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Article

MICROBIOLOGICAL METHODS

Development of a Selective Agar for Improving *Campylobacter jejuni* Detection in Food

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Abstract

Background: *Campylobacter jejuni* is a major gastroenteritis-causing foodborne pathogen. However, it is difficult to isolate when competing bacteria or cold-damaged cells are present.
Objective: Herein, a medium (*Campylobacter* selective agar, CSA) was developed and supplemented with catalase, L-serine, L-cysteine, and quercetin for the selective detection of *C. jejuni* in food.
Method: The *C. jejuni*-detection efficiency in media broth and chicken tenders was evaluated. The pathogen was enumerated on modified charcoal–cefoperazone–deoxycholate agar (mCCDA), CSA supplemented with 4 μM catalase (CSA-C4), 8 μM catalase (CSA-C8), 20 mM L-serine (CSA-S20) or 50 mM L-serine (CSA-S50), and mCCDA supplemented with 0.5 mM L-cysteine (mCCDA-LC0.5), 1 mM L-cysteine (mCCDA-LC1), 40 μM quercetin (mCCDA-Q40) or 320 μM quercetin (mCCDA-Q320). The detection efficiency was then evaluated by counting colonies on the selective agar media. Quantitative assessment was also performed using chicken and duck carcasses.
Results: The *C. jejuni* detection efficiencies were higher ($P < 0.05$) in the groups CSA-C4 or CSA-C8, and CSA-S20 or CSA-S50, than mCCDA, and the detection efficiencies were maintained even in the presence of *Acinetobacter baumannii*, a competing bacterium. In the quantitative test, CSA-C8 and CSA-S50 demonstrated higher *C. jejuni*-detection efficiencies than mCCDA (control).
Conclusions: Therefore, CSA-C8 and CSA-S50 improved the detection efficiency of *C. jejuni* in poultry products by promoting the recovery of cold-damaged cells.
Highlights: When using CSA-C8 or CSA-S50 developed in this study for detection of *C. jejuni* in food, detection efficiency was higher than mCCDA.

Campylobacter jejuni is one of the most common primary foodborne pathogens globally (1), possibly owing to its low infectious dose (100 to 500 cells). According to the Foodborne Diseases Active Surveillance Network (FoodNet), from 2011 to 2015, approximately 14 cases reported per 100 000 individuals presented with food poisoning caused by *C. jejuni* (2). In Korea, foodborne illness caused by *C. jejuni* was reported in seven cases and in 194 individuals in 2019 (3). Generally, *C. jejuni* infection is characterized by gastroenteritis, fever, and diarrhea. In severe cases, it may lead to the development of Guillain-Barre syndrome, which

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affects the nervous system (4). Therefore, the reliable detection of *C. jejuni* in food and the prevention of *C. jejuni* outbreaks is much needed.

Poultry remains the most common source of *C. jejuni* contamination (5). However, it is difficult to isolate *C. jejuni* from poultry as it is sensitive to the presence of other competing bacteria and oxygen (6, 7). In addition, several food products, including poultry meat, are frozen or refrigerated. These storage conditions affect the detection efficiency, as *C. jejuni* weakened by the storage process may not be detectable using selective media (8). Therefore, it is crucial to develop an appropriate detection method that compensates for these complexities.

Unlike other bacteria, *C. jejuni* cannot utilize glucose as its sole carbon source, as it does not produce the glycolytic enzyme 6-phosphofructokinase (Pfk) (9). Therefore, it relies on glucogenic amino acids as growth substrates (10). Accordingly, the presence of these amino acids in any selective medium is a critical determinant of the successful growth of this pathogen (11). In addition, since *C. jejuni* is a microaerophile, it is important to remove superoxide (SO) generated in the presence of oxygen (12) from the media; hence, the addition of an enzyme that can catalyze the breakdown of superoxide is helpful.

Previous studies have shown that antibiotic supplementation in selective agar improved the selectivity of the media for *C. jejuni* (13, 14). In particular, cefoperazone, a third-generation cephalosporin, has been widely used for the detection of *C. jejuni* in food samples. Antibiotics are added at high concentrations to form modified charcoal–cefoperazone–deoxycholate agar (mCCDA) (15). However, since the number of antibiotic-resistant bacterial strains is increasing (16, 17), only supplementation with antibiotics alone is insufficient for developing novel selective media. Therefore, for the effective detection of *C. jejuni*, it is preferable to add antibiotics along with other substances that promote the rapid recovery of damaged cells or facilitate growth.

The objective of this study was to develop a selective agar for the detection of *C. jejuni* in food by supplementation of the media with *C. jejuni* growth-promoting components.

METHOD

Experimental

Preparation of Inocula.—*C.jejuni* NCTC11168 (National Collection of Type Cultures, London, UK) was stored at –70°C using beads enclosed in vials (AES Chemunex, Combourg, France). A single bead was inserted into the end of the loop, which was then used to streak a Columbia blood agar (BioMérieux, Marcy-l'Étoile, France) plate, which was incubated at 42°C for 48 h under microaerobic conditions (CO_2: 2.5–9.5%, O_2: 6.2–13.2%) established using the CampyGen gas-generating system (Oxoid Ltd, Basingstoke, UK). Colonies from these plates were then collected using a loop and restreaked onto Columbia agar with a loop, and the plates were incubated at 42°C for 48 h under microaerobic conditions. The colonies formed were then collected using a loop and suspended in 5 mL phosphate buffered saline (PBS; pH 7.4; 0.2 g KH_2PO_4, 1.5 g $Na_2HPO_4{\cdot}7H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 L distilled water). These suspensions were centrifuged at 1912 × *g* and 4°C for 15 min, and the cell pellets were washed twice with PBS. The supernatants were then discarded, and the cell pellets were resuspended in PBS and diluted to 3–5 log CFU/mL, which was determined by plating the suspension on mCCDA. *Acinetobacter baumannii* KACC12454 (Korean Agricultural Culture Collection, Wanju, South Korea) was cultured in 10 mL Luria-Bertani broth (LB; Becton, Dickinson and Company, Sparks, MD) at 35°C for 24 h. Aliquots (0.1 mL) of the cultures were transferred to 10 mL fresh LB broth and subcultured at 35°C for 24 h. The culture media were centrifuged at 1912 × *g* and 4°C for 15 min, and the cell pellets were washed twice with PBS. The suspensions were then diluted to 3–4 log CFU/mL with PBS.

Evaluation of Supplementation in Agar Media

The *Campylobacter* selective agar (CSA) was formulated by mixing 12 g agar, 3 g casein hydrolysate, 4 g charcoal, 25 g nutrient broth, and 1 g sodium deoxycholate in 1 L distilled water, then CSA was supplemented individually with the following components to evaluate their effects on *C. jejuni* detection.

Catalase.—Similarto the role played by sodium pyruvate in mCCDA (Oxoid), catalase prevents the accumulation of hydrogen peroxide in growth media (24). Accordingly, 10 mg catalase (Sigma-Aldrich Corp., St. Louis, MO) was dissolved in 10 mL potassium phosphate buffer (PPB; 50 mM). To produce 0.1 M PPB, 615 μL 1 M K_2HPO_4, 375 μL 1 M KH_2PO_4, and 10 mL distilled water were mixed. Following this, an equal volume of distilled water was added to produce 50 mM PPB, which was used for the dissolution of catalase. This solution was then sterilized by filtration using a 0.2 μm filter (Hyundai Micro, Seoul, South Korea) and used to supplement CSA at concentrations of 4 μg/mL (CSA-C4) or 8 μg/mL (CSA-C8).

L-serine.—*Campylobacter*can utilize several glucogenic amino acids such as serine, aspartate, glutamate, and proline. L-serine plays an important role in the growth of *C. jejuni* as a carbon source. Its role is the same as that of ferrous sulfate (18) in mCCDA. Accordingly, 25 mg L-serine (Sigma-Aldrich) was dissolved in 100 mL sterile distilled water, and the suspension was sterilized by filtration using a 0.2 μm filter. Following this, the solution was added to CSA at a final concentration of 20 mM (CSA-S20) or 50 mM (CSA-S50).

L-cysteine.—L-cysteinehas been shown to play an important role as a nitrogen source for *C. jejuni* and should be added to any selective media at a concentration of at least 0.2 mM (19). In this study, L-cysteine (Sigma-Aldrich) was dissolved in sterilized distilled water and used to produce 0.5 and 1 mM solutions, which were sterilized by filtration using a 0.2 μm filter. The 0.5 and 1 mM L-cysteine solutions were added to mCCDA to produce mCCDA-LC0.5 and mCCDA-LC1, respectively, after autoclaving.

Quercetin.—Quercetinis known to exert an antimicrobial effect against *A. baumannii* (20). As the efficiency of *Campylobacter* detection decreases when *A. baumannii* is present in food (21), *A. baumannii* growth should be controlled. Quercetin (1.9 mg; Sigma-Aldrich) was dissolved in 1 mL ethyl alcohol (SAMCHUN, Seoul, South Korea) and added to mCCDA at a concentration of 40 μM (mCCDA-Q40) or 320 μM (mCCDA-Q320) after autoclaving. Following this, a CCDA selective supplement (SR0155; Oxoid) composed of 0.01 g/L amphotericin B and 0.032 g/L cefoperazone was added to the agar immediately before the medium was poured. The mCCDA, CSA-C4, CSA-C8, CSA-S20, CSA-S50, mCCDA-LC0.5, mCCDA-LC1, mCCDA-Q40, and mCCDA-Q320

plates were inoculated with *C. jejuni* and incubated at 42°C for 48 h under microaerobic conditions. The colonies formed on the plates were counted manually, and the number of colonies formed were compared between the plates. The two media with the highest *C. jejuni* recovery rates were selected for further evaluation.

Evaluation of the Selectivity of the Developed Agar Media

The selectivity of the CSA-C8 and CSA-S50 media (Table 1) was further evaluated based on the specificity for *C. jejuni*. To evaluate the selectivity of the developed agar media for *C. jejuni*, a suspension of *A. baumannii* and *C. jejuni* (in 1:1 ratio) inocula was prepared at 3–4 log CFU/mL. The mixture was then plated on CSA-8 and CSA-S50 plates. The plates were incubated at 42°C for 48 h under microaerobic conditions, and the number of colonies formed were determined.

Assessment of C. jejuni *Contamination in Food Using CSA-C8 and CSA-S50*

Twenty-five gram portions of raw chicken tenders were placed in sterile filter bags and inoculated with 0.1 mL 5 log CFU/mL aliquots of *C. jejuni*. The inoculated samples were then hand-rubbed to spread the cells evenly on the surface. Fifty milliliters 0.1% buffered peptone water (BPW) were added to half the number of samples, and the samples were homogenized in a BagMixer (Interscience, St. Nom, France) for 60 s. The homogenates were 10-fold diluted using BPW, and 100 μL of each diluent was plated on the developed agar media. The plates were incubated at 42°C for 48 h under microaerobic conditions, and the number of colonies formed were determined. Half the number of samples were maintained continuously at 4°C for 2 days, and plating was performed following the procedure described above. For the quantitative test, 10 chicken and 22 duck carcasses were purchased from a local market and transferred to zipper bags. To these, 400 mL (chicken) or 800 mL (duck) of BPW was added and shaken for 1 min. Finally, 0.1 mL aliquots from the samples were plated on the developed media, followed by incubation at 42°C for 48 h under microaerobic conditions and determination of the number of colonies formed.

Table 1. Compositions of modified charcoal–cefoperazone–deoxycholate agar (mCCDA), *Campylobacter* selective agar (CSA)-C8 (CSA supplemented with 8 μg/mL catalase), and CSA-S50 (CSA supplemented with 50 mM L-serine)

	Composition per L, unit: g		
Ingredients	mCCDA	CSA-C8	CSA-S50
Agar	12	12	12
Casein hydrolysate	3	3	3
Catalase	–	8 μg/mL	–
Charcoal	4	4	4
Ferrous sulfate	0.25	0.25	–
L-serine	–	–	50 mM
Nutrient broth	25	25	25
Sodium deoxycholate	1	1	1
Sodium pyruvate	0.25	–	0.25
Amphotericin B	0.01	0.01	0.01
Cefoperazone	0.032	0.032	0.032

Statistical Analysis

Experimental data were analyzed using the general linear model (GLM) from SAS® version 9.3 software (SAS Institute, Inc., Cary, NC), and the mean values were compared using the pairwise *t*-test at $\alpha = 0.05$.

Results and Discussion

Selection of Supplements and the Appropriate Concentrations

The number of *C. jejuni* CFUs was higher when the aliquots were plated on CSA supplemented with 4 μg/mL (CSA-C4) or 8 μg/mL (CSA-C8) catalase ($P < 0.05$) than the number of CFUs formed on mCCDA (Figure 1A). Similar to catalase, sodium pyruvate, a component of mCCDA, is known to decompose hydrogen peroxide, resulting in the prevention of the accumulation of hydrogen peroxide produced during bacterial respiration. Catalase plays a

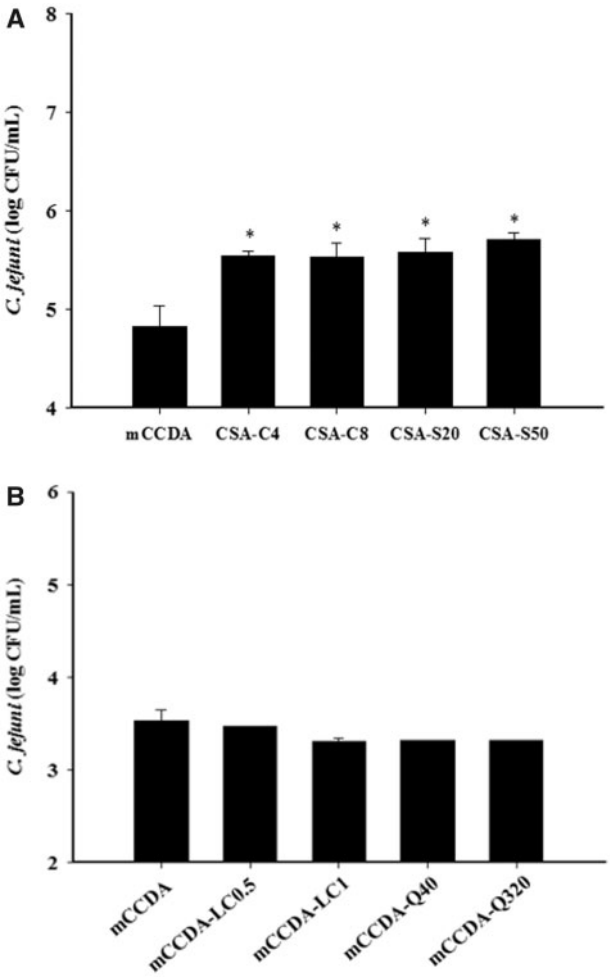

Figure 1. Evaluation of *C. jejuni* detection using (A) mCCDA, CSA-C4, CSA-C8, CSA-S20, CSA-S50, and (B) mCCDA, mCCDA-LC0.5, mCCDA-LC1, mCCDA-Q40, mCCDA-Q320. mCCDA= modified charcoal–cefoperazone–deoxycholate agar; *Campylobacter* selective agar (CSA)-C4= CSA supplemented with 4 μg/mL catalase; CSA-C8= CSA supplemented with 8 μg/mL catalase; CSA-S20= CSA supplemented with 20 mM L-serine; CSA-S50= CSA supplemented with 50 mM L-serine; mCCDA-LC0.5= mCCDA supplemented with 0.5 mM L-cysteine; mCCDA-LC1= mCCDA supplemented with 1 mM L-cysteine; mCCDA-Q40= mCCDA supplemented with 40 μM quercetin; mCCDA-Q320= mCCDA supplemented with 320 μM quercetin. * =Significant difference with mCCDA ($P < 0.05$).

critical role in the oxidative defense mechanism by decomposing hydrogen peroxide into water and oxygen (22). According to Gamer and Elsanousi (23), compared to sodium pyruvate supplementation, catalase supplementation leads to the recovery of a greater number of bacterial cells. In a study by Martin et al. (24), the colonies formed after supplementation with catalase and sodium pyruvate in the same medium were counted, and the results were compared. A higher number of colonies were formed in the medium supplemented with catalase in cases of both injured and non-injured cells. This suggests that catalase supplementation increases the number of viable cells in the inoculum, and thus, improves the detection efficiency.

The addition of L-serine at 20 mM (CSA-S20) or 50 mM (CSA-S50) also led to significantly higher ($P < 0.05$) *C. jejuni* cell counts compared to those observed in mCCDA (Figure 1A). Serine plays a crucial role in *C. jejuni* metabolism and is frequently detected in chicken excreta (25). Pyruvate forms the primary link between the Embden–Meyerhof–Parnas pathway and the citric acid cycle. However, as *C. jejuni* cannot produce Pfk, it cannot produce pyruvate from glucose (26, 27). Instead, *Campylobacter* utilizes several glucogenic amino acids (28) to produce this critical intermediate. Serine is one of the glucogenic amino acids used, and as shown in several studies, supplementation with serine augments *C. jejuni* growth (29). *C. jejuni* expresses L-serine dehydratase (SdaA), which can break down L-serine into pyruvate. Several studies have shown that this catabolic process is critical to intestinal clustering (30). SdaA and SdaC (serine transporter) are important for L-serine metabolism and play a role in intestinal colonization in chickens (18). The role played by serine is similar to that of ferrous sulfate, which augments the growth and colonization of *C. jejuni*. Even though L-serine is not essential for *C. jejuni* growth, it is highly beneficial for its growth when added as a key carbon source to the media following exposure to various environmental factors (18). Therefore, the relationship between *C. jejuni* growth and L-serine is well-documented.

L-cysteine and quercetin were added to mCCDA to compare the growth of *C. jejuni* in their presence and absence. There were no significant differences observed in the cell counts between the two groups (Figure 1B). Therefore, the agar media formulated using CSA with 8 μM catalase (CSA-C8) or 50 mM L-serine (CSA-S50) were selected for the subsequent experiments.

Media Selectivity

After mixing *A. baumannii* and *C. jejuni* inocula, which are primarily detected jointly on mCCDA (31), the inocula were spread on mCCDA, CSA-C8, and CSA-S50 plates. Higher *C. jejuni* cell counts were observed in CSA-C8 and CSA-S50 than in mCCDA, even in the presence of competing bacteria (Table 2). CSA-S50 particularly promoted *C. jejuni* growth and exhibited a colony count that was 0.5 log CFU/mL higher than that on mCCDA.

Table 2. Evaluation of *C. jejuni* detection in the presence of *A. baumannii* using mCCDA, CSA-C8, and CSA-S50

Selective medium[a]	Mean ± SD, log CFU/mL
mCCDA	3.23 ± 0.21[b]
CSA-C8	3.69 ± 0.48[b]
CSA-S50	4.09 ± 0.42[c]

[a]mCCDA = Modified charcoal-cefoperazone-deoxycholate agar; *Campylobacter* selective agar (CSA)–C8 = CSA supplemented with 8 μg/mL catalase; CSA–S50 = CSA supplemented with 50 mM L–serine.
[b,c]Means in the same column with different superscript capital letters are significantly different ($P < 0.05$).

Assessment of Food Samples Using the Developed Agar Media

The detection efficiency of the developed media for *C. jejuni* was assessed using inoculated chicken tenders. The *C. jejuni* cell counts on CSA-S50 were higher ($P < 0.05$) than those on mCCDA (Table 3). Additionally, CSA-C8 also showed higher cell counts than mCCDA. The *C. jejuni* cell counts were also significantly higher ($P < 0.05$) in CSA-S50 than in mCCDA after storage at 4°C for 2 days (Table 4). Therefore, serine was confirmed to promote the recovery of *C. jejuni* cells. It was observed that the detection efficiencies of CSA-C8 and CSA-S50 media were higher than that of mCCDA when studied using food samples. Since *Campylobacter* exists in a viable but non-culturable (VBNC) state or it suffers from cold damage at low temperatures (32), this finding is of relevance for enhancing the recovery potential of agar medium for cold-damaged bacteria.

Quantitative Assessment of the Developed Agar Media

C. jejuni was detected in 1 of the 10 chicken and 7 of the 22 duck carcasses. Comparing the cell counts obtained from the different developed agar media, the detection level (2.8 log CFU/g) of bacteria in chicken carcasses was the highest when the samples were cultured in CSA-S50 (Figure 2). For duck samples, the *C. jejuni* cell counts were higher by approximately 1 log CFU/g when cultured on either CSA-C8 or CSA-S50 than when cultured on mCCDA (Figure 2). As approximately 2 log CFU/g of *C. jejuni* has been detected in poultry products (33), an increase in sensitivity by 1 log CFU/g could be critical for both the surveillance and prevention of *C. jejuni* infections.

Table 3. Evaluation of *C. jejuni* detection in chicken tenders using mCCDA, CSA-C8, and CSA-S50

Selective medium[a]	Mean ± SD, log CFU/g
mCCDA	3.57 ± 0.19[b]
CSA-C8	3.79 ± 0.07[b]
CSA-S50	3.82 ± 0.07[c]

[a]mCCDA = Modified charcoal-cefoperazone-deoxycholate agar; *Campylobacter* selective agar (CSA)–C8 = CSA supplemented with 8 μg/mL catalase; CSA–S50 = CSA supplemented with 50 mM L–serine.
[b,c]Means in the same column with different superscript capital letters are significantly different ($P < 0.05$).

Table 4. Evaluation of the cell counts of *C. jejuni* recovered from a chicken tender after storage at 4°C for 2 days using mCCDA, CSA-C8, and CSA-S50

Selective medium[a]	Mean ± SD, log CFU/g
mCCDA	3.09 ± 0.11[b]
CSA-C8	3.11 ± 0.16[b]
CSA-S50	3.35 ± 0.16[c]

[a]mCCDA = Modified charcoal-cefoperazone-deoxycholate agar; *Campylobacter* selective agar (CSA)–C8 = CSA supplemented with 8 μg/mL catalase; CSA–S50 = CSA supplemented with 50 mM L–serine.
[b,c]Means in the same column with different superscript capital letters are significantly different ($P < 0.05$).

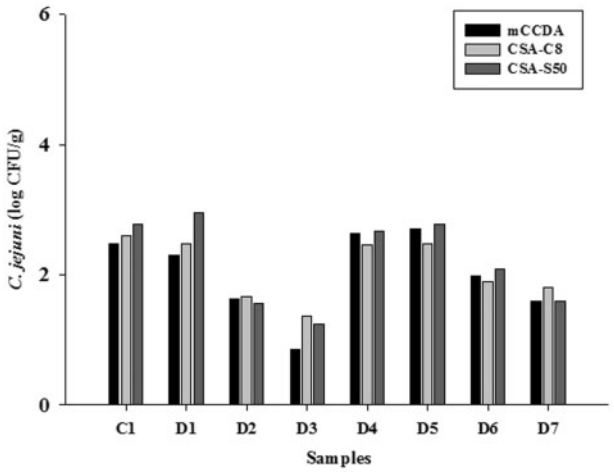

Figure 2. Quantitative evaluation of *C. jejuni* detection in chicken and duck carcasses using mCCDA, CSA-C8, and CSA-S50. mCCDA= modified charcoal–cefoperazone–deoxycholate agar; *Campylobacter* selective agar (CSA)-C8 = CSA supplemented with 8 μg/mL catalase; CSA-S50= CSA supplemented with 50 mM L-serine. C1= chicken sample 1; D1= duck sample 1; D2= duck sample 2; D3= duck sample 3; D4= duck sample 4; D5= duck sample 5; D6= duck sample 6; D7= duck sample 7.

Unlike other bacteria, *C. jejuni* does not use glucose as an energy source, and instead produces energy by utilizing glucogenic amino acids. It is also sensitive to oxygen toxicity. The findings of this study indicate that supplementation of CSA with 8 μg/mL catalase (CSA-C8) or 50 mM L-serine (CSA-S50) improved *C. jejuni* recovery and detection. Additionally, compared to mCCDA, CSA-C8 and CSA-S50 exhibited higher detection efficiencies for *C. jejuni* in food samples.

Conclusions

In conclusion, CSA-C8 and CSA-S50 may improve the efficiency of *C. jejuni*-detection in food and may be useful for detecting *C. jejuni* cells present in the cold-damaged state.

Acknowledgments

None.

Conflict of Interest

The authors declare no conflicts of interest.

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