's exact test).

dilutions. Thus, CAC is a reliable selective and differential media to enumerate *Campylobacter* cfu/g from intestinal contents.

Growth of C. jejuni *from distal ileal samples*

Previously, *C. jejuni* was shown to differentially colonize the distal ileum of chickens (Chaloner et al., 2014). Isolates NCTC 11,168 and NADC 20,827 were recovered from distal ileal samples on CLA-S and CAC media, but the number of *Campylobacter* significantly decreased ($P < 0.05$) at d 7 and 21 (Figures 2a,b). The number of poults positive for ileal *Campylobacter* colonization at d 21 was significantly different ($P = 0.007$ and 0.015) for CLA-S when comparing at d 3 and 7, and significantly different ($P = 0.001$ and 0.01) for CAC when comparing at d 3 and 7 (Table 1). There was no significant difference in the number of *C. jejuni* recovered from the same distal ileal sample cultured on CLA-S and CAC. In a previous study, ileum was identified as the primary site of colonization by *C. jejuni,* which led to colonization of the jejunum and ceca (Lacharme-Lora et al., 2017). Perhaps initial colonization occurs in the distal ileum of turkeys, but the declining number of *C. jejuni* recovered over time suggests that the turkey distal ileum is only transiently colonized by *C. jejuni.*

Growth of C. jejuni *from colon samples*

Isolates NCTC 11,168 and NADC 20,827 colonized the colon of poults. However, the number of NADC 20,827 recovered at d 21 on both CLA-S and CAC was significantly less ($P < 0.01$) than that cultured at d 3 and 7 (Figures 3a,b). The number of poults positive for *C. jejuni* at d 21 on CAC media was significantly different ($P = 0.045$ for both) than d 3 and 7 (Table 1). It is unclear why fewer poults colonized with NADC 20,827 had positive colon contents when cultured on CAC and compared to CLA-S. Our results are similar to a previous study (Chaloner et al., 2014) in which different isolates of *C. jejuni* colonized at dissimilar levels in the colon of chickens. There was no significant difference in the number of *C. jejuni* recovered from the same colon sample cultured on CLA-S and CAC. At d 7 and 14 post colonization, the colon of poults colonized with NCTC 11,168 grossly appeared reddened, but colon content appearance was similar to poults colonized with NADC 20,827. These data indicate that *C. jejuni* isolate NCTC 11,168 appears to be better adapted than NADC 20,827 to colonize the colon.

Growth of C. jejuni *from cecal samples*

Isolates NCTC 11,168 and NADC 20,827 highly colonized the cecum of poults (Figure 4a,b; Table 1). The number of NCTC 11,168 cultured on CLA-S and CAC varied by the d with a significant difference ($P < 0.05$) in the number grown on CLA-S and CAC at d 7 versus d 3 and 21 post colonization. The amount of isolate NADC 20,827 grown on CLA-S and CAC was not statistically different throughout the study, demonstrating that this isolate persistently colonized the cecum

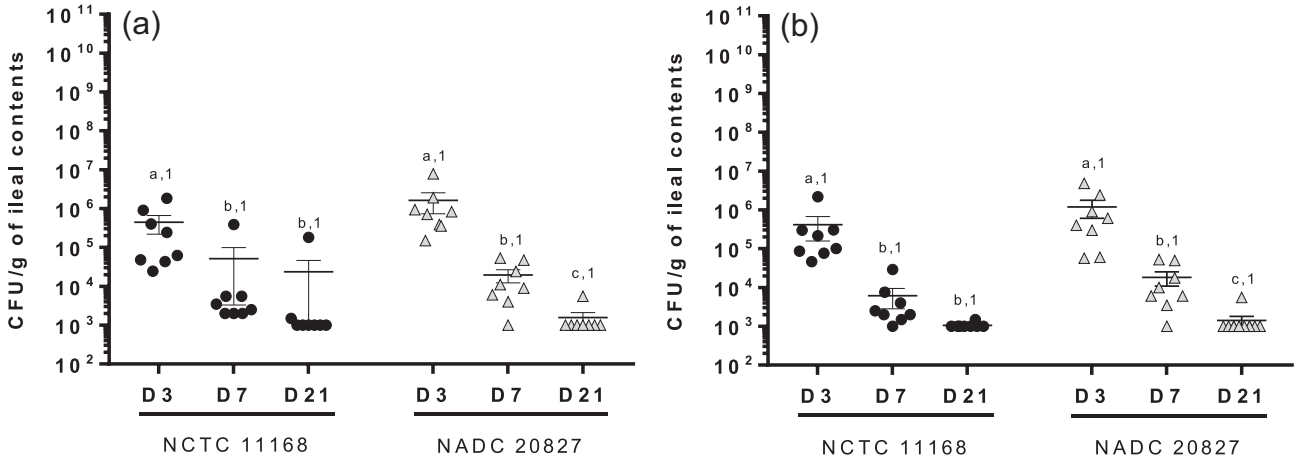

Figure 2. Enumeration of different *C. jejuni* isolates NCTC 11,168 and NADC 20,827 from distal ileal contents of turkey poults. Scatter plot data represent the mean enumeration of *Campylobacter* per g of distal ileal contents per poult, and the mean (solid bar) ± SEM for the group of animal at d 3 (D3), 7 (D7), and 21 (D21) post colonization cultured on (a) Campy Line agar with sulfamethoxazole and (b) CHROMagar *Campylobacter*. Statistical differences in the number of enumerated *Campylobacter* cfu were determined using one-way ANOVA followed by a post-hoc multiple comparisons test (Tukey). Significant differences ($P < 0.05$) for each isolate between time points on the same medium are represented by different letters, and the same time points between different media are represented by different numbers.

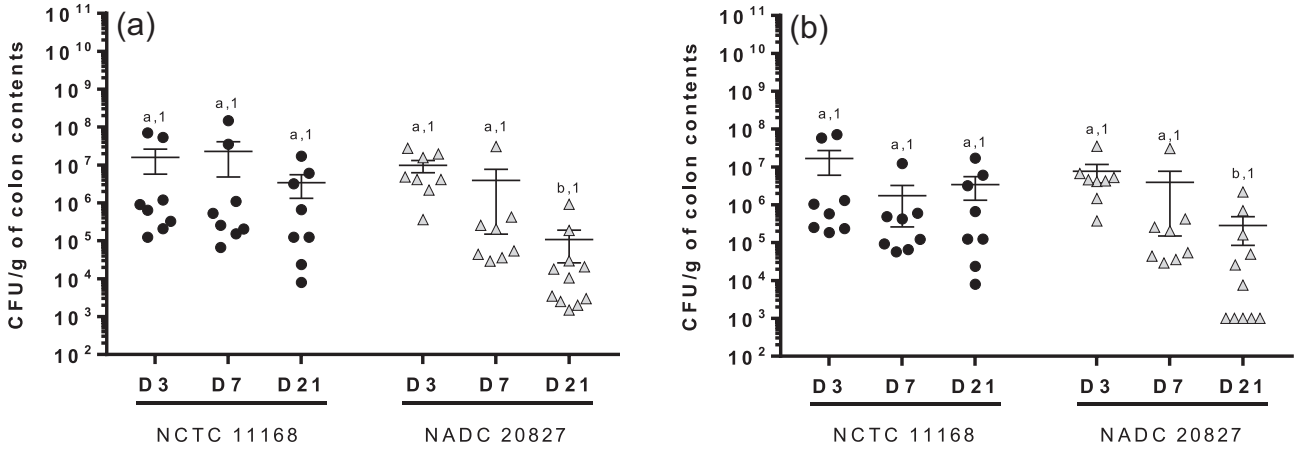

Figure 3. Enumeration of different *C. jejuni* isolates NCTC 11,168 and NADC 20,827 from colon contents of turkey poults. Scatter plot data represent the mean enumeration of *Campylobacter* per g of colon contents per poult, and the mean (solid bar) ± SEM for the group of animal at d 3 (D3), 7 (D7), and 21 (D21) post colonization cultured on (a) Campy Line agar with sulfamethoxazole and (b) CHROMagar *Campylobacter*. Statistical differences in the number of enumerated *Campylobacter* cfu were determined using one-way ANOVA followed by a post-hoc multiple comparisons test (Tukey). Significant differences ($P < 0.05$) for each isolate between time points on the same medium are represented by different letters, and the same time points between different media are represented by different numbers.

of turkey poults. *C. jejuni* colonizes within the mucus layer between villi of the cecum of chickens (Beery et al., 1988). Thus, it is possible that sampling cecal contents alone may not represent the total number of *Campylobacter* present. Future experiments will focus on comparing lumen and mucosa-associated *C. jejuni* in colonized poults. Colonization with NCTC 11,168 produced watery and foamy cecal contents at d 3, 7, and 21 in the study, with reddened cecal mucosa present at d 7 and 21. Colonization of young chicks <1 wk old produced morbidity in the form of watery diarrhea (Sanyal et al., 1984; Welkos, 1984), which also was seen in poults experimentally colonized with *C. jejuni* (Wallace et al., 1998). In contrast, cecal contents from NADC 20,827 were thick and lacked foam, suggesting that this isolate behaved similar to a poultry-adapted *Campylobacter* (Lam et al., 1992). Regardless of the difference in cecal content appearance, intestinal colonization by NCTC 11,168 and NADC 20,827 produced no morbidity (e.g., watery diarrhea, depression, or anorexia) or mortalities.

Continued laboratory passage of *C. jejuni* reduces the colonization potential in chickens (Ringoir and Korolik, 2003) and has been noted for strain NCTC 11,168 (Ringoir and Korolik, 2003; Gaynor et al., 2004). NCTC 11,168-O, the original strain preserved by Skirrow, is an excellent colonizer of poults and invader of human epithelial cells. These abilities decline as it genetically modifies into strain NCTC 11,168-GS, which occurs after laboratory adaptation (Gaynor et al., 2004). Poor colonization by NCTC 11,168-GS, due to laboratory adaptation, was not restored after in vivo passage in

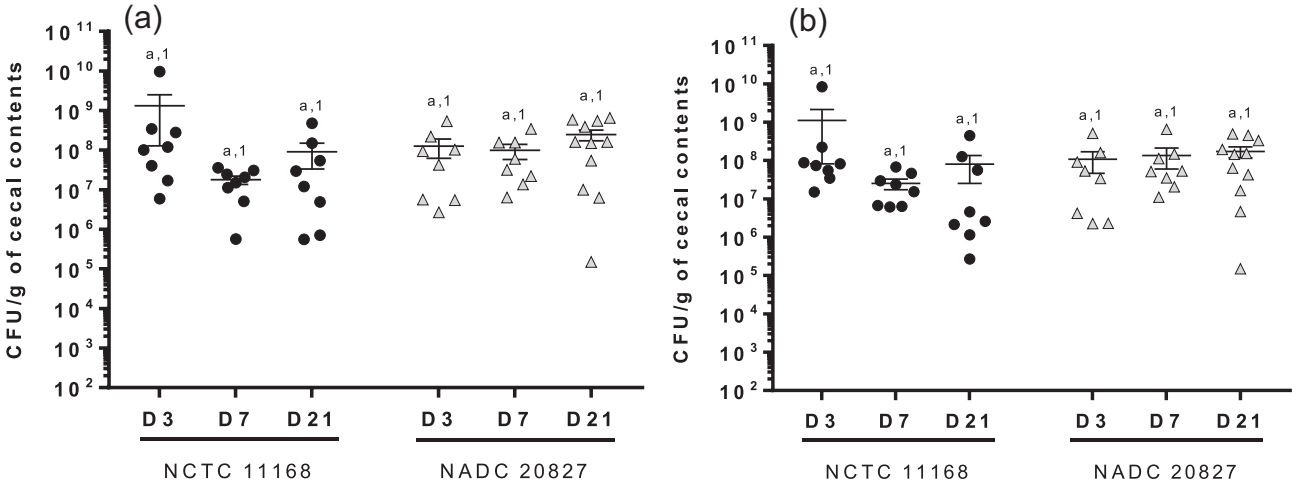

Figure 4. Enumeration of different *C. jejuni* isolates NCTC 11,168 and NADC 20,827 from cecal contents of turkey poults. Scatter plot data represent the mean enumeration of *Campylobacter* per g of cecal contents per poult, and the mean (solid bar) ± SEM for the group of animal at d 3 (D3), 7 (D7), and 21 (D21) post colonization cultured on (a) Campy Line agar with sulfamethoxazole and (b) CHROMagar *Campylobacter*. Statistical differences in the number of enumerated *Campylobacter* cfu were determined using one-way ANOVA followed by a post-hoc multiple comparisons test (Tukey). Significant differences ($P < 0.05$) for each isolate between time points on the same medium are represented by different letters, and same time points between different media are represented by different numbers.

Table 2. Post-culture validating using *C. jejuni hipO* and *Campylobacter* 16S qPCR.

Isolate	DNA source	*C. jejuni hipO*	*Campylobacter* 16S
C. jejuni NCTC 11,168	Pure culture on CLA-S	Pos	Pos
	Isolate from cecal sample on CLA-S	Pos	Pos
E. coli NADC 123	Pure culture on LB agar	Neg	Neg
C. jejuni NADC 20,827	Pure culture on CLA-S	Pos	Pos
	Isolate from cecal sample on CLA-S	Pos	Pos

Pos = positive for qPCR reaction.
Neg = negative for qPCR reaction.

chickens (Ahmed et al., 2002). Based on these findings and its ability to grow in laboratory media, it was unexpected that NCTC 11,168 would persistently colonize the cecum of turkey poults. Likewise, it was unexpected that isolate NADC 20,287, with low in vitro passage and poor growth in laboratory media, was the most consistent and highest cecal colonizer. Thus, results of in vitro growth do not appear predictive of the ability of *C. jejuni* to colonize poults. Results of the present study demonstrate that *C. jejuni* isolates NCTC 11,168 and NADC 20,827 were enumerable from turkey cecal contents similar to the range (10^6 to 10^9 cfu/g) described in experimentally colonized chickens (Wyszynska et al., 2004; Meade et al., 2009; Buckley et al., 2010; Kobierecka et al., 2016; Nothaft et al., 2016). Human origin *C. jejuni* isolates may colonize poults less successfully than poultry-origin isolates (Korolik et al., 1998); however, our data indicate no difference between NCTC 11,168 and turkey-origin isolate NADC 20,827 to colonize the ceca of poults. Both isolates will be useful to evaluate the biology of *C. jejuni* in turkeys and test pre-harvest strategies to reduce *C. jejuni* colonization in turkeys and promote food safety.

Post-culture validation of recovered colonies using PCR

Because selective *Campylobacter* media may afford growth of organisms that resemble, but are not, *Campylobacter*, colonies recovered from the present study were tested in different PCR reactions to verify identity. Multiplex *Campylobacter* PCR of colonies grown on CLA-S and CAC validated that that colonies recovered from intestinal samples on either CLA-S or CAC were *C. jejuni*, and not *C. coli* (Table 1) (Cloak and Fratamico, 2002). Additional post-culture qPCR was performed on colonies recovered from CLA-S, CAC or pure cultures of *C. jejuni* to test for *hipO*, a *C. jejuni*-specific gene, and *Campylobacter* 16S rRNA DNA (de Boer et al., 2015). Results of the qPCR for *C. jejuni* hipO and *Campylobacter* 16S DNA (Table 2) corroborate the multiplex PCR data and demonstrate that colonies recovered on CLA-S and CAC were *C. jejuni*.

In conclusion, we have identified that *C. jejuni* isolates NCTC 11,168 and NADC 20,827 persistently colonize the cecum of commercial turkey poults and that selective *Campylobacter* media CLA-S and CAC were both suitable for enumeration of *C. jejuni* from

intestinal samples. Future work will focus on the host response in intestinal tissues in poults colonized with isolates NCTC 11,168 and NADC 20,827. These data may lead to a better understanding of the commensal relationship of *C. jejuni* in the cecum of turkeys, and evaluate pre-harvest strategies to reduce its colonization and promote food safety.

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